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Nanoparticle-based immunomagnetic assay of plasma biomarkers for differentiating dementia and prodromal states of Alzheimer's disease – A cross-validation study

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Abstract

Blood-based biomarker assays of plasma β-amyloid (Aβ) and tau have the advantages of cost-effective and less invasive for the diagnosis of Alzheimer's disease (AD). We used two independent cohorts to cross-validate the clinical use of the nanoparticle-based immunomagnetic assay of plasma biomarkers to assist in the differential diagnosis of early AD. There were in total 160 subjects in the derivation cohort, and 242 in the validation cohort both containing controls, mild cognitive impairment due to AD and AD dementia diagnosed according to the 2011 NIA-AA guidelines. The cutoff value for plasma $A\beta_{1-42}$ (16.4 pg/ml) performed the best in differentiating between controls and patients with prodromal or clinical AD, with 92.5% for positive percent agreement (PPA), negative percent agreement (NPA), and overall rate of agreement (ORA). $A\beta_{1-42}$ × tau (642.58) was useful for separating patients with dementia and prodromal states of AD, with 84.9% PPA, 78.8% NPA and 83% ORA.
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Key words: Blood-based biomarkers; Immunomagnetic reduction assay; Plasma $A\beta_{1-42}$; Plasma tau; Mild cognitive impairment

Compared to neuropsychological tests (NPT), neuroimaging, or biomarker detection in the cerebrospinal fluid (CSF) for the diagnosis of Alzheimer's disease (AD), blood-based biomarker assays have the advantages of being time- and cost-effective, less invasive, and well accepted by the patient population.[1,2] Clinical scientists and pharmaceutical researchers recommend blood-based detection not only for AD screening but also for clinical course prediction and treatment response follow-up.[2] The most convincing biomarkers in the blood for AD are currently β-amyloid (Aβ) and tau protein because amyloid plaques and neurofibrillary tangles are pathological hallmarks in the brains of patients with AD.[3–6] Other promising biomarkers, e.g.,

Abbreviation: Aβ, beta amyloid; AD, Alzheimer's disease; AUC, area under the curve; ANOVA", analysis of variance; CDR, clinical dementia rating; CE, Conformité Européene; CSF, cerebrospinal fluid; ELISA, enzyme linked immunosorbent assay; GDS, geriatric depression scale; IMR, immunomagnetic reduction; IVD, in vitro diagnostics; MCI, mild cognitive impairment; NPA, negative percent agreement; ORA, overall rates of agreement; PPA, positive percent agreement; NPT, neuropsychological test; ROC, receiver operating characteristics; SIMOA, single molecular array; TMSE, Taiwanese mental state examination

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Competing interests

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neurofilament light, also known as apolipoprotein J, have also been proposed.[7–9] However, the levels of these biomarkers in human blood are extremely low, often on the level of pg/ml. Thus, typical or conventional assays used in clinical practice are neither sensitive nor reliable enough to detect these blood-based biomarkers. Substantial increases in the sensitivity and reliability of the measurement of blood-based biomarkers have been made possible by the development in recent decades of ultrasensitive assay technologies, such as modified enzyme-linked immunosorbent assay (ELISA),[10] multiplex electrochemiluminescence,[11,12] surface-based fluorescence intensity distribution analysis,[13] multiplexed flowmetrix analysis,[14,15] selected reaction monitoring mass spectrometry,[16] single-molecule array (SIMOA)[17,18] and immunomagnetic reduction (IMR).[19,20] Many cross-sectional studies using these ultrasensitive assays have revealed the feasibility of assaying blood-based biomarkers to differentiate between healthy subjects and patients with mild cognitive impairment (MCI) due to AD or those with AD dementia.[21–25] However, to the best of our knowledge, until now, all these ultrasensitive assay technologies have been tools used only for research. There is a lack of clinical validation following the Good Clinical Practice regulatory framework for diagnosing AD via blood-based biomarker detection by using any of these assay technologies. In this work, a cross-sectional study with two independent cohorts was conducted to verify the clinical use of the IMR assay of plasma Aβ and tau protein to assist in the differential diagnosis of early AD.

Therefore, it was our aim to cross-validate the usefulness of the blood-based biomarkers in assisting with the differential diagnosis of early AD in clinical settings by recruiting two independent cohorts, a derivation cohort recruited from a single tertiary referral medical center and a validation cohort recruited from 4 different hospitals located in Northern Taiwan.

Methods

Study design

This is a cross-sectional observation study by using stratified sampling with disproportionate allocation.

Recruitment of subjects

This study adhered to the diagnostic criteria of the National Institute on Aging/Alzheimer's Association Diagnostic Guidelines for MCI due to AD and dementia due to AD.[26–28] The subjects were required to have had least 6 years of formal education or to be capable of reading and writing Chinese in order to complete the comprehensive NPT battery. The subjects who were younger than 50 years of age or who had major systemic diseases, neuropsychiatric disorders, or visual or auditory problems that were sufficiently severe to interfere with the cognitive function tests were excluded. The demographic data and basic clinical information, including age, gender, years of education, Geriatric Depression Scale (GDS-15) score, Taiwanese Mental State Examination (TMSE) score, and Clinical Dementia Rating (CDR) score, were collected. Those who had a GDS score equal to or greater than 8 were excluded.

Table 1
Demographic, clinical and biomarker information of subjects enrolled in the derivation cohort.

Group	Healthy control	MCI due to AD	AD dementia
Numbers	67	34	59
Age (year)	63.3 ± 8.9	68.8 ± 10.7	71.8 ± 10.4
Gender (F/M)	41/26	13/21	32/27
Education (year)	13.2 ± 3.3	11.8 ± 4.3	9.3 ± 4.9
CDR (0/0.5/1/2/3)	67/0/0/0/0	0/34/0/0/0	0/31/20/5/3
TMSE	29 ± 1.4	26.9 ± 2.8	21.6 ± 5.7
ApoE4 (%)	20	29.4	47.2
$A\beta_{1-40}$ (pg/ml)	60.3 ± 7.2	40.6 ± 4.3	43.9 ± 22.6
$A\beta_{1-42}$ (pg/ml)	15.8 ± 0.7	17.6 ± 1.8	23.5 ± 18.8
Tau (pg/ml)	13. 3 ± 6.9	30.5 ± 9.3	47.6 ± 19.2
$A\beta_{1-42}/A\beta_{1-40}$	0.267 ± 0.038	0.437 ± 0.673	0.557 ± 0.231
$A\beta_{1-42}$ X tau	212.0 ± 113.6	536.6 ± 173.1	1075.3 ± 821.4

Gender (F/M) female/male; CDR: clinical dementia ranking; TMSE: mini-mental state examination; AD: Alzheimer's disease; MCI: mild cognitive impairment.

A neuropsychological test battery was performed by a board-certified clinical neuropsychologist to help demarcate mild changes in the cognition and normal daily function of those subjects with MCI. The battery of neuropsychological tests included the Wisconsin Card Sorting Test, Trail Making Test parts A and B (or Color Trail Test I and II), subtests of the Wechsler Adult Intelligence Scale Version III (digit span, matrix reasoning, block design, and digit-symbol substitution) and a semantic verbal fluency test. In addition, subjects were administered at least two subtests of the Wechsler Memory Scale Version III, including logical memory and visual reproduction (in the validation cohort); the word sequence learning test; the visual recognition test; the 3-D block design test; and a basic language evaluation. Subjects with abnormal performances on the NPT, defined as scores on more than one task in memory performance lower than the 4th percentile of their age- and education-matched controls[29] and/or in other cognitive domains and normal activities of daily living were defined as the MCI group. We also applied image biomarkers to increase the certainty level of MCI due to AD following our previous studies.[30,31]

The inclusion and exclusion criteria for the derivation and validation cohorts were the same, identifying and enrolling subjects in the healthy control, MCI due to AD, or AD dementia groups.

In total, 160 subjects, including 67 controls (age 63.3 ± 8.9 years), 34 patients with MCI due to AD (age 68. ± 10.7 years), and 59 patients with AD dementia (age: 71.8 ± 10.4 years), were enrolled in the derivation cohort at the memory clinic of the Department of Neurology, National Taiwan University Hospital from 2010 to 2014. The demographic, clinical and biomarker data for the derivation cohort are listed in Table 1. An additional 240 subjects, including 134 controls (age 68.4 ± 7.7 years), 33 patients with MCI due to AD (age: 72.6 ± 9.0 years), and 73 patients with AD dementia (age: 78.1 ± 8.7 years), were enrolled from the memory clinics of the Taipei Medical University Shuang-Ho Hospital (New Taipei City), the Renai Branch of Taipei City Hospital (Taipei City), En Chu Kong Hospital (New

Table 2
Demographic, clinical and biomarker information of subjects enrolled in the validation cohort.

Group	Healthy control	MCI due to AD	AD dementia
Numbers	134	33	73
Age (year)	68.4 ± 7.7	72.6 ± 9.0	78.1 ± 8.7
Gender (F/M)	89/45	23/10	37/36
Education (year)	12.6 ± 3.7	10.5 ± 3.6	9.0 ± 3.9
CDR (0/0.5/1/2/3)	134/0/0/0/0	0/34/0/0/0	0/21/36/13/3
TMSE	28.2 ± 1.7	24.2 ± 2.9	17.1 ± 5.74
ApoE4 (%)	19	26.3	36.7
$A\beta_{1-40}$ (pg/ml)	62.2 ± 1.4	52.81 ± 19.44	42.73 ± 8.04
$A\beta_{1-42}$ (pg/ml)	14.65 ± 1.45	18.6 ± 2.5	20.0 ± 3.0
Tau (pg/ml)	20.2 ± 10.5	30.5 ± 11.1	40.9 ± 14.4
$A\beta_{1-42}/A\beta_{1-40}$	0.225 ± 0.082	0.384 ± 0.121	0.486 ± 0.107
$A\beta_{1-42}$ X tau	299.8 ± 166.4	576.2 ± 244.6	814.8 ± 293.4

Gender (F/M) female/male; CDR: clinical dementia ranking; TMSE: Taiwanese mini-mental state examination; AD: Alzheimer's disease; MCI: mild cognitive impairment.

Taipei City) and the National Taiwan University Hospital (Taipei City) in the validation cohort from 2015 to 2016. The demographic, clinical and biomarker data of the validation cohort are listed in Table 2. All study subjects and/or their principle caregivers provided written informed consent prior to participation in this investigation, and the study was approved by the ethics committees and institute review boards of the involved hospitals (NTUH: 201406125DS; ECK: 1040102; TCH: 1031203; TMU:201408011).

Preparation of human plasma

Subjects were asked to provide a 10-ml nonfasting venous blood sample (K3 EDTA, lavender-top tube). Researchers in the laboratory were blind to the status of the subject associated with each sample. The blood samples were centrifuged (1500–2500 g for 15 minutes) within 1 hour of being collected, and the plasma was aliquoted into cryotubes and stored at −20 °C.

Assays of plasma biomarkers

All human plasma samples were blindly assayed for $A\beta_{1-40}$, $A\beta_{1-42}$ and tau protein using IMR with three different kinds of reagents. Each kind of reagent is consisted of magnetic nanoparticles dispersed in a pH -7.2 phosphoryl buffer solution. These reagent nanoparticles are produced by immobilizing antibodies against $A\beta_{1-40}$ (Sigma/A3981), $A\beta_{1-42}$ (Abcam/ab34376), and tau protein (Sigma/T9450) respectively on magnetic nanoparticles. The mean diameter of antibody-functionalized magnetic nanoparticles is 50–60 nm. The magnetic concentration of each kind of reagent is 12 mg-Fe/ml. For a given human plasma sample, 80 μl of $A\beta_{1-40}$ reagent (MF-AB0–0060, MagQu) was mixed with 40 μl of room temperature human plasma, 60 μl of $A\beta_{1-42}$ reagent (MF-AB2–0060, MagQu;) was mixed with 60 μl of room temperature human plasma, and 80 μl of tau reagent (MF-TAU-0060, MagQu) was mixed with 40 μl of room temperature human plasma (Supplementary data). The reduction in the ac magnetic susceptibility, i.e., the IMR signal, of the reagent after being mixed with a sample was measured using a superconducting quantum interference devices-based ac magnetic susceptometer (XacPro-S, MagQu). The detected IMR signals were converted to the concentrations of biomarkers via logistic functions. Duplicate measurements of IMR signals were performed for each human plasma sample. The mean value of the duplicated measurements for a given biomarker in a human plasma sample was used for the statistical analysis.

Statistical analysis

Comparisons between/among groups were performed using one-way analysis of variance (ANOVA). Continuous variables are presented as mean ± SD. Continuous variables were compared using Student's tests.

Receiver operating characteristic (ROC) curve analysis was performed to identify possible useful cutoff values for the biomarker concentrations in the derivation cohort. Through the identified cutoff values, sensitivities, specificities, accuracy and area under the curve (AUC) for the selected plasma biomarkers for use in discriminating controls from patients with MCI due to AD or in differentiating between patients with MCI due to AD and patients with AD dementia were further computed.

The positive percent agreement (PPA), negative percent agreement (NPA), and overall rates of agreement (ORA) were calculated for the plasma biomarkers in the validation cohort to evaluate ability of these cutoff values of plasma biomarkers to differentiate between patients with MCI due to AD and patients with AD.

The validation cohort was enrolled to clarify for discriminating between controls and patients with MCI due to AD or those with AD dementia or for differentiating between patients with MCI due to AD and patients with AD dementia by using the cutoff values obtained from the derivation cohort.

Results

Demographic and clinical information of the two cohorts

The demographic, clinical and biomarker information of the derivation and validation cohorts are shown in Tables 1 and 2 respectively.

The distributions of gender (*Pearson chi*^{2} = 2.951, P = 0.099) and ApoE4 carrier status (*Pearson chi*^{2} = 1.987, P = 0.159) were not significantly different between the derivation and validation cohorts. In addition, the distributions of CDR in the patient group (those with MCI due to AD plus those with AD dementia) were not significantly different between the derivation and validation cohorts (*Pearson chi*^{2} = 7.242, P = 0.065). In both the derivation and validation cohorts, the CDR distribution of patients with AD dementia showed that most of the patients had either with very mild (CDR = 0.5, 34/59) or mild (CDR =1, 31/59) dementia; those two categories of patients accounted for 86.4% of all patients with dementia in the derivation cohort (Table 1) and 78.1% (CDR = 0.5, 20/73; CDR =1, 31/59) of all patients with dementia in the validation cohort (Table 2). These findings imply that the clinical severity of dementia and genetic risk loading were approximately equivalent in the derivation and

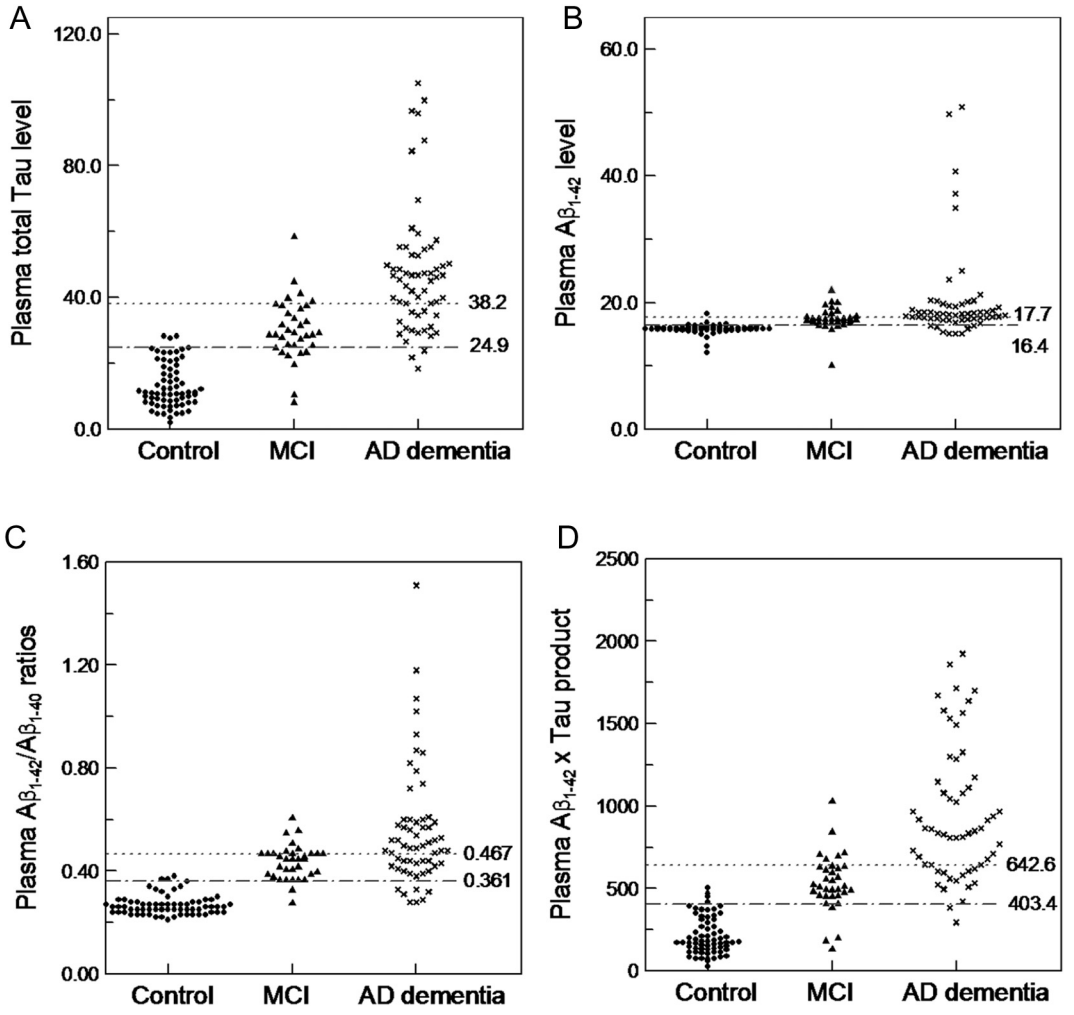


Figure 1. (A) Scattered dot plot of the plasma levels of total tau (pg/ml) in the three groups in the derivation cohort. (B) Scattered dot plot of the plasma levels of $A\beta_{1–42}$ (pg/ml) in the three groups in the derivation cohort. (C) Scattered dot plot of the $A\beta_{1–42}$/ $A\beta_{1–40}$ ratios in the three groups in the derivation cohort. (D) Scattered dot plot of the $A\beta_{1–42}$ × tau products in the three groups in the derivation cohort. The lower horizontal lines (dashed lines) indicate the cutoff values for differentiating between controls and patients (both MCI due to AD and AD dementia), and the upper horizontal lines (dotted lines) indicate the cutoff values for differentiating between patients with MCI due to AD and patients with AD dementia.

validation groups. However, we did not intend to control for or match other demographic or clinical conditions such as age, education or TMSE scores in this validation study because of the issue of generalization to the real-life population that we encounter in the clinical setting.

Plasma levels of the derivation cohort

The measured concentrations of plasma $A\beta_{1–40}$, $A\beta_{1–42}$ and total tau protein in the derivation cohort are shown in Table 1. It was found that the levels of these plasma biomarkers were distributed between 10 pg/ml and 100 pg/ml, which means that they were below the lower limit of detection of the assays currently used in clinical setting.

We found lower plasma levels of $A\beta_{1–40}$ in subjects with either MCI due to AD (40.72 ± 4.74 pg/ml) or AD dementia (43.78 ± 22.41 pg/ml) than in the controls (60.93 ± 6.50, both $P < 0.001$; Table 1). However, there was no significant difference in the plasma $A\beta_{1–40}$ levels between patients with MCI due to AD and those with AD dementia ($P > 0.05$). For the plasma levels of $A\beta_{1–42}$, the group with MCI due to AD had higher levels (17.52 ± 1.22 pg/ml) than the healthy controls (15.79 ± 0.62 pg/ml; $P < 0.001$); the AD dementia group had even higher levels (23.39 ± 18.65 pg/ml) than the MCI due to AD group ($P < 0.05$). A similar escalating trend in the plasma levels of total tau was also found, starting with low levels in the healthy control group (13.83 ± 6.50), elevated levels in the group with MCI due to AD (33.33 ± 7.77) and even higher levels in the group with AD dementia (47.47 ± 19.10). Significant differences were found for the plasma levels of total tau between the healthy control group and the group with MCI due to AD and between the group with MCI due to AD and the group with AD dementia (both $P < 0.001$).

The ratio derived from $A\beta_{1–42}$ divided by $A\beta_{1–40}$ ($A\beta_{1–42}$/ $A\beta_{1–40}$) and product of $A\beta_{1–42}$ multiplied by tau ($A\beta_{1–42}$ × tau) were calculated in the derivation cohort as additional biomarkers; these biomarkers also showed increasing trends, with the lowest

Table 3
Cutoff values, area-under-the-curve, sensitivity, specificity and accuracy of differentiating both controls from patients and patients with MCI due to AD from patients with AD dementia in the derivation cohort.

	Tau	$A\beta_{1-42}$	$A\beta_{1-42}/A\beta_{1-40}$	$A\beta_{1-42}$X tau
Normal controls versus MCI due to AD and AD dementia				
Cutoff value	24.90	16.41	0.361	403.41
AUC	0.919	0.919	0.983	0.975
Sensitivity	0.892	0.882	0.903	0.935
Specificity	0.955	0.925	0.955	0.940
Accuracy	0.961	0.919	0.925	0.938
MCI due to AD versus AD dementia				
Cutoff value	38.18	17.68	0.467	642.58
AUC	0.824	0.670	0.688	0.849
Sensitivity	0.712	0.661	0.627	0.763
Specificity	0.853	0.676	0.676	0.824
Accuracy	0.793	0.677	0.624	0.785

AUC: area-under-the-curve.

values in the controls, intermediate values in those with MCI due to AD, and the highest values in those with AD dementia (Table 1). Both ratio and product of biomarkers had significant between-group differences (both $P < 0.001$) among the three clinical groups (all post hoc tests showed $P < 0.01$). The dot plots of the biomarkers and ratio and product of biomarkers in the derivation cohort are shown in Figure 1, *A–D*.

ROC curve analyses using the cutoff points in the validation group

The cutoff values of plasma biomarkers or their ratio and product of biomarkers for differentiating between controls and patients (MCI due to AD and AD dementia) and between patients with MCI due to AD and patients with AD dementia were identified through the ROC curve analyses and are displayed in Table 3. The cutoff values for $A\beta_{1-40}$ are not available since the AUCs were rather low, 0.088 for differentiating between controls and patients and 0.555 for differentiating between patients with MCI due to AD and patients with AD dementia. The cutoff values for the plasma levels of total tau and $A\beta_{1-42}$ and the ratio of, $A\beta_{1-42}/A\beta_{1-40}$ and product of $A\beta_{1-42}$ × tau, are shown by the two horizontal lines on the dot plots (Figure 1, *A–D*). The lower horizontal lines indicate the cutoff values for differentiating between controls and patients (both MCI due to AD and AD dementia), and the upper horizontal lines indicate the cutoff values for differentiating between patients with MCI due to AD and patients with AD dementia.

As previously mentioned, with the exception of $A\beta_{1-40}$, all cutoff values of biomarkers or ratio and product of biomarkers performed very well in differentiating between controls and patients (both MCI due to AD and AD dementia) with AUCs, sensitivities, specificities and accuracies all close to or above 0.9 (Table 3, upper panel; Figure 2, *A*). On the other hand, only the cutoff values for the plasma level of total tau and the product of $A\beta_{1-42}$ × tau performed adequately for differentiating between patients with MCI due to AD and patients with AD dementia (Table 3, lower section; Figure 2, *B*).

Plasma levels of the validation group

The measured plasma levels of $A\beta_{1-40}$, $A\beta_{1-42}$ and total tau and the $A\beta_{1-42}/A\beta_{1-40}$ ratio and the product of $A\beta_{1-42}$ × tau, of the validation cohort are shown in Table 2 (Figure 3, *A–D*). As in the derivation cohort, the plasma levels of $A\beta_{1-40}$ show a decreasing trend from the highest levels in the healthy controls, to intermediate levels in patients with MCI due to AD, and finally to the lowest levels in patients with AD dementia, while the plasma levels of $A\beta_{1-42}$ and total tau demonstrated the opposite trend. Both $A\beta_{1-42}/A\beta_{1-40}$ ratio and the product of $A\beta_{1-42}$ × tau, showed a similar increasing trend as that of the plasma level of $A\beta_{1-40}$. These data were used to validate the cutoff values of the plasma biomarkers obtained from the derivation cohort.

Performance of biomarkers

The cutoff value of $A\beta_{1-42}$ performed the best at differentiating between controls and patients with MCI due to AD and patients with AD dementia in the validation cohort, with the PPA, NPA and ORA all equal to 92.5% (Table 4). The performance of $A\beta_{1-42}$ was better than those of ratio and product of biomarkers and total tau, although the other three cutoff values still had ORAs ranging from 85% to 88.8%. On the other hand, only the cutoff value of $A\beta_{1-42}$ × tau could differentiate between patients with MCI due to AD and patients with AD dementia, with 84.9% PPA, 78.8% NPA and 83% ORA.

The manufacturer also provides their in-house reference values for the IMR system, which uses both ratio and product of biomarkers (0.325 for $A\beta_{1-42}/A\beta_{1-40}$ and 455.5 for $A\beta_{1-42}$ × tau) for differentiating between controls and patients with MCI due to AD/patients with AD dementia. The control group was reported to be more than 400 persons with ages as young as 20 years. The NPA was 86.2%, the PPA was 84.3% and the ORA was 92.1% for the cutoff value of 0.325 for $A\beta_{1-42}/A\beta_{1-40}$. For $A\beta_{1-42}$ × tau, the cutoff value of 455.5 provided 84.8% NPA, 91% PPA and 93.9% ORA. In summary, these are approximately the same as the values obtained when evaluating the performance of the cutoff values of both ratio and product of biomarkers determined from the derivation cohort in this study.

Discussion

In this study, we found that the plasma levels of $A\beta_{1-42}$ and tau increased from the lowest levels in the healthy controls to intermediate levels in the patients with MCI due to AD and then to the highest levels in patients with AD dementia.

The plasma levels of total tau and p-tau 181 were found to be higher in patients with AD dementia than the cognitively unimpaired controls; in previous studies, these levels were shown to be associated with both Aβ and tau PET.[24,32] In contrast, another previous study reported that compared to the levels in healthy controls, the plasma level of CSF $A\beta_{1-42}$ was lower in patients with AD dementia when assayed using the ultrasensitive digital ELISA methodology (SIMOA assay)[17] and the ratio of $A\beta_{1-42}/A\beta_{1-40}$ was also lower in patients with AD dementia.[33] However, in our previous studies, we found that the

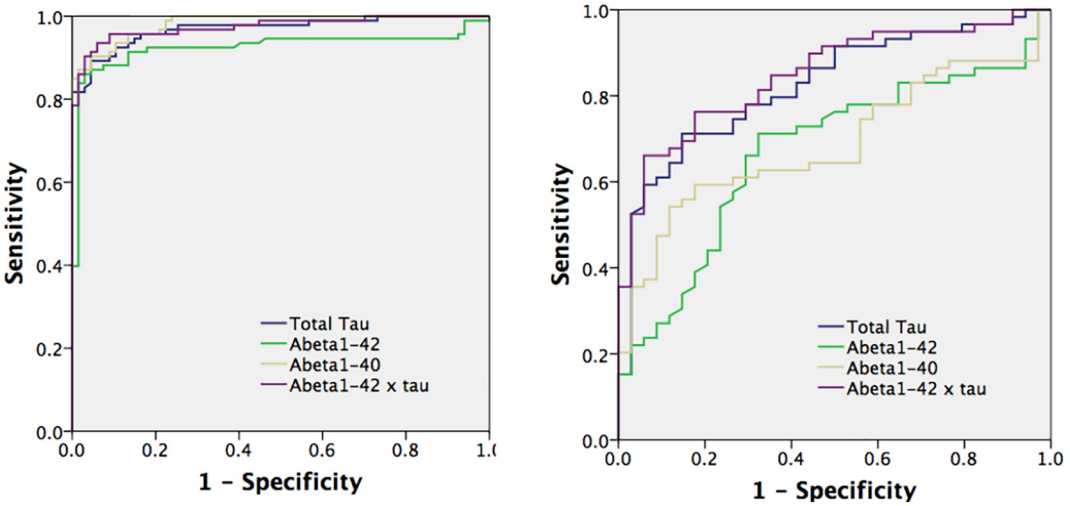


Figure 2. (A) ROC curves of the two biomarkers, the plasma levels of $A\beta_{1-42}$ and tau, and the two surrogate biomarkers, the $A\beta_{1-42}/A\beta_{1-40}$ ratio and the product of $A\beta_{1-42}$ × tau, computed from the derivation cohort for differentiating between controls and patients with MCI due to AD/AD dementia. (B) ROC curves of the two biomarkers, the plasma levels of $A\beta_{1-42}$ and tau, and the two surrogate biomarkers, the $A\beta_{1-42}$/ $A\beta_{1-40}$ ratio and the product of $A\beta_{1-42}$ × tau, computed from the derivation cohort for differentiating between patients with MCI due to AD and patients with AD dementia.

plasma levels of both $A\beta_{1-42}$ and total tau were elevated in patients with MCI due to AD and those with AD dementia compared to healthy controls.[19,21] These findings were further confirmed by a recent report that demonstrated that the plasma $A\beta_{42}$ levels are negatively correlated with the CSF $A\beta_{1-42}$ levels in patients with AD,[34] while the CSF $A\beta_{1-42}$ levels are lower in patient with AD dementia than in healthy controls, which is considered nearly a consensus diagnostic biomarker of AD.[35] There may be several reasons for the elevated plasma levels of $A\beta_{1-42}$ such as the different designs of technological platforms used; possible detection of oligomerized $A\beta_{1-42}$, as suggested in a recent report[36]; and finally, the huge quantity of carrier proteins, e.g., albumin, in the plasma.[34]

Several studies on CSF biomarkers showed that the combined use of $A\beta_{1-40}$, $A\beta_{1-42}$ and tau protein instead of the use of an individual biomarker could increase the sensitivity and specificity of the clinical diagnosis of AD.[37–39] Our previous work also revealed that using either $A\beta_{1-42}/A\beta_{1-40}$ ratio or the $A\beta_{1-42}$ × tau product enhanced differential diagnosis among healthy controls, patients with MCI due to AD, and patients with AD dementia.[21] However, in this validation study there was no need to use ratio or product of biomarkers to differentiating between the controls and the patients with MCI due to AD or between the controls and patients with AD dementia. The use of the cutoff value for the plasma level of $A\beta_{1-42}$ alone provided an overall rate of agreement greater than 90% (92.5%) and high levels of positive and negative percent agreement (both 92.5%).

However, the determination of AD severity is very important in clinical practice because there are different clinical responses to various disease modifying therapies and biological follow-up of disease progression at various stages of AD. Therefore, we propose using a combination of plasma amyloid and tau protein levels by computing the product of $A\beta_{1-42}$ × tau $(pg/ml)^2$, which in this study, provided a reasonable clinical ability to differentiate between patients with MCI due to AD and those with AD dementia, with an overall rate of agreement of 83%, 84.9% positive percent agreement and 78.8% negative percent agreement. The product of $A\beta_{1-42}$ × tau outperformed the cutoff values of any single biomarker such as the plasma levels of total tau (57.5% overall rate of agreement) and $A\beta_{1-42}$ (68.9% overall rate of agreement).

As previously mentioned, amyloid plaques and neurofibrillary tangles are the pathological hallmarks of AD.[3–6] This implies that both plasma amyloid and tau protein should be taken into account when diagnosing AD and evaluating the severity of dementia due to AD.

There is a very interesting temporal agreement among the plasma levels of the biomarkers and the product of $A\beta_{1-42}$ × tau and the findings from amyloid and tau PET. In the model of biomarkers in the AD cascade, amyloid pathology (CSF amyloid or amyloid PET) appears first.[26] This was later supplemented by a study on dominantly inherited Alzheimer's disease that demonstrated that amyloid imaging precedes clinical symptoms of dementia by a period of 15 years.[40] However, the Aβ accumulation reaches its plateau early in the prodromal stage of AD.[41] On the other hand, tau lesions continuously spread from the hippocampus to the limbic system and to the entire neocortex as the disease progresses from normal aging to advanced AD.[42] In addition to the transynaptic spreading of tau,[43] the accumulation of Aβ may precipitate tau accumulation and aggravate neuronal degeneration.[44] Therefore, tau PET images can be more useful than amyloid PET data as objective indices for the severity and progression of the disease.[44] Our plasma biomarker study agreed with the aforementioned cascade in terms of amyloid and tau pathology. Thus, the $A\beta_{1-42}$ cutoff value alone is sensitive and specific enough to diagnose early stage (prodromal) AD and to differentiate between controls and patients. The product of $A\beta_{1-42}$ and tau, an indicator biomarker

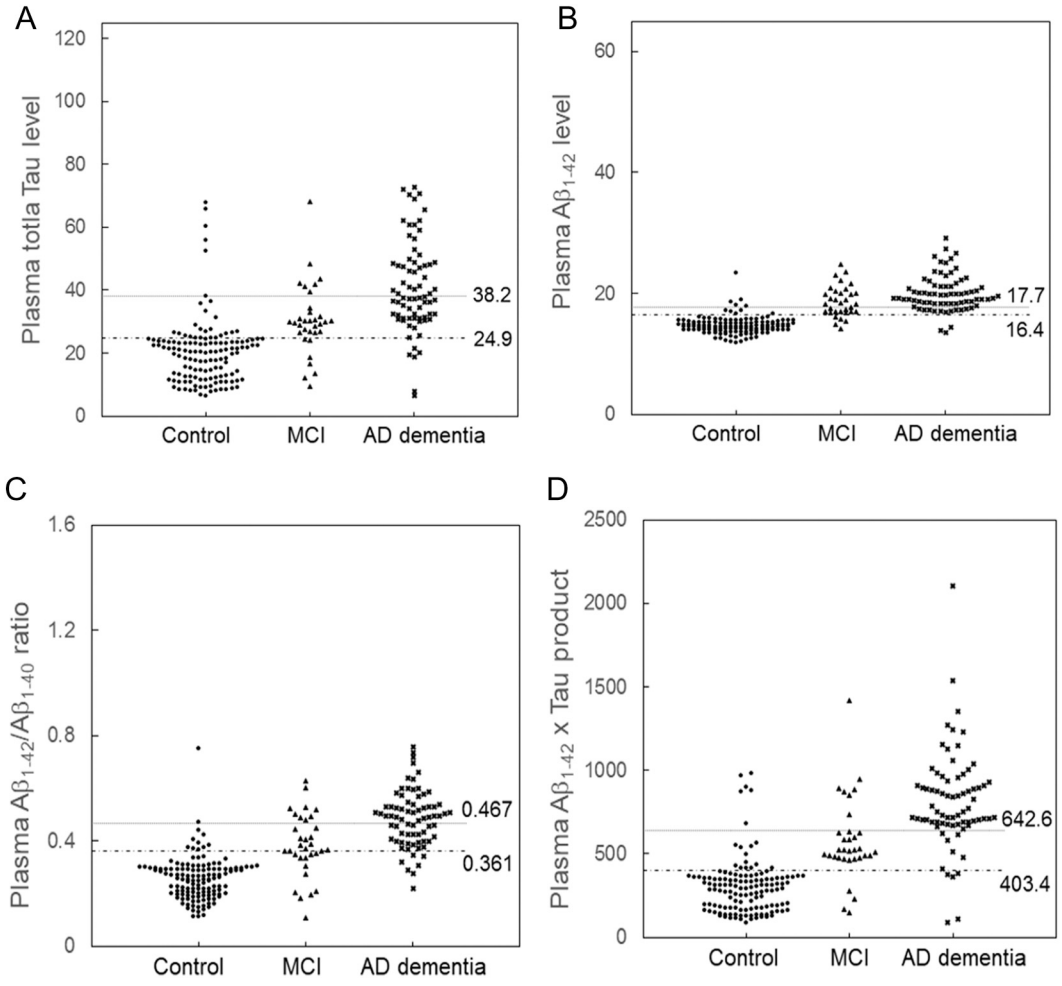


Figure 3. (A) Scattered dot plot of the plasma levels of total tau (pg/ml) in the three groups in the validation cohort. (B) Scattered dot plot of the plasma levels of $A\beta_{1–42}$ (pg/ml) in the three groups in the validation cohort. (C) Scattered dot plot of the $A\beta_{1–42}$/ $A\beta_{1–40}$ ratios in the three groups in the validation cohort. (D) Scattered dot plot of the $A\beta_{1–42}$ × tau products in the three groups in the validation cohort. The lower horizontal lines (dashed lines) indicate the cutoff values for differentiating between controls and patients (both MCI due to AD and AD dementia), and the upper horizontal lines (dotted lines) indicate the cutoff values for differentiating between patients with MCI due to AD and patients with AD dementia.

for the severity and progression of AD, is required to differentiate between patients with prodromal AD and those with AD dementia.

There were several limitations of the present study. First, we segregated the clinical groups on a clinical basis although we also used neuroimages to supplement the diagnosis. Second, basically this is a cross-sectional observation study. We do not know the relationship of future cognitive decline and the plasma levels of biomarkers. We will keep following up the participants of their clinical and cognitive status as well as the biomarker changes to fulfill validation on a temporal term.

There are strengths for the use of IMR in the clinical setting. Technically, the limits of detection for plasma $A\beta_{1–40}$, $A\beta_{1–42}$ and total tau protein are at or below the level of 1 pg/ml. The upper limit of the analytical measurement range for assaying these biomarkers is 1000–3000 pg/ml. The imprecision of assaying these biomarkers is less than 15%. Meanwhile, studies of the interference attributable to naturally existing biomaterials such as uric acid, rheumatoid factor or albumin; common diseases or clinical conditions such as hemolysis, jaundice or hypertriglyceridemia; and frequently used drugs or chemicals to treat inflammatory diseases, viral and bacterial infections, cancers or cardiovascular diseases, have shown the high specificity of assays of these biomarkers. These results demonstrate that IMR is able to precisely detect plasma $A\beta_{1–40}$, $A\beta_{1–42}$ and tau protein. In terms of adherence to regulations, the production of IMR reagents for $A\beta_{1–40}$, $A\beta_{1–42}$ and tau protein and the analyzer itself follow international quality management guidelines for in vitro diagnostics (IVD) medical devices, i.e., ISO13485 and Good Manufacture Practice. Moreover, the reagents and the analyzer have been certificated by CE (Conformité Européene) IVD. This means that the batch-to-batch variations in the qualities of reagents and analyzers can be well controlled. A high degree of reliability of the IMR reagents and the analyzer is therefore assured.

In conclusion, two independent cohorts were enrolled in Taiwan. All participants were administered neuropsychological tests and had plasma samples collected for an IMR assay of

Table 4
Positive, negative percent agreements and overall rates of agreement differentiating between MCI due to AD and AD dementia in the validation cohort by applying cutoff values obtained in the derivation cohort.

	Tau	$A\beta_{1-42}$	$A\beta_{1-42}/A\beta_{1-40}$	$A\beta_{1-42}$X tau
Normal controls versus MCI due to AD and AD dementia				
Positive percent agreement	87.7	92.5	79.2	91.5
Negative percent agreement	82.8	92.5	92.5	86.6
Overall rates of agreement	85.0	92.5	86.7	88.8
MCI due to AD versus AD dementia				
Positive percent agreement	78.8	80.8	64.4	84.9
Negative percent agreement	47.9	42.4	72.7	78.8
Overall rates of agreement	57.5	68.9	67.0	83.0

$A\beta_{1-40}$, $A\beta_{1-42}$, and tau protein. Each cohort included healthy subjects, patients with MCI due to AD, and patients with AD dementia. The cutoff values for the plasma biomarkers obtained in the derivational cohort were verified in the validation cohort. It was found that the cutoff value for the plasma level of $A\beta_{1-42}$ alone is capable of differentiating between controls and patients (both prodromal and clinical) with AD. The product of $A\beta_{1-42}$ and tau protein was able to distinguish between patients with MCI due to AD and those with AD dementia, indicating an indicator biomarker can differentiate among patients with different disease severities and levels of progression, which implies a potential application in monitoring therapeutic responses of disease modifying therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2020.102182.

References

1. Lista S, Faltraco F, Prvulovic D, Hampel H. Blood and plasma-based proteomic biomarker research in Alzheimer's disease. *Prog Neurobiol* 2013;**101-102**:1-17.
2. O'Bryant SE, Mielke MM, Rissman RA, Lista S, Vanderstichele H, Zetterberg H, et al. Blood-based biomarkers in Alzheimer disease: current state of the science and a novel collaborative paradigm for advancing from discovery to clinic. *Alzheimers Dement* 2017;**13**:45-58.
3. Brier MR, Gordon B, Friedrichsen K, McCarthy J, Stern A, Christensen J, et al. Tau and Abeta imaging, CSF measures, and cognition in Alzheimer's disease. *Science translational medicine* 2016;**8**338ra66.
4. O'Brien JT, Herholz K. Amyloid imaging for dementia in clinical practice. *BMC Med* 2015;**13**:163.
5. Okamura N, Harada R, Furumoto S, Arai H, Yanai K, Kudo Y. Tau PET imaging in Alzheimer's disease. *Curr Neurol Neurosci Rep* 2014;**14**:500.
6. Johnson KA, Minoshima S, Bohnen NI, Donohoe KJ, Foster NL, Herscovitch P, et al. Appropriate use criteria for amyloid PET: a report of the Amyloid Imaging Task Force, the Society of Nuclear Medicine and Molecular Imaging, and the Alzheimer's Association. *Alzheimer's & dementia : the journal of the Alzheimer's Association* 2013;**9**:1-16 e.
7. Bacioglu M, Maia LF, Preische O, Schelle J, Apel A, Kaeser SA, et al. Neurofilament light chain in blood and CSF as marker of disease progression in mouse models and in neurodegenerative diseases. *Neuron* 2016;**91**:494-6.
8. Gupta VB, Doecke JD, Hone E, Pedrini S, Laws SM, Thambisetty M, et al. Plasma apolipoprotein J as a potential biomarker for Alzheimer's disease: Australian imaging, biomarkers and lifestyle study of aging. *Alzheimers Dement* 2016;**3**:18-26.
9. Weinstein G, Beiser AS, Preis SR, Courchesne P, Chouraki V, Levy D, et al. Plasma clusterin levels and risk of dementia, Alzheimer's disease, and stroke. *Alzheimers Dement* 2016;**3**:103-9.
10. Xia W, Yang T, Shankar G, Smith IM, Shen Y, Walsh DM, et al. A specific enzyme-linked immunosorbent assay for measuring beta-amyloid protein oligomers in human plasma and brain tissue of patients with Alzheimer disease. *Arch Neurol* 2009;**66**:190-9.
11. Oh ES, Mielke MM, Rosenberg PB, Jain A, Fedarko NS, Lyketsos CG, et al. Comparison of conventional ELISA with electrochemiluminescence technology for detection of amyloid-beta in plasma. *Journal of Alzheimer's disease : JAD* 2010;**21**:769-73.
12. Kvartsberg H, Portelius E, Andreasson U, Brinkmalm G, Hellwig K, Lelental N, et al. Characterization of the postsynaptic protein neurogranin in paired cerebrospinal fluid and plasma samples from Alzheimer's disease patients and healthy controls. *Alzheimer's research & therapy* 2015;**7**:40.
13. Birkmann E, Henke F, Weinmann N, Dumpitak C, Groschup M, Funke A, et al. Counting of single prion particles bound to a capture-antibody surface (surface-FIDA). *Vet Microbiol* 2007;**123**:294-304.
14. Oliver KG, Kettman JR, Fulton RJ. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin Chem* 1998;**44**:2057-60.
15. Olsson A, Vanderstichele H, Andreasen N, De Meyer G, Wallin A, Holmberg B, et al. Simultaneous measurement of beta-amyloid(1-42), total tau, and phosphorylated tau (Thr181) in cerebrospinal fluid by the xMAP technology. *Clin Chem* 2005;**51**:336-45.
16. Kim JS, Ahn HS, Cho SM, Lee JE, Kim Y, Lee C. Detection and quantification of plasma amyloid-beta by selected reaction monitoring mass spectrometry. *Anal Chim Acta* 2014;**840**:1-9.
17. Janelidze S, Stomrud E, Palmqvist S, Zetterberg H, van Westen D, Jeromin A, et al. Plasma beta-amyloid in Alzheimer's disease and vascular disease. *Sci Rep* 2016;**6**:26801.
18. Mondello S, Buki A, Barzo P, Randall J, Provuncher G, Hanlon D, et al. CSF and plasma amyloid-beta temporal profiles and relationships with neurological status and mortality after severe traumatic brain injury. *Sci Rep* 2014;**4**:6446.
19. Chiu MJ, Yang SY, Chen TF, Chieh JJ, Huang TZ, Yip PK, et al. New assay for old markers-plasma beta amyloid of mild cognitive impairment and Alzheimer's disease. *Curr Alzheimer Res* 2012;**9**:1142-8.
20. Yang CC, Yang SY, Chieh JJ, Horng HE, Hong CY, Yang HC, et al. Biofunctionalized magnetic nanoparticles for specifically detecting biomarkers of Alzheimer's disease in vitro. *ACS Chem Neurosci* 2011;**2**:500-5.
21. Chiu MJ, Yang SY, Horng HE, Yang CC, Chen TF, Chieh JJ, et al. Combined plasma biomarkers for diagnosing mild cognition impairment and Alzheimer's disease. *ACS Chem Neurosci* 2013;**4**:1530-6.
22. Ruiz A, Pesini P, Espinosa A, Perez-Grijalba V, Valero S, Sotolongo-Grau O, et al. Blood amyloid beta levels in healthy, mild cognitive impairment and Alzheimer's disease individuals: replication of diastolic blood pressure correlations and analysis of critical covariates. *PloS one* 2013;**8**e81334.
23. Zetterberg H, Wilson D, Andreasson U, Minthon L, Blennow K, Randall J, et al. Plasma tau levels in Alzheimer's disease. *Alzheimer's research & therapy* 2013;**5**:9.
24. Mattsson N, Zetterberg H, Janelidze S, Insel PS, Andreasson U, Stomrud E, et al. Plasma tau in Alzheimer disease. *Neurology* 2016;**87**:1827-35.
25. Lui JK, Laws SM, Li QX, Villemagne VL, Ames D, Brown B, et al. Plasma amyloid-beta as a biomarker in Alzheimer's disease: the AIBL study of aging. *Journal of Alzheimer's disease : JAD* 2010;**20**:1233-42.

26. Sperling RA, Aisen PS, Beckett LA, Bennett DA, Craft S, Fagan AM, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 2011;**7**:280-92.
27. McKhann GM, Knopman DS, Chertkow H, Hyman BT, Jack Jr CR, Kawas CH, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 2011;**7**:263-9.
28. Jack Jr CR, Albert MS, Knopman DS, McKhann GM, Sperling RA, Carrillo MC, et al. Introduction to the recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 2011;**7**:257-62.
29. Lezak MD, Howieson DB, Loring DW. *Neuropsychological assessment (4th ed.)*. New York: Oxford University Press; 2004.
30. Chiu MJ, Chen YF, Chen TF, Yang SY, Yang FP, Tseng TW, et al. Plasma tau as a window to the brain-negative associations with brain volume and memory function in mild cognitive impairment and early Alzheimer's disease. *Hum Brain Mapp* 2014;**35**:3132-42.
31. Fan LY, Tzen KY, Chen YF, Chen TF, Lai YM, Yen RF, et al. The relation between brain amyloid deposition, cortical atrophy, and plasma biomarkers in amnesic mild cognitive impairment and Alzheimer's disease. *Front Aging Neurosci* 2018;**10**:175.
32. Mielke MM, Hagen CE, Xu J, Chai X, Vemuri P, Lowe VJ, et al. Plasma phospho-tau181 increases with Alzheimer's disease clinical severity and is associated with tau- and amyloid-positron emission tomography. *Alzheimers Dement* 2018;**12**:989-97.
33. Vogelgsang J, Shahpasand-Kroner H, Vogelgsang R, Streit F, Vukovich R, Wiltfang J. Multiplex immunoassay measurement of amyloid-beta42 to amyloid-beta40 ratio in plasma discriminates between dementia due to Alzheimer's disease and dementia not due to Alzheimer's disease. *Exp Brain Res* 2018;**236**:1241-50.
34. Teunissen CE, Chiu MJ, Yang CC, Yang SY, Scheltens P, Zetterberg H, et al. Plasma amyloid-beta (Abeta42) correlates with cerebrospinal fluid Abeta42 in Alzheimer's disease. *J Alzheimers Dis* 2018;**62**:1857-63.
35. Molinuevo JL, Blennow K, Dubois B, Engelborghs S, Lewczuk P, Perret-Liaudet A, et al. The clinical use of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's biomarkers standardization initiative. *Alzheimers Dement* 2014;**10**:808-17.
36. Wang MJ, Yi S, Han JY, Park SY, Jang JW, Chun IK, et al. Oligomeric forms of amyloid-beta protein in plasma as a potential blood-based biomarker for Alzheimer's disease. *Alzheimer's research & therapy* 2017;**9**:98.
37. Hulstaert F, Blennow K, Ivanoiu A, Schoonderwaldt HC, Riemenschneider M, De Deyn PP, et al. Improved discrimination of AD patients using beta-amyloid(1-42) and tau levels in CSF. *Neurology* 1999;**52**:1555-62.
38. Fagan AM, Roe CM, Xiong C, Mintun MA, Morris JC, Holtzman DM. Cerebrospinal fluid tau/beta-amyloid(42) ratio as a prediction of cognitive decline in nondemented older adults. *Arch Neurol* 2007;**64**:343-9.
39. Spies PE, Slats D, Sjogren JM, Kremer BP, Verhey FR, Rikkert MG, et al. The cerebrospinal fluid amyloid beta42/40 ratio in the differentiation of Alzheimer's disease from non-Alzheimer's dementia. *Curr Alzheimer Res* 2010;**7**:470-6.
40. Bateman RJ, Xiong C, Benzinger TL, Fagan AM, Goate A, Fox NC, et al. Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *N Engl J Med* 2012;**367**:795-804.
41. Jack Jr CR, Wiste HJ, Lesnick TG, Weigand SD, Knopman DS, Vemuri P, et al. Brain beta-amyloid load approaches a plateau. *Neurology* 2013;**80**:890-6.
42. Hoenig MC, Bischof GN, Seemiller J, Hammes J, Kukolja J, Onur OA, et al. Networks of tau distribution in Alzheimer's disease. *Brain : a journal of neurology* 2018;**141**:568-81.
43. DeVos SL, Corjuc BT, Oakley DH, Nobuhara CK, Bannon RN, Chase A, et al. Synaptic tau seeding precedes tau pathology in human Alzheimer's disease brain. *Front Neurosci* 2018;**12**:267.
44. Sperling R, Mormino E, Johnson K. The evolution of preclinical Alzheimer's disease: implications for prevention trials. *Neuron* 2014;**84**:608-22.