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Combined Plasma Biomarkers for Diagnosing Mild Cognition Impairment and Alzheimer's Disease

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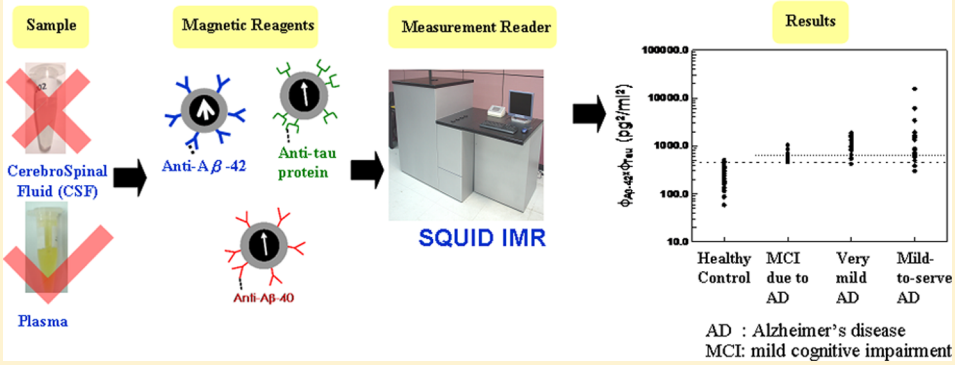

ABSTRACT: A highly sensitive immunoassay, the immunomagnetic reduction, is used to measure several biomarkers for plasma that is related to Alzheimer's disease (AD). These biomarkers include Aβ-40, Aβ-42, and tau proteins. The samples are composed of four groups: healthy controls (n = 66), mild cognitive impairment (MCI, n = 22), very mild dementia (n = 23), and mild-to-serve dementia, all due to AD (n = 22). It is found that the concentrations of both Aβ-42 and tau protein for the healthy controls are significantly lower than those of all of the other groups. The sensitivity and the specificity of plasma Aβ-42 and tau protein in differentiating MCI from AD are all around 0.9 (0.88−0.97). However, neither plasma Aβ-42 nor tau-protein concentration is an adequate parameter to distinguish MCI from AD. A parameter is proposed, which is the product of plasma Aβ-42 and tau-protein levels, to differentiate MCI from AD. The sensitivity and specificity are found to be 0.80 and 0.82, respectively. It is concluded that the use of combined plasma biomarkers not only allows the differentiation of the healthy controls and patients with AD in both the prodromal phase and the dementia phase, but it also allows AD in the prodromal phase to be distinguished from that in the dementia phase.

KEYWORDS: *Immunomagnetic reduction, plasma, biomarkers, mild cognition impairment, Alzheimer's disease*

Because of the rapid aging of the global population, neurodegenerative diseases have become a serious problem. Dementia is the most prevalent neurodegenerative disease. Its prevalence in those aged 60 years or more varies from 5% to 7% in most world regions, and it is estimated that 35.6 million people lived with dementia worldwide in 2010.[1] The World Health Organization has urged that all governments, policy-makers, and other stakeholders address the impact of dementia as an increasing threat and allocate all necessary resources to ready the health and social care system for the imminent increased occurrence of dementia.[2]

Patients with Alzheimer's disease (AD) comprise 50%−70% of the elderly population with dementia. The two cornerstones for the diagnosis of Alzheimer's disease are neuroimaging[3−5]

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and neurocognitive tests.[6−8] Although neurocognitive tests are more widespread, the results depend not only on the degree of neurodegeneration, but also on factors such as education, culture, and social economic level, so the interpretation of the neurocognitive tests requires care and should not be the only information that is used to reach a final diagnosis of Alzheimer's disease. Neuroimaging provides structural or functional data for a more objective diagnosis of Alzheimer's disease. For example, by using magnetic resonance imaging, hippocampal atrophy can be identified qualitatively (visual rating) or quantitatively (volumetrically), and amyloid or tau positron emission tomography (PET) shows amyloid plaques and neurofibrillary tangles in the brains of the patients that suffer from Alzheimer's disease. However, neuroimaging has a relatively high cost and limited availability, especially among general practitioners and local hospitals. These shortcomings motivate the development of other realistic technologies for the diagnosis of Alzheimer's disease.

Molecular diagnosis is a popular method for the in vitro diagnosis of Alzheimer's disease. The potential biomarkers include amyloids, tau protein, and their derivates.[9−13] Most of these biomarkers are in the cerebrospinal fluid (CSF). Lumbar puncture is necessary for the collection of CSF samples. However, the CSF sampling process is relatively risky and uncomfortable, so it is not suitable for screening on a large-scale or for repetitive sampling in the long term monitoring of disease progression or the effect of therapy. Therefore, biomarkers in types of body fluid other than CSF are necessary. One of the most promising body fluids is blood, which is the most reliable, convenient, and familiar clinical sample. However, the concentrations of biomarkers in blood are very low, in pg/mL. Ultrahigh-sensitivity assay technologies are needed for the detection of these ultralow concentrations of biomarkers.

An ultrahigh-sensitivity technology was developed for immunoassay in 2008.[14] This technology is referred to as a superconducting quantum interference device (SQUID) immunomagnetic reduction (IMR) assays.[14−16] The low-detection limit for amyloids and tau protein is found to be 1−10 pg/mL, using the SQUID based IMR,[17,18] which makes possible the measurement of plasma biomarkers for the diagnosis of Alzheimer's disease. In this work, the characterizations of SQUID IMR for assaying biomarkers in human plasma are explored.

In the prodromal stage of AD, patients usually suffer mild cognition impairment (MCI). The annual conversion rate of MCI to AD is around 10%,[19] and within 3 years, around 30%−50% of these develop dementia.[20] In a subgroup with cerebral amyloid positive MCI, the 3-year accumulated conversion rate increases to 82%. [21] In order to allow preventive intervention for AD dementia, MCI due to AD must be diagnosed using biomarker assays, as early as possible. This study explores the diagnostic parameters that are capable of distinguishing between healthy controls, MCI due to AD, and AD dementia according, using the results of plasma amyloid and tau-protein assays obtained using SQUID IMR.

RESULTS AND DISCUSSION

The concentration-dependent IMR signals, that is, IMR (%)−ϕ curves or characteristic curves, for Aβ-40, Aβ-42, and tau protein spiked in PBS are shown, with data points, in Figure 1. For a given biomarker, the data points can be well fitted with the logistic function

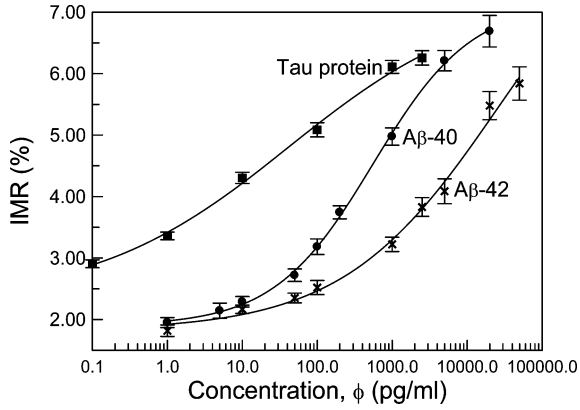

Figure 1. Concentration dependent IMR signals for Aβ-40 (●), Aβ-42 (×), and tau protein (■) with the error-bar from a triplicate measurement.

$$\text{IMR}\,(\%) = \frac{A - B}{1 + \left(\frac{\phi}{\phi_o}\right)^{\gamma}} + B \tag{1}$$

where A, B, ϕ_o and γ are fitting parameters. By fitting the data points to eq 1, these fitting parameters are determined, as tabulated in Table 3 for Aβ-40, Aβ-42, and tau protein. The fitting curves are plotted using solid lines in Figure 1. It is clear that the low-detection limits for assaying Aβ-40, Aβ-42, and tau protein are in pg/mL.

Table 1. Volumes of the Reagents and Plasma Used for the Detection of IMR Signals

biomarker	volume of reagent (μL)	volume of plasma (μL)
Aβ-40	80	40
Aβ-42	60	60
tau protein	80	40

Table 2. Demographic Characteristics of the Subjects[a]

	group		
	HC	MCI	ADD
numbers	66	22	45
female/male	32/34	11/11	23/22
age (years)	23−81	55−95	53−89

[a]HC, healthy controls; MCI, mild cognitive impairment due to Alzheimer's disease; ADD, Alzheimer's disease dementia, including those with very mild to severe (CDR = 0.5−3) dementia.

Table 3. Fitting Parameters in eq 1 for Aβ-40, Aβ-42, and Tau Protein

	Fitting parameter			
biomarker	A	B	ϕ_o	γ
Aβ-40	1.89	7.39	567.3	0.65
Aβ-42	1.91	8.09	14157.7	0.49
tau protein	2.28	7.34	39.03	0.33

Three concentration levels of Aβ-40, Aβ-42 and tau protein are used to test the coefficient variance (CV) of SQUID IMR method. The concentrations used for Aβ-40 and Aβ-42 are 1, 100, and 5000 pg/mL, and the concentrations used for tau protein are 0.1, 10, and 1000 pg/mL. The detected IMR signals are shown in Figure 2. The second run for the detection of IMR

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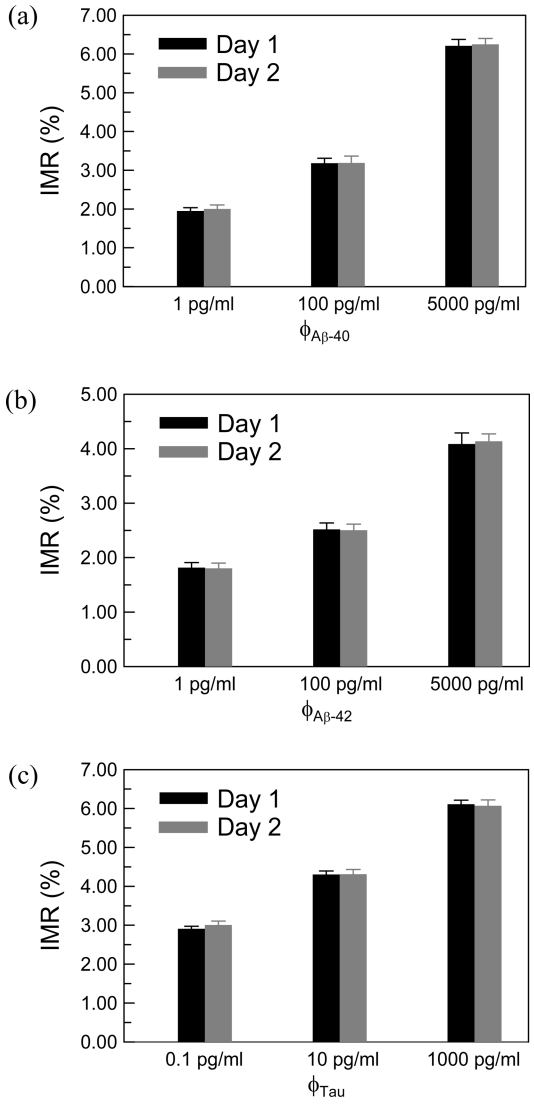

Figure 2. Variance tests for the assay of (a) Aβ-40, (b) Aβ-42, and (c) tau protein, using SQUID IMR; day 2 is 7 days later than day 1.

signals (labeled Day 2 in Figure 2) was performed 7 days after the first run (labeled day 1 in Figure 2). The results in Figure 2 show that both the interassay and intra-assay CV's are less than 8%. Therefore the reliability of the SQUID IMR method is satisfactory.

The Aβ-40 concentrations $\phi_{A\beta\text{-}40}$ for human plasma were measured using SQUID IMR. The results are shown in Figure 3. It is found that there is no significant difference in $\phi_{A\beta\text{-}40}$ between healthy controls, MCI due to AD, very mild AD dementia, and mild to severe AD dementia groups. This result shows that Aβ-40 in plasma is not a useful biomarker for the diagnosis of MCI or AD. This is also true for the concentrations of Aβ-40 for the CSF samples from the patients with AD dementia.

For Aβ-42, the detected concentrations, $\phi_{A\beta\text{-}42}$, in plasma are shown in Figure 4a. The concentrations of Aβ-42 for the healthy controls are relatively low, compared with those for patients with MCI due to AD and AD dementia groups. This observation is in contradiction with the results for the reduction in Aβ-42 concentration reported in CSF samples, which is usually measured using enzyme-linked immunosorbent assay (ELISA).[22−24] The reasons for this contradiction could be a

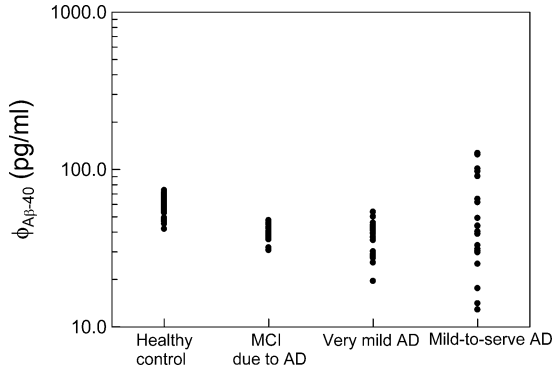

Figure 3. Concentrations of Aβ-40 in plasma from different clinical groups, detected using immunomagnetic reduction with the aid of Aβ-40 reagent.

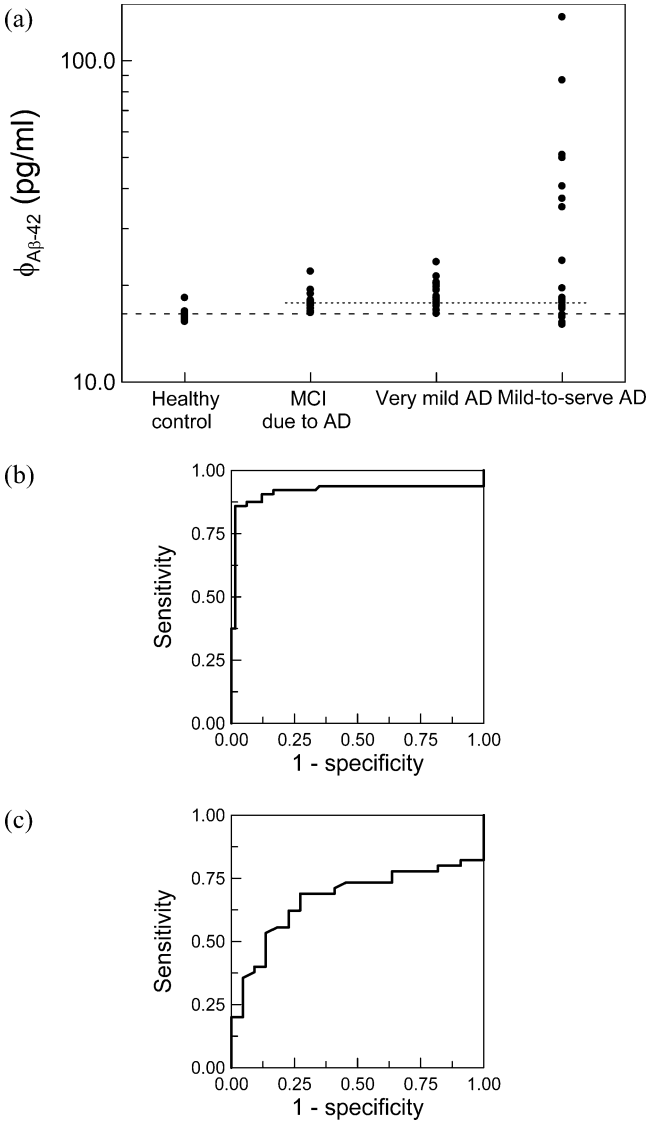

Figure 4. (a) Concentrations of Aβ-42 in plasma from different clinical groups, detected using immunomagnetic reduction with the aid of Aβ-42 reagent, and the ROC curves to distinguish (b) the healthy control group from the patient group (combining MCI due to AD, very mild AD, and mild-to-severe AD dementia) and to distinguish (c) the MCI due to AD and the AD dementia groups.

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Table 4. Thresholds, Sensitivity and Specificity for Differentiation of Healthy Controls, MCI due to AD, and AD Dementia, for Various Parameters[a]

parameter	groups	threshold	sensitivity	specificity
$\phi_{A\beta\text{-}42}$	HC vs Patients	16.33 pg/mL	0.91	0.88
	MCI vs ADD	17.65 pg/mL	0.69	0.68
ϕ_{tau}	HC vs Patients	23.89 pg/mL	0.97	0.91
	MCI vs ADD	38.18 pg/mL	0.78	0.82
$\phi_{A\beta\text{-}42} \times \phi_{Tau}$	HC vs Patients	455.49 $(pg/mL)^2$	0.96	0.97
	MCI vs AD	642.58 $(pg/mL)^2$	0.80	0.82

[a]HC, healthy controls; MCI, mild cognitive impairment due to Alzheimer's disease; ADD, Alzheimer's disease dementia, including those with very mild to severe (CDR = 0.5−3) dementia. ADD and MCI are combined to form Patients.

change in permeability of the blood brain barrier to amyloids in patients with Alzheimer's disease and extra-brain sources of plasma amyloids.[18] The difference with some previous studies, which reported decreased or no change of plasma Aβ-42 concentrations, is that the mechanism is probably due to the inhibition of oligomerization of Aβ-42 peptide in plasma, due to the iron-chelating effect of magnetic Fe_3O_4 nanoparticles in the reagent. Therefore, the concentration of Aβ-42 detected using IMR is higher in the MCI and AD groups, for this study.

In order to further characterize the diagnostic properties, MCI due to AD, very mild AD, and mild-severe AD groups are combined in one group, referred to as the patient group. In the receiver operating characteristic (ROC) curve analysis, shown in Figure 4b, the threshold, in terms of Aβ-42 concentration, between the healthy controls and the patient group is 16.33 pg/mL, which is shown with a dashed line in Figure 4a. The sensitivity and specificity in differentiating the healthy controls from the patients with MCI and dementia due to AD are found to be 0.91 and 0.88, using Aβ-42 concentration in plasma, as shown in Figure 4b. The specificity and sensitivity for differentiating MCI and dementia due to AD in the patient group are further examined. The ROC curve shown in Figure 4c reveals that the sensitivity and specificity are 0.69 and 0.68, respectively, with a threshold of 17.65 pg/mL, shown with the dotted line in Figure 4a. All of the results for threshold, sensitivity and specificity are listed in Table 4.

Figure 5a shows the concentrations, ϕ_{tau}, of plasma tau protein in the different clinical groups. A clear-cutoff threshold of 23.89 pg/mL is observed between the healthy controls and the patient group. The specificity and sensitivity are 0.97 and 0.91, respectively, as shown in Figure 5b. The threshold of 23.89 pg/mL for tau-protein concentration is plotted with a dashed line in Figure 5a. The increase in tau-protein concentrations in the plasma of the MCI and AD groups is consistent with the increase in tau-protein concentration in CSF, shown in many previous reports.[25−27] In the patient group, the ROC curves between the MCI due to AD group and the AD dementia group are further analyzed. The ROC curve is shown in Figure 5c. It shows that the sensitivity is 0.82 and the specificity is 0.80, with a threshold of 38.18 pg/mL. The threshold of 38.18 pg/mL for tau-protein concentration between the MCI group and the AD group is plotted with a dot line in Figure 5a.

The results shown in Figures 4a and 5a suggest that Aβ-42 and tau protein in plasma exhibit high sensitivity and specificity in identifying patients with either MCI or AD. However, for differentiating between MCI due to AD and AD dementia, a better parameter is necessary. Since the concentrations of both Aβ-42 and tau protein are higher in the patient group than those of the healthy controls, it is reasonable to use the product

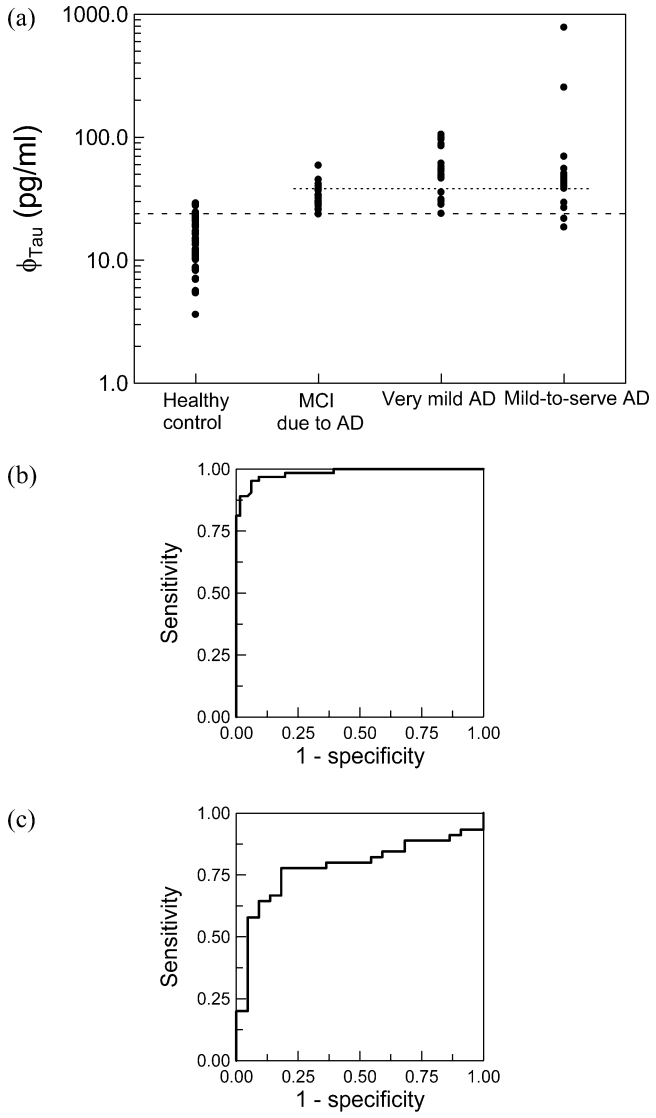

Figure 5. (a) Concentrations of tau protein in plasma from different clinical groups detected using immunomagnetic reduction with the aid of tau-protein reagent, and the ROC curves to distinguish (b) the healthy control group from the patient group (combining MCI due to AD, very mild AD, and mild-to-severe AD dementia) and to distinguish (c) the MCI due to AD and the AD dementia groups.

of Aβ-42 and tau-protein concentrations as a potential diagnostic parameter to improve the differentiation between AD dementia and MCI due to AD. The concentration products of Aβ-42 and tau protein for the MCI due to AD group, the AD dementia group (including very mild AD and mild-to-serve

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AD), and the healthy controls are plotted in Figure 6a. The ROC analysis shows a threshold of 642.89 $(pg/mL)^2$ for

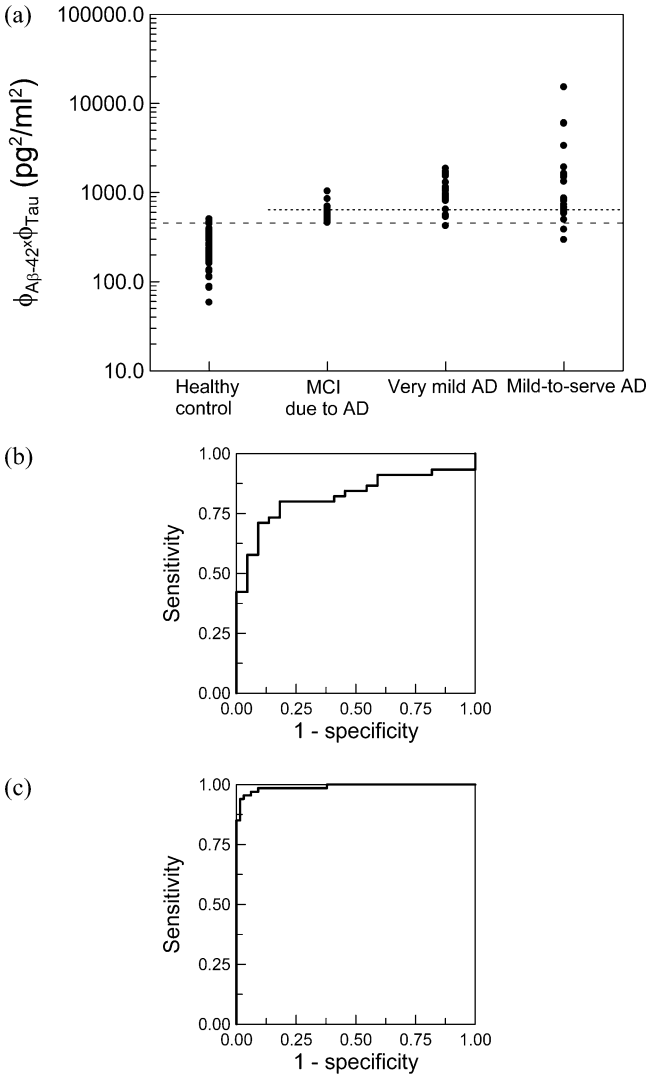

Figure 6. (a) Concentration products of Aβ-42 and tau protein in plasma from different clinical groups, detected using immunomagnetic reduction, and the ROC curves to distinguish (b) the healthy control group from the patient group (combining MCI due to AD, very mild AD, and mild-to-severe AD dementia) and to distinguish (c) the MCI due to AD and the AD dementia groups.

differentiating MCI due to AD from AD dementia, which results in a sensitivity of 0.8 and a specificity of 0.82, as shown in Figure 6b. The product of Aβ-42 concentration and tau-protein concentration gives better results for the differential diagnosis of MCI due to AD and AD dementia than using Aβ-42 concentration or tau-protein concentration alone. The concentration product yields a higher accuracy and improves the diagnosis for the patient group. The ROC curve analysis shows a sensitivity of 0.96 and a specificity of 0.97, with a threshold of 455.49 $(pg/mL)^2$, as shown in Figure 6c. The results shown in Figure 6 demonstrate that the concentration product of Aβ-42 and tau protein in plasma is a superior diagnostic parameter to either of the individual biomarkers, Aβ-42 or tau protein.

Table 2 shows that the healthy controls are younger that those in the patient group. In order to determine the effect of age, the concentrations of serum Aβ-42 and tau protein for subjects of healthy controls were determined. The results are shown in Figure 7. For the age dependent Aβ-42 concentration,

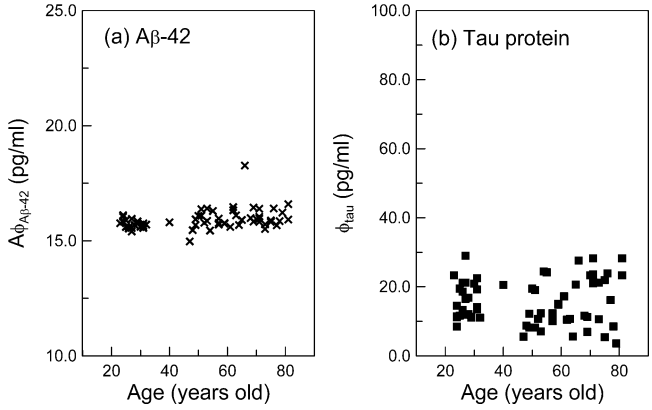

Figure 7. Age-dependent (a) serum Aβ-42 concentration and (b) serum tau-protein concentration for the healthy controls.

$\phi_{A\beta-42}$, in Figure 7a, the age varies from 23 to 81 years old and the detected $\phi_{A\beta-42}$ is distributed within the range from 15 to 17.5 pg/mL, except for one subject with 18.27 pg/mL. The results shown in Figure 7a demonstrate that age is not a crucial factor to the increase in the serum Aβ-42 concentration observed for the patient group. A similar finding for age-dependence for serum tau-protein concentration is obtained, as evidenced by the results shown in Figure 7b. The serum tau-protein concentrations for the healthy controls from 23 to 81 years old are less than 30 pg/mL, and are not dependent on age.

This study is not without limitations. There are wide variations in the clinical and pathological findings for Alzheimer's disease. The wide ranges for the plasma Aβ-42 and tau-protein levels are compatible with these findings. However, the plasma Aβ-42 and tau-protein levels can still serve as useful biomarkers to differentiate the healthy controls from the patients (combining MCI and AD patients). For Aβ-42, the sensitivity is 0.91 and the specificity is 0.88 (Figure 4b), and for the tau-protein the sensitivity is 0.91 and the specificity is 0.97 (Figure 5b). In terms of similar clinical severity, there is a wide range of pathological variations. For example, in patients with higher education or a better occupation, the severity of the hippocampal atrophy is greater at the moment of the onset of dementia.[32] However, the amyloid PET also shows limited progression, along with the severity of clinical dementia, but the extent of amyloid PET does not correlate well with the clinical severity. Therefore, this study does not propose the use of plasma biomarkers as a tool for differentiating clinical severity beyond mild dementia. These biomarkers are better used to differentiate healthy controls from patients with MCI or AD.

■ CONCLUSIONS

Instead of an individual biomarker, such as Aβ-42 or tau protein, the concentration product of plasma Aβ-42 and plasma tau protein improves the sensitivity and specificity of an AD diagnosis and allows the differentiation of MCI due to AD from AD dementia with a sensitivity of 0.8 and a specificity of 0.82. In addition to this greater accuracy, the proposed method also assays plasma samples other than CSF samples. The safety and accessibility of the assay is significantly improved by utilizing

SQUID IMR. Therefore, the SQUID IMR assay of plasma biomarkers is a promising diagnostic aid, not only for the detection of AD dementia but also for the identification of preclinical AD at the stage of MCI.

METHODS

Subjects. The subjects with AD were recruited from the memory clinic at the National Taiwan University Hospital. After routine tests at the memory clinic, each participant was subjected to a comprehensive clinical check that included a review of the medical history, physical and neurological examinations, laboratory tests, and neuroimaging studies. All of the patients with dementia met the diagnostic guidelines for probable AD dementia proposed by the National Institute on Aging-Alzheimer's Association (NIA-AA) workgroups in 2011.[28] The diagnosis of MCI due to AD also followed the recommendations from the NIA-AA, in terms of diagnostic guidelines.[29] For the diagnosis of MCI due to AD, a formal cognitive test was used, with a cutoff value at or below the fourth percentile (lower than 1.5 SD) of the scale score for the age and education matched control. The healthy controls were selected from a group of healthy volunteers in an MCI project.[30,31] In this study, the elder volunteers were given a medical checklist, to identify any major systemic diseases, operations, and/or hospitalizations. Volunteers who reported experiencing certain uncontrolled medical conditions, including heart failure, recent myocardial infarction (within the past 6 months), malignancy (during the past 2 years), or poorly controlled diabetes (Hb A1C > 8.5) were excluded from the study. The volunteers were also subjected to physical and neurological examinations and they were scored on a short-form Geriatric Depression Scale (GDS-S). Subjects with GDS-S scores greater than 9 were also excluded from the study. The healthy volunteers had normal cognitive function, confirmed by the mini mental state examination (MMSE) and clinical dementia rating (CDR). All of the study subjects or their primary caregivers provided informed consent prior to participation in this investigation, and the study was approved by the ethics committee and the institute review board of the university hospital.

In total, plasma from 45 patients with AD dementia of various severity, from very mild (CDR 0.5), mild (CDR 1), to moderate (CDR 2), to severe (CDR 3), and 66 healthy controls were collected for the assays for amyloids and tau protein using SQUID IMR.

Specimen Collection and Preparation. The subjects were asked to provide a 10 mL nonfasting venous blood sample (K3 EDTA, lavender-top tube). Each sample was assigned a registration number following the sampling sequence, so the laboratory operators were blind to the clinical status and the demographic data of the subjects. The blood samples were centrifuged (2500*g* for 15 min) within 1 h of collection, and plasma was aliquoted into cryotubes and stored at −80 °C, until thawed for measurement.

Magnetic Reagent. Three types of reagents (MF-AB0-0060, MF-AB2-0060, and MF-TAU-0060, MagQu) were used to, respectively, assay the biomarkers, Aβ-40, Aβ-42, and tau protein. Each type of reagent consists of magnetic nanoparticles dispersed in phosphate buffered saline (PBS, pH 7.2). By immobilizing antibodies against Aβ-40 (A3981, Sigma), Aβ-42 (A8354, Sigma), and tau protein (T9450, Sigma) on the magnetic nanoparticles, three types of reagents were obtained. The mean diameter of the antibody-functionalized magnetic nanoparticles was 50−60 nm. The magnetic concentration of each type of reagent was 12 mg-Fe/mL.

IMR Measurement. The volumes of the reagents and the to-be-detected samples used for the measurements of IMR signals are listed in Table 1. Each mixture was put into a SQUID-based alternative-current (ac) magnetosusceptometer (XacPro-S, MagQu), in order to determine the time dependent ac magnetic susceptibility. Because of the association between the antibody-functionalized magnetic nanoparticles and the target biomarkers, the ac magnetic susceptibility of the mixture was reduced. This reduction in the magnetic susceptibility is referred to as the IMR signal. For each to-be-detected sample, the sample was divided into three parts, for which IMR signals were detected individually. Therefore, three IMR signals were obtained for each sample. The mean value, standard deviation and the coefficient of variation (inter-run) of the IMR signals were analyzed for the three IMR signals.

In this experiment, several solutions with various concentrations of Aβ-40/Aβ-42/tau protein were prepared. These solutions were used as to-be-detected samples, to establish the relationships between the IMR signal and the Aβ-40/Aβ-42/tau protein. These relationships are referred to as characteristic curves. The IMR signals from human plasma for Aβ-40, Aβ-42, and tau protein were then detected and converted to the concentrations of Aβ-40, Aβ-42, and tau protein, using the characteristic curves. The demographic features of 66 healthy controls, 22 patients with MCI due to AD, 23 patients with very mild AD, and 22 patients with mild-to-serve AD dementia are listed in Table 2.

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Notes

The authors declare no competing financial interest.

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