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Diagnostic values of cerebrospinal fluid t-tau and $A\beta_{42}$ using Meso Scale Discovery assays for Alzheimer's disease

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Abstract

Background—Meso Scale Discovery (MSD) recently established electrochemiluminescence-based assays to measure cerebrospinal fluid (CSF) levels of total tau (t-tau) and amyloid beta 1–42 peptide ($A\beta_{42}$) that can aid in the diagnosis of Alzheimer's disease (AD). The goal of this investigation is to independently evaluate this platform and establish cut-off values of these biomarkers for AD diagnosis.

Objective—To validate the analytical and clinical performance of the MSD t-tau and $A\beta_{42}$ kits and propose diagnostic cut-off values for the field.

Methods—The analytical performance of the CSF t-tau and $A\beta_{42}$ assays was determined, followed by assessment of diagnostic performance of CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ in three clinically characterized cohorts.

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Results—Both MSD assays demonstrated consistent and stable analytical performance, as well as resistance to several important pre-analytic variables. Diagnostically, t-tau/$A\beta_{42}$ performed the best.

Conclusions—Our results independently confirm the analytical and clinical performance of the MSD CSF t-tau and $A\beta_{42}$ assays. Based on a large, multi-center, clinically diagnosed cohort, we propose for the first time candidate diagnostic cut-offs for MSD measured CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$. However, these values needs to be refined as more subjects are included and the assays are tested by other laboratories.

Keywords

$A\beta_{42}$; tau; cerebrospinal fluid; Alzheimer's disease; Meso Scale Discovery

1. Introduction

Alzheimer's disease (AD), the most common cause of geriatric dementia, lacks effective treatment. In this regard, there is a growing need for biomarkers to aid in drug development for AD by improving the accuracy of clinical diagnosis, as well as enabling pre-dementia diagnosis and tracking of disease progression [1]. Compared to the "gold" standard of neuropathologic evaluation, the clinical diagnosis of AD has been reported to have a sensitivity of 70 – 90 % and a specificity of 45 – 75 % in large studies, particularly the recent one by the National Alzheimer's Coordinating Center (NACC) [2]. Importantly, the diagnostic accuracy can be increased by analysis of key cerebrospinal fluid (CSF) biomarkers, especially amyloid beta peptide 1–42 ($A\beta_{42}$), total tau (t-tau), and tau phosphorylated at residue 181 (p-tau_{181}). Therefore, the use of these core biomarkers has been incorporated in the latest National Institute of Aging (NIA) and International Working Group (IWG) research clinical diagnostic criteria for AD dementia [3, 4]. CSF tau and $A\beta_{42}$ levels have also been shown to be strong predictors of progression of mild cognitive impairment (MCI) to AD [5], and are one tool suggested for the detection of preclinical AD [6].

Until recently, CSF tau and $A\beta_{42}$ for AD diagnostic or research purposes have been measured mostly by singleplex ELISA (INNOTEST) or multiplex xMAP Luminex (INNO-BIA AlzBio3) assays manufactured by Innogenetics (now Fujirebio Europe, Ghent, Belgium). Although these assays are widely used and have been shown to perform well diagnostically in clinical and research settings [7–9], their analytical performance is less robust. A significant matrix effect has been reported for both platforms [10, 11] and the assays show substantial inter-laboratory variability, possibly originating from pre-analytical, analytical, post-analytical and kit manufacturing sources [12–14]. This might be one of the reasons that diagnostic cut-off values vary considerably between studies [15] such that currently, with the absence of absolute reference standards, each laboratory is recommended to have their own validated cut-offs to ensure adequate sensitivity and specificity [16]. With extraordinary attention to detail, including testing kits from manufactured lots for consistency, single laboratories can maintain robust measurement accuracy with these assays [17].

Meso Scale Discovery (MSD, Rockville, MD, USA) has recently developed and validated new electrochemiluminescence-based assays for CSF $A\beta_{42}$ and t-tau designed specifically to address the challenges with the existing AD biomarker assays (for a description of the MSD platform Supplemental methods). A major strength of the MSD platform is its high sensitivity, allowing dilution of samples to minimize possible matrix effects [12]. Attention to manufacturing and reagents has led to sufficient consistency that these assays have been proposed to be suitable for clinical use, however, in contrast to the INNOTEST and INNO-BIA AlzBio3 assays [7–9, 18–20], the clinical performance and proposed cut-off values for the MSD $A\beta_{42}$ and t-tau assays have not yet been investigated independently. In this study, we validate the analytical performance of both MSD assays, followed by systematic evaluation of the diagnostic performance (AD dementia vs. healthy controls) of CSF $A\beta_{42}$, t-tau or the t-tau/$A\beta_{42}$ ratio when measured on the MSD platform. Finally, we propose for the first time diagnostic cut-off values for these MSD measured biomarkers based on a large, multi-center cohort of participants diagnosed with AD dementia vs. healthy controls.

2. Materials and methods

2.1 Subjects and cohorts

A total of 488 subjects from three cohorts (2 single-sites and 1 multi-site) were included in the study. The first cohort consisted of 54 control and 45 AD dementia CSF samples obtained from the University of California at San Diego (UCSD) that had never been thawed after initial freezing and storage at −80 ° C. The second cohort comprised 98 control and 85 AD dementia CSF samples obtained from Oregon Health and Sciences University (OHSU). Like the samples in the first cohort, these samples had never been thawed after collection. The third cohort consisted of 104 control and 102 AD dementia archived CSF samples that were collected as part of several studies at multiple sites, including UCSD, OHSU, the Seattle VA, the University of California at Davis (UC Davis), Indiana University and the University of Pennsylvania (UPENN). These samples had previously been thawed, treated with 10 % protease inhibitor cocktail (PIC; Sigma, St Louis, MO, USA) and aliquoted before once again being frozen and stored at −80° C. The Human Subject Institutional Review Boards of each institution listed above, as well as the University of Washington (UW) approved the study. All individuals provided informed consent and underwent detailed clinical evaluations that consisted of medical history, laboratory tests, neurological examinations and neuropsychological assessment, including tests for memory and cognition [21]. Control subjects were community volunteers in good health with normal cognitive function and AD dementia patients were diagnosed with probable AD according to NINDS-ADRDA criteria [22]. Individuals with neurological disorders (multiple sclerosis, epilepsy, stroke, Parkinson’s disease etc.), major psychiatric disorders (schizophrenia and bipolar affective disorders), unstable or poorly controlled medical conditions, excessive alcohol abuse, or use of illegal drugs were excluded. All UCSD patients and controls also underwent magnetic resonance imaging and any subjects with significant cerebrovascular changes (e.g. lacunes, stroke(s) and substantial white matter hyperintensities) were excluded.

2.2 CSF collection and analysis

CSF samples were obtained by lumbar puncture in the morning according to standard procedures. For individual clinical samples, the first 2 mL of CSF was sent to a local laboratory for determination of protein, glucose and cell count. CSF samples with 500 or more red blood cells per mL were excluded. Up to 25 ml CSF was then taken from each subject, aliquoted into polypropylene cryotubes (0.5 mL per aliquot) and stored at −80 °C. Reference CSF was obtained from the clinical laboratory at Harborview Medical Center (Seattle, WA, USA). Individual samples were pooled, split in half and either directly aliquoted (pooled reference CSF without PIC treatment) or first treated with 10 % PIC (pooled reference CSF with PIC treatment) before being aliquoted and stored at −80 °C. Three batches of pooled reference CSF (A, B & C) with and without PIC were used in the analytical performance studies. In the clinical studies, an aliquot of one batch of pooled reference CSF (B) treated with PIC was run in duplicate on each plate as an internal standard. To control for plate-to-plate variation in the clinical study, results of a particular plate were normalized if the internal standard varied more than 10 % from the average of all the plates as performed previously [23–27]. To avoid over or erroneous correction, any outliers (by Chauvenet's criterion) were removed when calculating the normalization factors where all internal references are considered.

All CSF samples were analyzed for t-tau and $A\beta_{42}$ levels using the MSD Human Total Tau (K151LAG) and Human $A\beta_{42}$ (K151LBG) V-PLEX Plus kits according to the manufacturer’s protocol (see Supplemental methods for details).

2.3 Analysis of MSD assay analytical performance

The analytical performance of the MSD t-tau and $A\beta_{42}$ assays in terms of parallelism, sensitivity, accuracy, precision, lot-to-lot variability, selectivity and freeze/thaw stability was assessed according to published guidelines [28–30] (see Supplemental methods for details). The effect of PIC treatment on all of these aspects was also investigated. Lot-to-lot variability was assessed with aliquots of three batches of pooled reference CSF (with and without PIC treatment) using three different kit lots, performed in one day by the same technician. All other analytical performance experiments were done using aliquots of one to three batches of pooled reference CSF (with and without PIC treatment) in a minimum of three experiments performed by the same technician.

2.4 APOE Genotyping

APOE genotyping was performed by several different methods, including restriction digestion, single nucleotide polymorphism genotyping and sequencing [31–33].

2.5 Statistical analysis

Statistical analysis was performed using PASW 18.9 Statistics software (SPSS, Inc., Chicago, IL, USA). For the analyses of data in Table 1, Mann-Whitney U or Kruskal-Wallis and Chi square tests were applied to continuous and qualitative variables, respectively. Receiver operating characteristic (ROC) curves were used to calculate the relationship between sensitivity and specificity for the AD group versus controls, and hence evaluate the

diagnostic performance of the CSF biomarkers. The optimum cut-off value from the ROC curve is the point at which the sum of sensitivity and specificity is maximal (Youden index). Average unbiased estimates of biomarker diagnostic performance were obtained by identifying the optimal cutoff using one cohort and then applying that cutoff to the other two cohorts. Statistical tests were two-sided and significance was set at $p< 0.05$.

3. Results

3.1 Analytical performance

The t-tau and $A\beta_{42}$ assays have been fully validated for use in human CSF by MSD according to the principles outlined in "Fit-For-Purpose Method Development and Validation for Successful Biomarker Measurement" by Lee and colleagues [28]. Both assays also were shown to be tolerant of up to 1.6 mg/mL hemoglobin, which is equivalent to 1 % blood contamination in the CSF sample and can easily be identified by eye (http://www.mesoscale.com). In the current study, we independently confirmed the analytical performance of the t-tau and $A\beta_{42}$ MSD assays. Parallelism was demonstrated for both assays, with a minimum required dilution (MRD) of 1:4 and 1:8 for t-tau and $A\beta_{42}$, respectively (Supplemental figure 1 A & B). Assay sensitivity was good, with a lower limit of detection (LLOD) range of 7.02 – 14.60 pg/mL for t-tau and 0.09 – 0.48 pg/mL for $A\beta_{42}$. Both assays also had a broad working range with low lower limits of quantification (LLOQs) (t-tau: 9.48 pg/mL; $A\beta_{42}$: 0.5 pg/mL) and high upper limits of quantification (ULOQs) (t-tau: 9680 pg/mL; $A\beta_{42}$: 3100 pg/mL; Supplemental table 1). Our results indicated that both the intra-plate (average coefficient of variation (CV) < 10 %)) and inter-plate (average CV < 20 %) precision of the MSD CSF t-tau and $A\beta_{42}$ assays were in line with that reported by MSD. In our hands, both MSD assays also demonstrated good accuracy, with measured concentrations within 80 – 120 % of expected concentration for kit controls (Supplemental table 2 & 3), and selectivity, with spike recovery rates of 70–130 % (Supplemental table 4). Finally, we found the lot-to-lot variability of both assays to be good (inter-lot CV < 20 %; Supplemental table 5).

Importantly, we showed that the addition of PIC to pooled reference CSF after the first thaw did not significantly affect either assay's performance at the recommended sample dilution factor (4 fold for t-tau; 8 fold for $A\beta_{42}$). Furthermore, we found that pooled reference CSF samples (with or without PIC added after the first thaw) were stable for up to three (t-tau assay) or two ($A\beta_{42}$ assay) additional freeze/thaw cycles (Supplemental figure 2 A & B). Finally, we monitored the in-study performance (sensitivity, accuracy and precision) of both assays in the 8 clinical sample plates that were run and found their performance to be consistent with our results from the analytical study, apart from slightly lower inter-plate precision (Supplemental table 6 & 7).

3.2 Clinical performance

3.2.1 Subjects included in the study—The clinical performance study included three separate cohorts with a combined total of 256 control and 232 AD dementia subjects. Demographic, clinical and CSF biomarker data of the studied subjects are summarized in Table 1. The diagnostic groups differed significantly in terms of gender ($p = 0.002$), years of

education ($p = 0.004$) and, as expected, Apolipoprotein E (*APOE)* ε4 allele presence ($p < 0.001$), mini-mental state examination (MMSE) score ($p < 0.001$), t-tau ($p < 0.001$), $A\beta_{42}$ ($p < 0.001$) and t-tau/$A\beta_{42}$ ($p < 0.001$). The decrease in CSF $A\beta_{42}$ and increase in CSF t-tau and t-tau/$A\beta_{42}$ levels as measured by MSD assay were in line with the majority of studies published previously using singleplex ELISA or multiplex xMAP Luminex immunoassays [7–9, 20]. The analysis also revealed some inter-cohort differences. AD dementia patients in the three cohorts differed significantly in terms of *APOE* ε4 allele presence ($p = 0.033$), years of education ($p = 0.029$) and CSF levels of all three biomarkers (t-tau: $p = 0.034$; $A\beta_{42}$: $p = 0.017$; t-tau/$A\beta_{42}$: $p = 0.002$). With regard to control subjects, there were significant differences in age ($p < 0.001$), as well as in CSF t-tau ($p = 0.001$), $A\beta_{42}$ ($p = 0.001$) and t-tau/$A\beta_{42}$ ($p < 0.001$) levels between cohorts 1, 2 and 3.

3.2.2 ROC analysis of individual cohorts—ROC analysis demonstrated that CSF levels of both t-tau and $A\beta_{42}$ as measured by the MSD assay performed reasonably well in distinguishing AD dementia patients from controls when the optimum cut-off (maximum sensitivity and selectivity) was selected in each of the three cohorts (Figure 1 A, B, & C; Table 2). In each cohort, based on the ROC analysis, numerically, the best overall diagnostic performance was obtained with the CSF t-tau/$A\beta_{42}$ ratio, with sensitivity of 86 – 87 % and specificity of 72 – 84 %, depending on the cohort. However, obtaining a cut-off from a cohort and applying it to the same cohort to may lead to an overestimation (bias) of a biomarker's diagnostic performance. Therefore, we determined the optimum cut-off from each of the cohorts and applied it to the other two cohorts to obtain unbiased estimates of diagnostic performance for each CSF biomarker (Table 3). Once again, the t-tau/ $A\beta_{42}$ ratio performed the best of the three biomarkers (sensitivity 82 %; specificity 74 %).

3.2.3 ROC analysis of combined cohort—ROC analyses of the combined cohort (Figure 2) provided cut-off values for the CSF biomarkers at the maximum sensitivity and specificity. The ROC parameters and diagnostic test performance for each of the 3 CSF biomarkers are summarized in Table 4. For CSF t-tau, optimum cut-offs resulted in a sensitivity and specificity of 70 % and 80 %, respectively, whereas CSF $A\beta_{42}$ showed 78 % sensitivity and 68 % specificity. As was the case in the individual cohorts, the best overall diagnostic performance in the combined cohort was achieved with the CSF t-tau/$A\beta_{42}$ ratio (sensitivity 79 %; specificity 82 %).

4. Discussion

CSF biomarkers are increasingly important in AD diagnosis [3] and clinical trials [1, 34]. However, their use is currently limited by methodological variability and subsequent inconsistency in the cut-off values used to interpret results [35]. Although intense efforts to standardize preanalytical and analytical procedures are under way [13, 14, 36], some variability in biomarker quantification may remain due to inherent characteristics of existing commercial immunoassays, such as matrix interference and lot-to-lot variability [12, 13]. In response, MSD has recently developed a new electrochemiluminescence-based platform for measuring CSF t-tau and $A\beta_{42}$, which they claim successfully address these challenges. This is the first study to assess independently the practical use of MSD CSF t-tau and $A\beta_{42}$ assays in AD in terms of analytical and clinical performance.

Precision and accuracy of the analytical method are fundamental prerequisites before the clinical utility of a biomarker can be established. In addition, a biomarker assay needs to be sensitive and dynamic enough to be able to measure a broad range of analyte concentrations in clinical samples. Results from our analytical performance study indicated that the intra-assay and inter-assay precision of the MSD CSF t-tau and $A\beta_{42}$ assays were in line with that reported by MSD. Inter-assay CVs in the clinical study however, were slightly higher although still acceptable, perhaps reflecting additional variability introduced in this part of the study such as multiple technicians performing the assays. In our hands, both MSD assays also demonstrated good accuracy. Furthermore, although we did not perform a head-to-head comparison between the different assay platforms, our results support the claim by Kang et al. [12] that the sensitivity and working range of the MSD assays are better than that of other existing assays (INNOTEST and INNO-BIA AlzBio3). Importantly, the increased sensitivity allows for dilution of samples to minimize possible matrix effect, as evidenced by the good selectivity of both assays. Finally, we found that the addition of PIC does not affect the performance of either MSD assay and that both assays were resistant to at least 2 additional freeze/thaw cycles, with no apparent increase in variability as has been reported for INNOTEST ELISAs [37]. This has important practical implications for biomarker studies where samples may have been treated with PIC and/or exposed to more than one freeze/thaw cycle.

Having confirmed the analytical performance of the MSD CSF t-tau and $A\beta_{42}$ assays, we set out to investigate their clinical performance in terms of classification of AD dementia patients vs. healthy control subjects. For this purpose, we analyzed 3 cohorts individually and found that the optimum cut-off values, as well as the diagnostic performance (sensitivity and specificity) of the three biomarkers varied depending on the cohort analyzed, with the t-tau/$A\beta_{42}$ ratio having the best and most consistent performance. As all the CSF samples were analyzed in the same laboratory, these results may reflect the heterogeneity of the cohorts in terms of demographic factors (e.g. age, *APOE* ε4 allele presence and years of education as identified in our descriptive analysis), as well as slight differences in subject diagnosis and/or sample collection and handling between sites. Of note, similar inter-site differences have been reported for CSF tau and $A\beta_{42}$ measured by INNOTEST assays [38]. Additionally, a recent paper has shown that CSF levels of both t-tau and $A\beta_{42}$ and their cut-off values as determined by ROC are significantly affected by the source of polypropylene tubes used for CSF collection [39]. To this end, although the CSF collection protocol is identical in all three sites involved, the materials of collection are slightly different, which might also have contributed to this site variation. These results emphasize again the critical need for standardization of the procedures and materials used when comparing biomarker levels between sites.

It is well known that deriving biomarker cut-offs from the cohort under study risks overestimating its diagnostic performance. Therefore, we obtained the optimum cut-off in each individual cohort and applied it to the other 2 cohorts to get estimates of unbiased sensitivity and specificity. Once again, the CSF t-tau/ $A\beta_{42}$ ratio had the highest unbiased sensitivity and specificity of the three biomarkers.

Finally, we analyzed the combined cohort in an attempt to avoid some of the risks associated with a single-center cohort study (low number and narrow range of subjects). In the combined cohort, as in the individual cohorts, the best clinical performance was obtained with the CSF t-tau/$A\beta_{42}$ ratio. Of note is that these "biased" estimates of clinical performance obtained in the combined cohort are quite similar to the "unbiased" estimates obtained by applying cut-offs derived from one of the individual cohorts to the other two. The clinical performance of these 3 CSF biomarkers in distinguishing AD subjects from healthy controls as assessed by the MSD platform were in the range of values for other existing commercial assays, as published in a recent meta-review [40]. It should be mentioned that this review included studies where the diagnostic groups were classified based on clinical diagnosis alone, as well as by neuropathologic evaluation of brain autopsy. As the clinical diagnosis of AD dementia is estimated to have a sensitivity of 70.9 – 87.3 % and a specificity of 44.3 – 70.8 % compared to the "gold" standard of postmortem neuropathologic evaluation [2], it is not surprising that autopsy based studies generally report higher sensitivity and specificity for CSF tau and $A\beta_{42}$ [8, 20]. Indeed, a recent study reported that the use of clinical instead of neuropathologic diagnosis led to a 14 – 17 % underestimation of CSF tau and $A\beta_{42}$ biomarker accuracy [41]. It is well known that around one third of cognitively normal elderly display AD-like changes in their CSF, on amyloid Positron emission tomography (PET) scans, or in their brain at autopsy, suggesting the possible existence of preclinical AD in these subjects [42]. Furthermore, a percentage of clinically diagnosed AD patients may in fact suffer from a different or additional disease that contributes to dementia such as vascular brain injury or Parkinson's disease [43–46], which are known to have different tau and $A\beta_{42}$ profiles than AD [25, 47–49]. Therefore, since clinical diagnosis faces challenges to accuracy compared to postmortem, neuropathological confirmation, it is probably not possible to obtain a much higher sensitivity and specificity than 85 % for a biomarker based on clinically characterized groups. Thus, the clinical performance (sensitivity 79.3 %; specificity 82.4 %) that we obtain for CSF t-tau/$A\beta_{42}$ as measured by the MSD platform is close to the maximum that can reasonably be expected for this cohort.

In addition to a lack of pathologic diagnosis for our subjects, the effect of pharmacotherapy is a caveat of the current study, as most subjects were not drug-free at the time of sample collection. Furthermore, for an assay to be suitable for long term multi-center testing, low interlaboratorty variability is vital. Therefore, the lack of assessment of interlaboratory variability of the MSD CSF t-tau and $A\beta_{42}$ assays represents another limit of the current study. Furthermore, to truly compare the MSD assays with the existing widely used commercial assays, a head to head comparison in the same cohort is needed. Finally, the lack of an MSD p-tau_{181} assay is a major drawback of the platform, as p-tau_{181} has been shown to be a valuable AD biomarker, especially with regard to differential diagnosis [50–52].

In summary, our results indicate that the MSD CSF t-tau and $A\beta_{42}$ assays are robust in terms of analytical and clinical performance. Based on a large, heterogeneous group of subjects we propose for the first time cut-offs for MSD measured CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ for the diagnosis of AD dementia compared to healthy controls, with good sensitivity and specificity. For future studies, we propose that the interlaboratory variability of the MSD

platform be assessed and compared head-to-head to that of other existing commercial assays. Our results should also be confirmed in an independent cohort, preferably with tau and Aβ PET data and/or neuropathologic evaluation. It also would be important to investigate the clinical performance and establish cut-offs for MSD measured CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ in distinguishing AD from MCI and other dementias.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MSD	Meso Scale Discovery
AD	Alzheimer's disease
CSF	Cerebrospinal fluid
t-tau	total tau
$A\beta_{42}$	Amyloid beta 1–42 peptide
p-tau_{181}	tau phosphorylated at residue 181
NIA	National Institute of Aging
MCI	mild cognitive impairment
UCSD	University of California at San Diego
OHSU	Oregon Health and Sciences University
UC Davis	University of California at Davis
UPENN	University of Pennsylvania
UW	University of Washington
PIC	protease inhibitor cocktail
ROC	Receiver operating characteristic
MRD	minimum required dilution
LLOD	lower limit of detection
LLOQ	lower limit of quantification
ULOQ	upper limit of quantification
CV	coefficient of variance

APOE ε4	Apolipoprotein E epsilon 4 allele
MMSE	mini-mental state examination
PET	Positron emission tomography.

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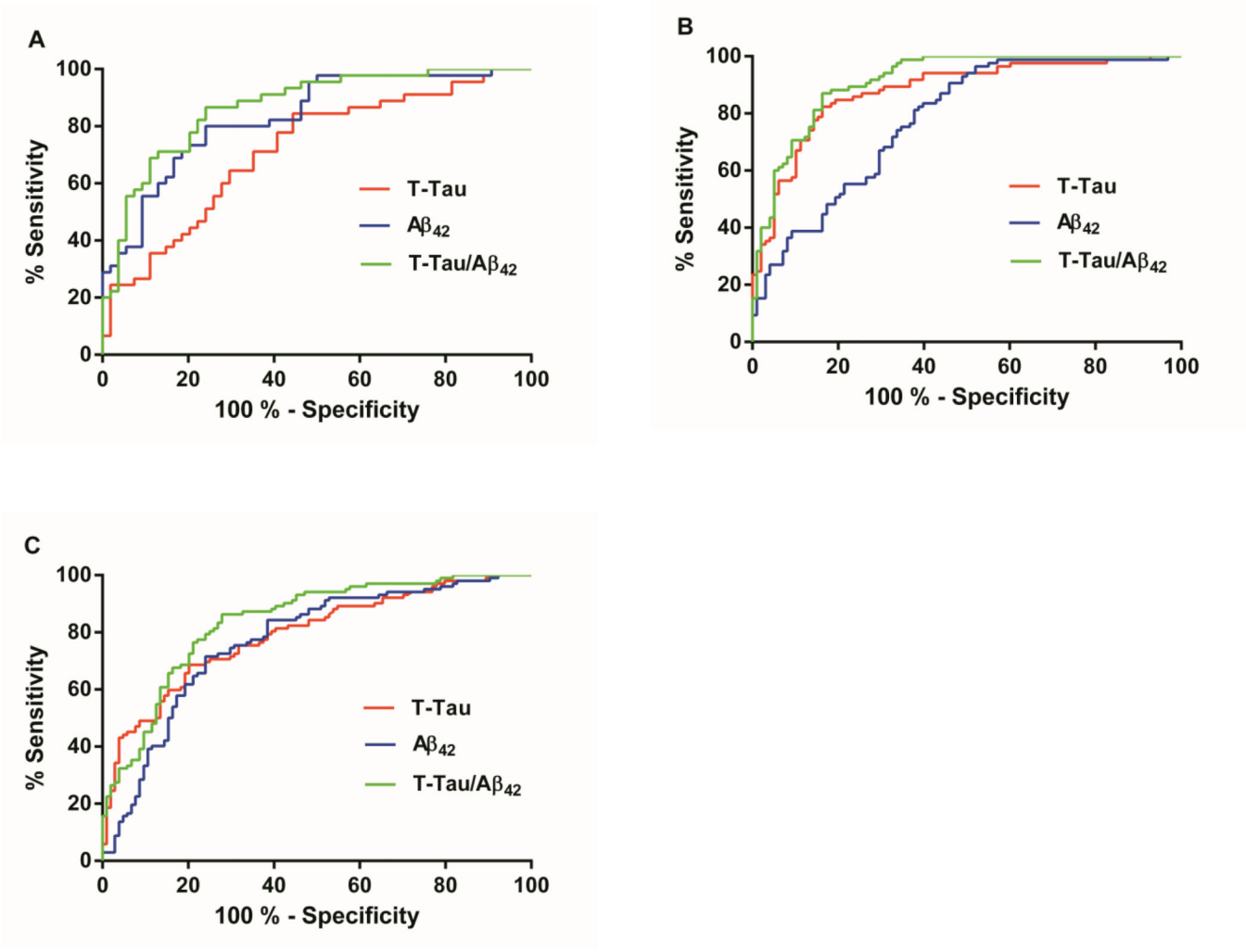

Figure 1.

Receiver operating characteristic (ROC) curves of CSF t-tau, Aβ42 and t-tau/$A\beta_{42}$ measured by MSD for the classification of AD vs controls in cohort 1 (A), 2 (B) and 3 (C).

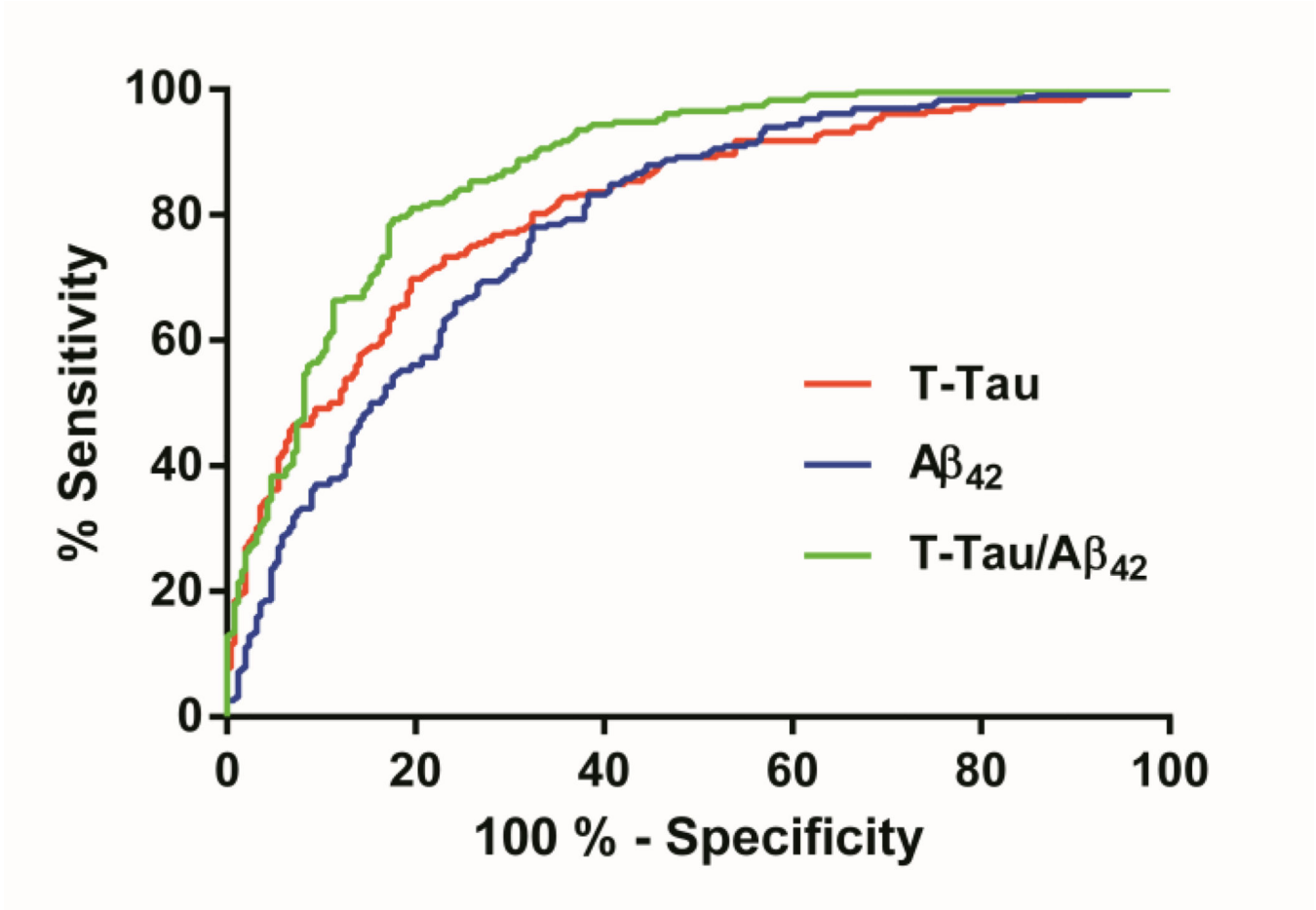

Figure 2.
Receiver operating characteristic (ROC) curves of CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ measured by MSD for the classification of AD vs controls in the combined cohort.

Table 1

Summary of subjects and cohorts included in the study.

	Cohort 1		Cohort 2		Cohort 3		Combined cohort		*p* value		
	CTL n=54	**AD n=45**	**CTL n=98**	**AD n=85**	**CTL n=104**	**AD n=102**	**CTL n=256**	**AD n=232**	**CTL vs. AD**	**CTL cohorts**	**AD cohorts**
Age in years	71.9 (6.7)	72.2 (10.1)	67.4 (10.4)	69.7 (8.5)	73.7 (6.0)	71.0 (10.0)	70.9 (8.6)	70.7 (9.5)	0.921	<0.001	0.364
Gender M/F (% M)	18/36 (33.3)	23/22 (51.1)	41/57 (41.8)	53/32 (62.4)	51/53 (49.0)	56/46 (54.9)	110/146 (43.0)	132/100 (56.9)	0.002	0.160	0.404
***APOE* ε4** $\varepsilon4^+/\varepsilon4^-$ (% $\varepsilon4^+$)	16/38 (29.6)	25/19 (55.6)	32/42 (32.7)	59/16 (69.4)	33/71 (31.7)	62/33 (60.8)	81/151 (31.6)*	146/68 (62.9)†	<0.001	0.184	0.033
MMSE	29.2 (1.4)	20.6 (3.9)	29.3 (1.0)	19.1 (6)	29.1 (1.1)	21.1 (5.4)	29.2 (1.2)‡	20.2 (5.4)§	<0.001	0.176	0.107
Years education	16.4 (2.5)	14.4 (3.6)	15.7 (2.7)	14.9 (2.9)	16.0 (2.4)	16.0 (3.2)	16.0 (2.5)¶	15.2 (3.2)#	0.004	0.228	0.029
T-tau pg/mL	308.7 (222.6–499.4)	501.4 (357.6–777.1)	279.1 (175.9–374.2)	709.7 (471.8–952.4)	337.2 (262.5–445.5)	596.0 (400.0–863.5)	307.3 (226.0–433.7)	596.0 (417.6–893.3)	<0.001	0.001	0.034
$A\beta_{42}$ pg/mL	305.9 (205.9–465.5)	163.0 (118.6–203.2)	244.7 (143.1–353.6)	134.7 (98.9–193.2)	213.2 (155.8–311.3)	125.6 (91.0–169.8)	232.5 (162.3–356.1)	135.2 (97.6–186.3)	<0.001	0.001	0.017
T-tau/ $A\beta_{42}$	0.8 (0.6–1.7)	3.8 (2.0–4.7)	0.9 (0.6–2.2)	5 (3.2–7.9)	1.4 (1.0–2.6)	5.4 (2.9–8.0)	1.1 (0.7–2.2)	4.6 (3.0–7.4)	<0.001	<0.001	0.002

Data of normally distributed variables (age, MMSE and education) are presented as mean (SD). Data of non-normally distributed variables (t-tau, $A\beta_{42}$ and t-tau/Aβ42) are presented as median (1^{st} – 3^{rd} quartile).

**APOE* genotype missing for 24 subjects;

†*APOE* genotype missing for 18 subjects;

§MMSE missing for 8 subjects;

‡MMSE missing for 21 subjects;

¶Education missing for 11 subjects;

#Education missing for 19 subjects.

P values obtained by Mann-Whitney U or Kruskal-Wallis tests and Chi square tests for continuous and qualitative variables, respectively.

APOE ε4, Apoliporptein E epsilon 4 allele; MMSE, mini-mental state examination; T-tau, total-tau; $A\beta_{42}$, amyloid beta 1–42 peptide..

J Alzheimers Dis. Author manuscript; available in PMC 2015 July 28.

Table 2

Receiver operating characteristic (ROC) curve parameters for CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ as measured by MSD assays in distinguishing AD patients from controls in the individual cohorts.

	Cohort 1			Cohort 2			Cohort 3		
	T-tau	**$A\beta_{42}$**	**T-tau/ $A\beta_{42}$**	**T-tau**	**$A\beta_{42}$**	**T-tau/ $A\beta_{42}$**	**T-tau**	**$A\beta_{42}$**	**T-tau/ $A\beta_{42}$**
AUC (95% CI)	0.718 (0.617–0.819)	0.833 (0.753–0.912)	0.87 (0.80–0.939)	0.878 (0.827–0.929)	0.778 (0.712–0.844)	0.915 (0.876–0.954)	0.791 (0.73–0.852)	0.77 (0.705–0.836)	0.838 (0.784–0.892)
p	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cut-off	325 pg/mL	206 pg/mL	1.72	440 pg/mL	230 pg/mL	2.72	470 pg/mL	155 pg/mL	2.13
% Sensitivity (95% CI)	84 (70–93)	80 (65–90)	87 (73–95)	82 (73–90)	91 (82–96)	87 (78–93)	69 (59–77)	72 (62–80)	86 (78–92)
% Specificity (95% CI)	56 (41–69)	76 (62–87)	76 (62–87)	84 (75–90)	54 (44–64)	84 (75–90)	80 (71–87)	76 (67–84)	72 (62–80)

AUC, area under the curve; CI, confidence interval; T-tau, total-tau; $A\beta_{42}$, amyloid beta 1–42 peptide.

Table 3

Estimates of unbiased sensitivity and specificity of CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ measured by MSD for classification of AD vs. controls. Optimum cut-offs from each cohort was applied to the other two cohorts and the mean and 95 % CI of the sensitivity and specificity calculated.

Parameter	T-tau	$A\beta_{42}$	T-tau/$A\beta_{42}$
% Mean sensitivity (95% CI)	74 (68–79)	74 (68–80)	82 (78–87)
% Mean specificity (95% CI)	69 (63–74)	64 (58–70)	74 (68–79)

CI, confidence interval; T-tau, total-tau; $A\beta_{42}$, amyloid beta 1–42 peptide.

Table 4

Receiver operating characteristic (ROC) curve parameters for CSF t-tau, $A\beta_{42}$ and t-tau/$A\beta_{42}$ as measured by MSD assays in distinguishing AD patients from controls in the combined cohort.

Parameter	T-Tau	$A\beta_{42}$	T-tau/$A\beta_{42}$
ROC AUC (95% CI)	0.813 (0.776–0.851)	0.783 (0.742–0.823)	0.873 (0.842–0.904)
p	<0.001	<0.001	<0.001
Cut-off	470 pg/mL	190 pg/mL	2.75
% Sensitivity (95% CI)	70 (64–76)	78 (72–83)	79 (74–84)
% Specificity (95% CI)	80 (75–85)	68 (62–73)	82 (77–87)

ROC, receiver operating characteristic; AUC, area under the curve; CI, confidence interval; T-tau, total-tau; $A\beta_{42}$, amyloid beta 1–42 peptide.