




Investigation of the Number of Tests Required for Assaying Plasma Biomarkers Associated with Alzheimer's Disease Using Immunomagnetic Reduction

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ABSTRACT

Introduction: Concentrations of plasma biomarkers associated with Alzheimer's disease have been reported to be as low as several tens of picograms/milliliter (pg/ml). However, in assays measuring these biomarkers, it is likely that repeated measurements are necessary to obtain reliable values.

Methods: We performed assays as a single test or as duplicate, quadruplicate, fivefold and tenfold repeated tests, on samples spiked with different concentrations of amyloid β 1–40 ($A\beta_{1-40}$; 1–1000 pg/ml), $A\beta_{1-42}$ (1–30,000 pg/ml) and total Tau protein (T-Tau; 0.1–1000 pg/ml), with the aim to calculate the coefficients of variation (CVs).

Results: The results demonstrated common changes in the CVs with changes in the number of tests for a given sample: the CVs decreased with increases in the number of tests from one to ten. All CV values were distributed within the range of 0.35 to 15.5%; as such, the CV values were all lower than the acceptable value of 20%.

Conclusion: Based on this study, a single assay of $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau, respectively, provides reliable results in terms of the measurement of that plasma biomarker.

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Keywords: Plasma biomarkers; Alzheimer's disease; Immunomagnetic reduction

Key Summary Points

Why carry out this study?

Ultrasensitive systems are increasingly being used to detect dementia by detecting proteins at ultralow concentrations in blood samples. However, to date there are no scientific data to show how many analytical replicates are needed to ensure reliable plasma biomarker measurements.

In this study, we applied the immunomagnetic reduction (IMR) platform to investigate how many measurements are necessary to obtain reliable values.

What was learned from the study?

The plasma total Tau protein (T-Tau) concentration determined in a single test was found to have the same clinical significance for left hippocampus atrophy as that obtained in duplicate tests.

The measured plasma T-Tau concentrations determined in a single test showed equivalent significance for monitoring hippocampus atrophy to that obtained in duplicate tests.

For clinical samples, the effectiveness of a single IMR test was as effective as the duplicate test for discriminating normal subjects and patients with Alzheimer's disease.

feasible due to the successful development of ultrasensitive assay technologies [2–7], such as immunomagnetic reduction (IMR) [7]. Many research groups have validated the clinical significance of discriminating AD or amnesic mild cognitive impairment (aMCI) from normal controls (NCs) [8–10]. In addition, correlations between plasma $A\beta_{1-42}/A\beta_{1-40}$ and $A\beta$ plaques that were obtained using positron emission tomography (PET) have been demonstrated [11, 12]. The elevation of plasma T-Tau levels caused by atrophy of the brain (especially in the hippocampus) has also been observed [12, 13]. All of these previous results have demonstrated that assays of plasma $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau using IMR are useful for the diagnosis of AD in clinical practice. Consequently, assays of plasma $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau using IMR have been approved for clinical uses in Europe and Taiwan.

In previously published studies [7–13], biomarkers using IMR have been measured in duplicate. The original reason underlying the need for duplicate measurements using IMR was the ultralow concentrations of these plasma biomarkers, present approximately at concentrations of picograms per milliliter (pg/ml); to obtain reliable results, therefore, the measurement procedure was based on duplicate measurements. However, there are no scientific results to demonstrate that even duplicate measurements are needed for assaying such low biomarker concentrations; it may be that three or more measurements are necessary to obtain reliable results.

In the present study, we examined the coefficients of variation (CVs) of measured concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau in cases of a single test and tests carried out in duplicate, quadruplicate, fivefold and tenfold. The number of tests required to obtain reliable results was also determined. In addition, 242 subjects at four hospitals were enrolled in 2015 and 2016, including 134 cognitively normal controls and 108 patients with aMCI or AD [14]. The plasma $A\beta_{1-42}$, $A\beta_{1-40}$ and T-Tau concentrations of the duplicate tests have been previously reported. The correlations between hippocampal volume and measured plasma T-Tau concentrations, as well as the agreement

INTRODUCTION

Plasma biomarkers related to pathology have become important parameters for the assessment of Alzheimer's disease (AD) [1]. Although the concentrations of these biomarkers, such as amyloid β 1–40 ($A\beta_{1-40}$), $A\beta_{1-42}$ and total Tau protein (T-Tau), have been observed to be very low, assays of these plasma biomarkers are

between clinical diagnoses and plasma biomarker diagnoses, have also been investigated. In the present study, instead of using the results of duplicate measurements, we used the concentrations of a single test for $A\beta_{1-42}$, $A\beta_{1-40}$ and T-Tau to explore the correlations between hippocampal volume and measured plasma T-Tau concentrations, as well as the agreement between clinical diagnoses and plasma biomarker diagnoses.

METHODS

Preparation of Biomarker-Phosphate-Buffered Saline (PBS) Samples

Amyloid β 1–40 (A1075; Sigma-Aldrich, St. Louis, MO, USA), $A\beta_{1-42}$ (A9810; Sigma-Aldrich) and T-Tau (T7951; Sigma-Aldrich) were separately spiked into PBS solution (pH 7.4) to obtain the $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau samples. The spiked concentrations of the $A\beta_{1-40}$ samples ranged from 1 to 1000 pg/ml; those of the $A\beta_{1-42}$ samples ranged from 1 to 30,000 pg/ml; and the spiked concentrations of T-Tau samples ranged from 0.1 to 1000 pg/ml.

Analysis of CVs of Multiple Tests for Biomarker-Phosphate-Buffered Saline Samples

Immunomagnetic reduction reagents for assaying $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau in the biomarker-phosphate buffered saline (PBS) samples were used (MF-AB0-0060, MF-AB2-0060, MF-TAU-0060; MagQu Co. Ltd., New Taipei City, Taiwan). For each measurement, 80, 60 and 80 μ l of reagents were mixed with 40, 60 and 40 μ l, respectively, of biomarker-PBS samples. The concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau in a biomarker-PBS sample were detected using an IMR analyzer (XacPro-S; MagQu Co. Ltd.). For a given concentration of a biomarker, 20 measurements were performed (Table 1). With these two sets of data, which were denoted as s_n with $n = 1-20$, the CVs for a single test and for duplicate, quadruplicate,

fivefold repeated and tenfold repeated tests were calculated as follows:

In the case of a single test, the CV (%) was expressed as:

$$CV (\%) = \frac{SD \text{ of } \{s_n\}}{\text{Mean value of } \{s_n\}} \times 100\%, \quad (1)$$

where SD denotes the standard deviation ($n = 1-20$).

In the case of duplicate tests, the CV (%) was expressed as:

$$CV (\%) = \frac{SD \text{ of } \{d_n\}}{\text{Mean value of } \{d_n\}} \times 100\%, \quad (2)$$

where $d_n = (s_{2n} + s_{2n-1})/2$ and $n = 1-10$.

In the case of quadruplicate tests, the CV (%) was expressed as:

$$CV (\%) = \frac{SD \text{ of } \{q_n\}}{\text{Mean value of } \{q_n\}} \times 100\%, \quad (3)$$

where $q_n = (d_{2n} + d_{2n-1})/2$ and $n = 1-5$.

In the case of fivefold repeated tests, the CV (%) was expressed as:

$$CV (\%) = \frac{SD \text{ of } \{f_n\}}{\text{Mean value of } \{f_n\}} \times 100\%, \quad (4)$$

wherein $f_n = ((\sum_{s=1}^5 s_n)/5)$, and $n = 1-4$.

In the case of tenfold repeated tests, the CV (%) was expressed as:

$$CV (\%) = \frac{SD \text{ of } \{t_n\}}{\text{Mean value of } \{t_n\}} \times 100\%, \quad (5)$$

wherein $t_n = (f_{2n} + f_{2n-1})/2$ and $n = 1, 2$.

Recruitment of Subjects

A total of 242 subjects were enrolled from the memory clinics of the Taipei Medical University Shuang-Ho Hospital, the Renai Branch of Taipei City Hospital, the En Chu Kong Hospital and the National Taiwan University Hospital (master), from 2015 to 2016 [14]. Exclusion criteria were neurologic diseases that affect brain structure, such as epilepsy head injury or stroke;

medical diseases involving cardiopulmonary failure; hepatic or renal failure; and poor control of diabetes (HbA1c > 8.5). A neuropsychological test (NPT) 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. Those subjects who had a Geriatric Depression Scale (GDS) score ≥ 8 were excluded from the study. In addition, the subjects were required to have had at least 6 years of formal education or to be capable of reading and writing in Chinese to complete the comprehensive

NPT battery. The diagnostic criteria for MCI due to AD, as well as the criteria for dementia due to AD, followed the National Institute on Aging/Alzheimer's Association Diagnostic Guidelines [15–17]. All of the subjects and/or their primary caregivers provided written informed consent prior to their participation in this investigation. This study was approved by the ethics committees and institute review boards of the involved hospitals (National Taiwan University Hospital: 201406125DS; En Chu Kong Hospital: 1040102; Renai Branch of Taipei City Hospital: 1031203; Taipei Medical University Shuang-Ho

Table 1 Illustration of grouping of measured data in single, duplicate, quadruplicate, fivefold and tenfold repeated tests in Eqs. (1)–(5)

Test sequency no	Single test	Duplicate tests	Quadruplicate tests	Fivefold repeated tests	Tenfold repeated tests
1	s_1	d_1	q_1	f_1	t_1
2	s_2				
3	s_3	d_2			
4	s_4				
5	s_5	d_3	q_2		
6	s_6			f_2	
7	s_7	d_4			
8	s_8				
9	s_9	d_5	q_3		
10	s_{10}				
11	s_{11}	d_6		f_3	t_2
12	s_{12}				
13	s_{13}	d_7	q_4		
14	s_{14}				
15	s_{15}	d_8			
16	s_{16}			f_4	
17	s_{17}	d_9	q_5		
18	s_{18}				
19	s_{19}	d_{10}			
20	s_{20}				

S_n standard deviation of single test, d_n standard deviation of duplicate test, q_n standard deviation of quadruplicate test, f_n standard deviation of fivefold repeated tests, t_n standard deviation of tenfold repeated tests

Hospital: 201408011) and was conducted in accordance with the Helsinki Declaration of 1964 and its later amendments.

Preparation of Human Plasma

Blood samples were collected in a 6-ml EDTA blood collection tube. The collected blood was centrifuged at 1500–2500 *g* for 15 min at room temperature with a swing-bucket rotor, followed by transfers of each 1 ml of plasma to a 0.5-ml microcentrifuge tube and storage at –80 °C until biomarker assays were performed. All of the plasma samples were packaged in dry ice and shipped to the National Taiwan University Hospital in Taipei City, Taiwan, for blind testing of plasma biomarkers.

Assays of Plasma Biomarkers

Plasma biomarkers were assayed using IMR, with IMR kits MF-AB0-0060, MF-AB2-0060 and MF-TAU-0060 (MagQu Co. Ltd.) for assaying A β _{1–40}, A β _{1–42} and T-Tau, respectively, and IMR analyzer model XacPro-S (MagQu Co. Ltd.). For assaying A β _{1–40}, A β _{1–42} and T-Tau, 80, 60 and 80 μ l of reagents were mixed with 40, 60 and 40 μ l of plasma, respectively, for a single measurement. Duplicate measurements were conducted for each biomarker per sample and are denoted as the first measurement and second measurement hereafter.

Magnetic Resonance Imaging Scans

High-resolution structural brain magnetic resonance imaging (MRI) scans were acquired by using a 1.5 T MRI scanner (EXCITE; General Electric Co., Boston, MA, USA), as described in [13]. The MRI images were corrected for intensity inhomogeneities and reoriented, after which they were registered against the MNI 152 space by using FSL (FMRIB Software Library; Analysis Group, FMRIB, Oxford) tools [18]. The FIRST and SIENAX FSL tools were used to extract the relevant regions of interest and to calculate the volumes of the right and left hippocampus [19].

Statistical Methods

The continuous variables for each measurement are presented as the mean \pm standard deviation (SD). The continuous variables were compared by using Spearman correlation, *t* tests and *p* values.

RESULTS

Using Eqs. (1)–(5), the CVs (%), as functions of the number of tests for A β _{1–40}, A β _{1–42} and T-Tau at various concentrations, were calculated; these are shown in Fig. 1a–c, respectively. The number of tests ranged from a single test (*n* = 1) to the test repeated tenfold (*n* = 10). The curves in Fig. 1a–c can be seen to have common trends. First, all of the CVs (%) are < 20%. Second, CVs (%) obtained from a single test have the highest values; for example, the CV (%) for the single test of 1 pg/ml A β _{1–40} was 12.2%, that for 1 pg/ml A β _{1–42} was 15.0% and that for 0.1 pg/ml T-Tau was 15.5%. Third, the CV (%) decreased with increasing number of tests from one to five and remained almost unchanged as the number of tests increased to > 5; this indicated that the CV (%) was significantly suppressed by multiple measurements (for example, at the fivefold repeated test).

According to previously reported studies on bioanalytical method validation [20–22], the acceptable CV (%) for clinical use should be < 20–30%. As shown in Fig. 1a–c, the highest value of CV (%) for individual biomarkers occurred in the case of a single test, i.e. number of tests = 1. These highest values of CV (%) for these three biomarkers were < 20%, indicating that the measured results of a single test for A β _{1–40}, A β _{1–42} and T-Tau were reliable, even at ultralow concentrations in the pg/ml range.

A single test for assaying A β _{1–40} in human plasma was performed. Two rounds of single-test measurements were performed (i.e. first and second measurement, respectively). The correlation of the measured A β _{1–40} concentrations between the first measurement and second measurement is shown in Fig. 2a. The slope of the linearity in Fig. 2a was 0.99, and the coefficient of determination (*R*²) was 0.987; these

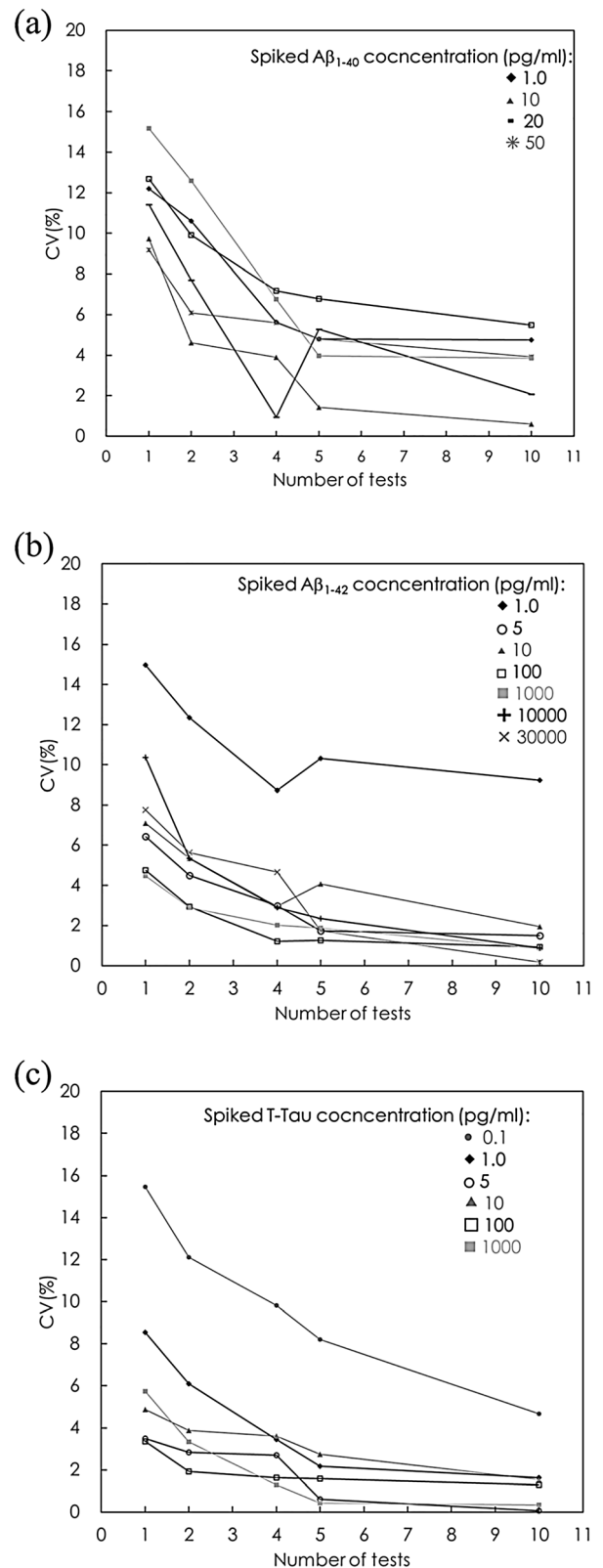
Fig. 1 CV (%) versus number of tests for A β _{1–40} (a), A β _{1–42} (b) and T-Tau (c) of various concentrations. CV Coefficient of variation

values demonstrated the high consistency between the two rounds of a single test for A β _{1–40} in human plasma by using IMR.

The consistency in the measured plasma A β _{1–42} and T-Tau concentrations between the first measurement and second measurement was also investigated. The results are shown in Fig. 2b, c. For plasma A β _{1–42} (Fig. 2b), the slope of the linearity was 0.99 and the R^2 was 0.995; for plasma T-Tau (Fig. 2c), the slope of the linearity was 0.96 and the R^2 was 0.950. These results demonstrate the high consistency between the two rounds of a single test for A β _{1–42} and T-Tau in human plasma using IMR. In a next step, we investigated whether the difference/performance is affected when the CV varies to the extremes of the concentration range. Analysis of the CVs of the lowest and highest three concentrations of biomarkers in the clinical samples demonstrated that: (1) for A β _{1–40} measurements, the CV values of the lowest and highest three concentrations were 4.37–8.62% and 6.90–12.58%, respectively, which are lower than the standard CV value of 20%; (2) for A β _{1–42} measurements, the CV values of the lowest and highest three concentrations were 1.32–1.68% and 1.50–14.51%, respectively, which are lower than the standard CV value of 20%; (3) for Tau measurements, the CV values of the lowest and highest three concentrations were 7.27–12.73% and 1.50–14.51%, respectively, which are lower than the standard CV value of 20%. These results show that for clinical samples with extreme values, the CV value can still be within 20% and, consequently, that the measured concentrations of A β _{1–40}, A β _{1–42} and T-Tau in a single test using IMR are reliable.

DISCUSSION

In studies utilizing IMR published so far [7–14], the concentration of plasma samples of A β _{1–40},



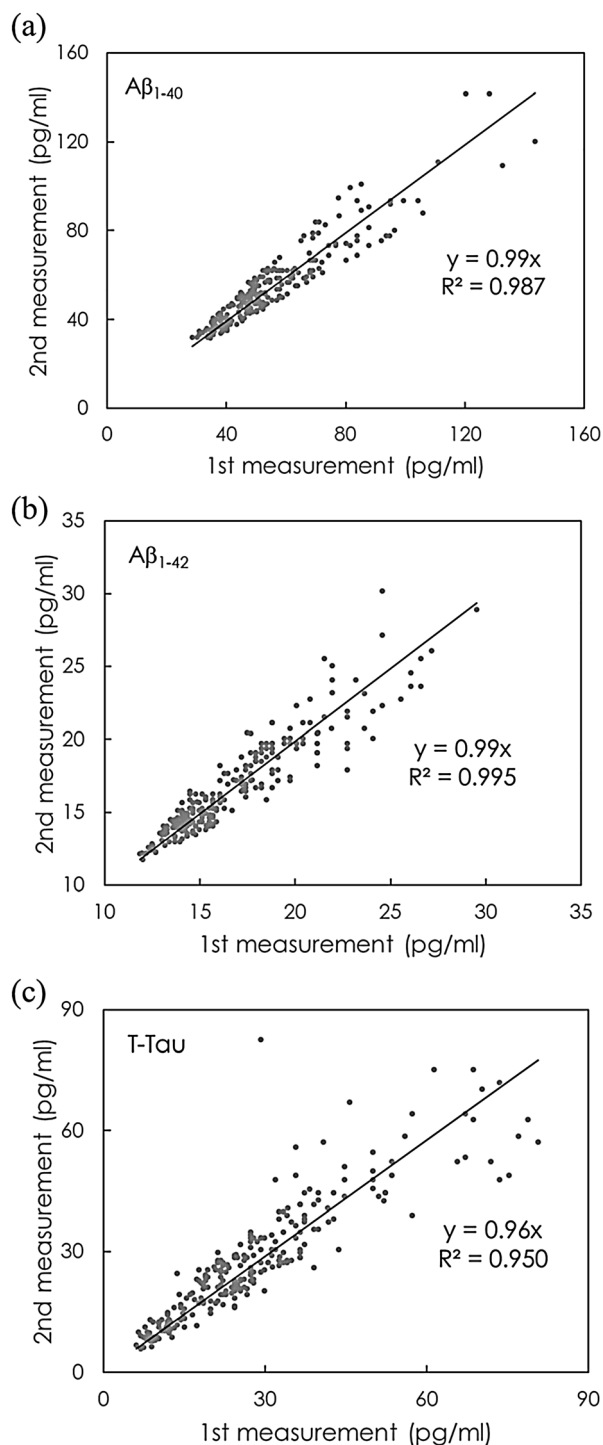


Fig. 2 Linearity of measured plasma Aβ₁₋₄₀ (a), Aβ₁₋₄₂ (b) and T-Tau (c) concentrations between two rounds of single tests, referred to as the 1st measurement and 2nd measurement, respectively

Aβ₁₋₄₂ or T-Tau represented the averaged value of duplicate measurements. It has also been observed that plasma T-Tau is negatively correlated with hippocampal volume [12, 13]. Aβ₁₋₄₂ × T-Tau or Aβ₁₋₄₂/Aβ₁₋₄₀ in plasma has been shown to discriminate NCs from subjects with aMCI and AD [8–10]. These results reveal the impacts of plasma biomarkers on clinical applications, such as in assisting in the diagnosis of AD, the prediction of cognitive decline in aMCI [23, 24], assessment of results from neurological imaging studies [11, 25] and the monitoring of interventions, among other impacts. The authors of an earlier study reported that there was a negative correlation between plasma and cerebral spinal fluid Aβ₁₋₄₂ levels in patients with AD ($r = -0.352$) [26], suggesting that IMR is no less useful than single molecule array (SIMOA; $r = 0.288$) testing in clinical applications [27]. According to the results shown in Figs. 1 and 2, reliable measurements of plasma Aβ₁₋₄₀, Aβ₁₋₄₂ or T-Tau concentrations were achieved in a single test by using IMR. This result was the underlying motivation to investigate the associations of plasma biomarkers that were obtained in a single test with the use of MRI and clinical diagnoses.

Two subjects withdrew their consent after blood sampling and diagnosis based on the MRI scan. A total of 240 enrolled subjects underwent MRI scans to analyze the right hippocampal volume and left hippocampal volume (Table 2). Two rounds of single-test T-Tau plasma concentrations with the use of IMR (referred to as the first and second measurement, respectively) were performed. The relationships between the left hippocampal volume and the first measurement of T-Tau concentration are plotted in Fig. 3a. The measured plasma T-Tau concentration was found to increase with decreasing hippocampal volume. Additionally, we observed a negative correlation between hippocampal volume and plasma T-Tau levels. This result is consistent with that reported in a previous study [13] and indicates that atrophy of the hippocampus results in an elevation of plasma T-Tau. The Spearman correlation coefficient in Fig. 3a was found to be -0.4723 ($p < 0.0001$). Figure 3b shows the relationship

between the left hippocampal volume and the second measurement of T-Tau concentration. The Spearman correlation coefficient was found to be -0.4446 ($p < 0.0001$). The measured T-Tau concentrations of the first and second measurements for a subject were averaged and referred to as duplicate measurements. The averaged T-Tau concentrations versus the left hippocampal volume are plotted in Fig. 3c. The Spearman correlation coefficient was found to be -0.4767 ($p < 0.0001$). The values of the Spearman correlation coefficient of the first measurement, second measurement and duplicate measurements were similar to each other (approximate range -0.4446 , -0.4767), which indicates that the plasma T-Tau concentration obtained in a single test has the same clinical significance for left hippocampal atrophy as that obtained in duplicate tests.

Fig. 3 Relationships between left hippocampal volume and measured plasma Tau concentrations in two rounds of a single test, referred to as the 1st measurement (a), 2nd measurement (b) and duplicate measurements (c). **d–f** Relationships between right hippocampal volume and measured plasma Tau concentrations in two rounds of single tests and duplicate measurements

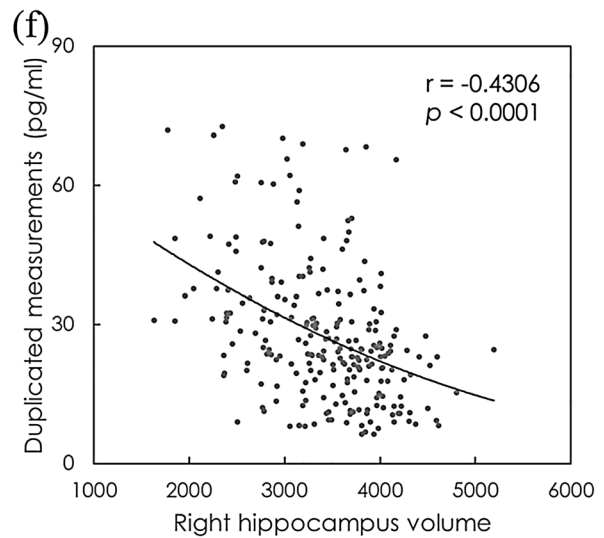
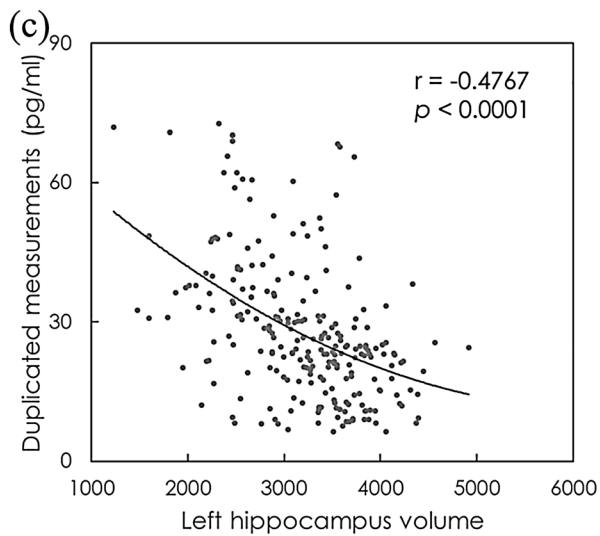
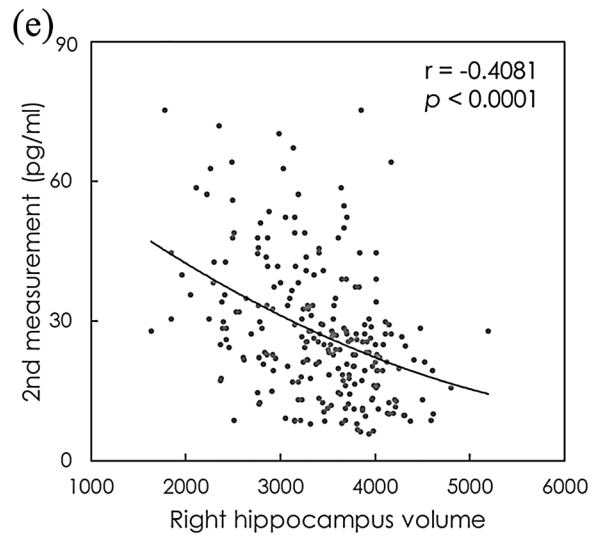
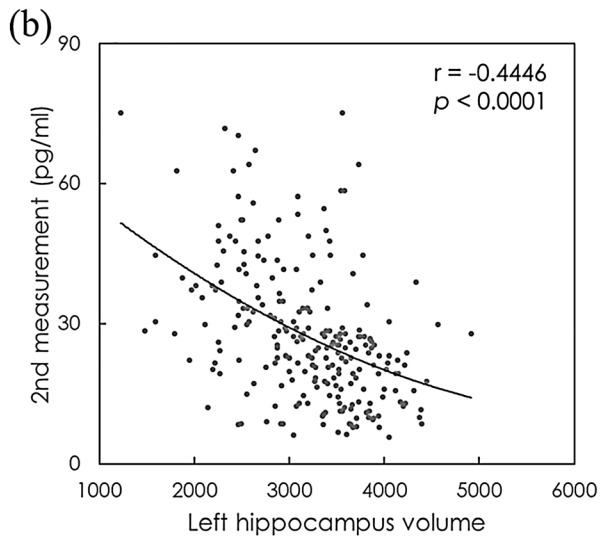
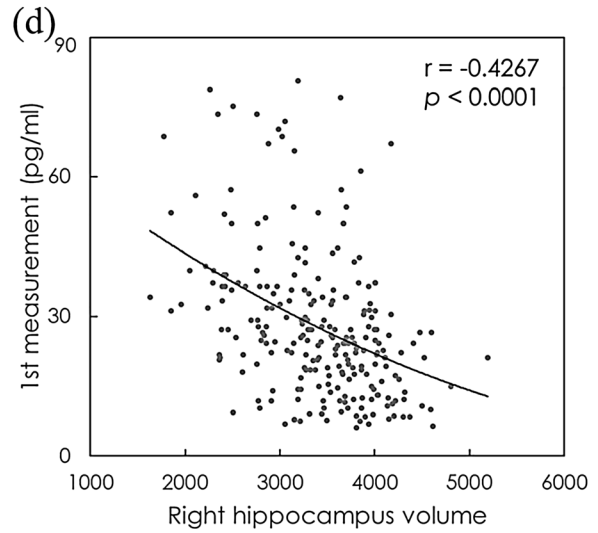
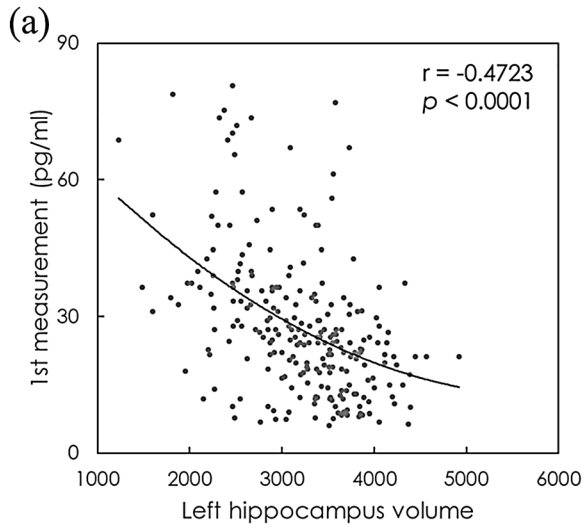
Figure 3d–f plot the relationships between the right hippocampal volume and plasma T-Tau in the first measurement, second measurement and duplicate measurements. Negative correlations were found in Fig. 3d–f, with Spearman correlation coefficients of -0.4267 , -0.4081 and -0.4306 ($p < 0.0001$), respectively. Thus, the measured plasma T-Tau concentrations obtained in a single test showed equivalent significance in monitoring

Table 2 Clinical and biomarker information on participants

Clinical and biomarker information	Healthy subjects (normal controls) ($n = 132$)	Subjects with MCI due to AD ($n = 34$)	Subjects with AD ($n = 74$)	p (ANOVA)
Age (years)	67.6 ± 7.7	71.5 ± 8.9	77.2 ± 8.9	
Clinical Dementia Rating (CDR) scale	0.17 ± 0.24	0.5 ± 0.25	1.1 ± 0.63	< 0.0001
Mini-Mental State Examination (MMSE) score	28.2 ± 1.7	24.4 ± 3.5	17.2 ± 5.7	< 0.0001
Gender, n (%)				
Male	45 (34.1%)	12 (35.3%)	36 (48.6%)	
Female	87 (65.9%)	22 (64.7%)	38 (51.4%)	
Hippocampal volume (mm^3)				
Left	3568.1 ± 442.2	3057.2 ± 550.7	2614.4 ± 561.0	< 0.0001
Right	3715.5 ± 491.2	3393.4 ± 557.8	2845.9 ± 571.2	< 0.0001
Protein level (pg/ml)				
$A\beta_{1-40}$	62.42 ± 18.55	52.81 ± 19.71	42.73 ± 8.04	< 0.0001
$A\beta_{1-42}$	14.66 ± 1.45	18.81 ± 2.82	19.00 ± 2.94	< 0.0001
T-Tau	19.99 ± 10.09	30.08 ± 11.72	40.91 ± 14.27	< 0.0001

Values are presented as the mean \pm standard deviation (SD)

$A\beta$ Amyloid β , AD Alzheimer's disease, ANOVA analysis of variance, MCI mild cognitive impairment, T-Tau total Tau



hippocampal atrophy to that obtained in duplicate tests.

Among the enrolled subjects, 132 were NCs (mean age 67.6 ± 7.7 years), 34 were patients with MCI due to AD (aMCI; mean age 71.5 ± 8.9 years) and 74 were patients with AD dementia (mean age: 77.2 ± 8.9 years). For the purposes of this study, we combined the aMCI and AD patients to represent the patient group (Table 3). According to a previously published study [8], the cutoff value of plasma $A\beta_{1-42} \times T\text{-Tau}$ using IMR that discriminates patients from NCs is 455.49 pg/ml^2 . Using the single-test concentrations of $A\beta_{1-42}$ and T-Tau in the plasma, the subjects showing $A\beta_{1-42} \times T\text{-Tau}$

values that are not lower than 455.49 pg/ml^2 were regarded as being positive, whereas subjects showing $A\beta_{1-42} \times T\text{-Tau}$ values that are lower than 455.49 pg/ml^2 were regarded as being negative. The agreement between the clinical diagnosis and the plasma biomarker diagnosis using single-test $A\beta_{1-42} \times T\text{-Tau}$ was analyzed, as shown in Table 3. Two rounds of single tests were performed (1st and 2nd measurements, respectively). Both the agreements of these two rounds of a single test and the duplicate tests are listed in Table 3. The $A\beta_{1-42}$ and T-Tau concentrations in the duplicate measurements are presented as the averaged values of those concentrations in the 1st measurement and 2nd measurement. Although the duplicate tests showed slightly higher agreements than those of the first measurement and second measurement, the agreements in all of the cases were > 0.85 (or 85%). Thus, the single-test plasma $A\beta_{1-42} \times T\text{-Tau}$ assay using IMR is a promising parameter to discriminate NCs from patients with MCI due to AD and AD.

In addition to discriminating between patients and NCs, plasma $A\beta_{1-42} \times T\text{-Tau}$ showed promise to differentiate AD from aMCI, with a cutoff value of 642.58 pg/ml^2 , as has been previously reported [8]. The data used in Table 3 were further analyzed to investigate the agreement between the clinical diagnosis and the plasma biomarker diagnosis using $A\beta_{1-42} \times T\text{-Tau}$ for discriminating AD from aMCI. In Table 4, the overall percentage agreement in the duplicate measurements was 82.4%, which was higher than those in the first measurement and second measurement by 5.5%. In fact, the overall percentage agreement in a single test was 76.9%, which is sufficient for the clinical purpose of discriminating AD from aMCI.

Another plasma biomarker index to differentiate NCs from patients with AD and aMCI is $A\beta_{1-42}/A\beta_{1-40}$. According to a previously published study, the cutoff value to discriminate NCs from patients was 0.325 [8]. Hence, subjects showing $A\beta_{1-42}/A\beta_{1-40}$ values that were not < 0.325 were regarded as being positive, and subjects with $A\beta_{1-42}/A\beta_{1-40}$ values that were < 0.325 were regarded as being negative. The agreements between the clinical diagnosis and the plasma biomarker diagnosis using single-

Table 3 Analysis of agreement between clinical diagnosis and plasma $A\beta_{1-42} \times T\text{-Tau}$ obtained in a single test (1st measurement and 2nd measurement) and duplicate tests for discriminating patients from healthy subjects (normal controls)

Measurement	Clinical diagnosis plasma $A\beta_{1-42} \times$ T-Tau	Patients <i>n</i>	Healthy subjects (normal controls) <i>n</i>
First measurement	Positive	<i>n</i> = 94	<i>n</i> = 12
	Negative	<i>n</i> = 14	<i>n</i> = 122
	Negative percentage agreement = 0.870		
	Positive percentage agreement = 0.910		
Overall percentage agreement = 0.893			
Second measurement	Positive	<i>n</i> = 92	<i>n</i> = 11
	Negative	<i>n</i> = 16	<i>n</i> = 123
	Negative percentage agreement = 0.851		
	Positive percentage agreement = 0.918		
Overall percentage agreement = 0.888			
Duplicate measurements	Positive	<i>n</i> = 98	<i>n</i> = 11
	Negative	<i>n</i> = 10	<i>n</i> = 123
	Negative percentage agreement = 0.907		
	Positive percentage agreement = 0.918		
Overall percentage agreement = 0.913			

test $A\beta_{1-42}/A\beta_{1-40}$ were also analyzed, as shown in Table 5. Notably, two rounds of single tests were performed in this study (1st measurement and 2nd measurement). The averaged values of those tests in the first measurement and second measurement were reported as $A\beta_{1-42}$ and T-Tau concentrations in the duplicate tests. In Table 5, the agreement between the two rounds of single tests (1st measurement and 2nd measurement), duplicate tests and clinical diagnosis are shown. Regardless of whether a single test or duplicate tests were performed, the agreements were > 0.85 (or 85%). Similar to the case of plasma $A\beta_{1-42} \times$ T-Tau, single-test plasma $A\beta_{1-42}/A\beta_{1-40}$ using IMR represents a promising parameter for

differentiating NCs from patients with MCI due to AD and AD.

There are several limitations in this study. First, the clinical samples were re-analyzed using the original duplicate data. More subjects with single measurements should be enrolled to further validate the discrimination in patients with AD and NCs. Second, this is a cross-sectional study. Therefore, a longitudinal study is needed to confirm whether the feasibility is as good as suggested by the results of this cross-sectional study.

Table 4 Analysis of agreement between clinical diagnosis and plasma $A\beta_{1-42} \times$ T-Tau obtained in a single test (1st measurement and 2nd measurement) and duplicate tests for discriminating AD from amnesic mild cognitive impairment

Measurement	Clinical diagnosis plasma $A\beta_{1-42} \times$ T-Tau	Patients with AD	Patients with amnesic mild cognitive impairment (aMCI)
First measurement	Positive	<i>n</i> = 59	<i>n</i> = 10
	Negative	<i>n</i> = 15	<i>n</i> = 24
	Negative percentage agreement = 0.797		
	Positive percentage agreement = 0.706		
Overall percentage agreement = 0.769			
Second measurement	Positive	<i>n</i> = 58	<i>n</i> = 9
	Negative	<i>n</i> = 16	<i>n</i> = 25
	Negative percentage agreement = 0.784		
	Positive percentage agreement = 0.735		
Overall percentage agreement = 0.769			
Duplicate measurements	Positive	<i>n</i> = 62	<i>n</i> = 7
	Negative	<i>n</i> = 12	<i>n</i> = 27
	Negative percentage agreement = 0.838		
	Positive percentage agreement = 0.794		
Overall percentage agreement = 0.824			

Table 5 Analysis of agreement between clinical diagnosis and plasma $A\beta_{1-42}/A\beta_{1-40}$ obtained in a single test (1st measurement and 2nd measurement) and duplicate tests for discriminating patients from healthy subjects (normal controls)

Measurement	Clinical diagnosis plasma $A\beta_{1-42}/A\beta_{1-40}$	Patients	Healthy subjects (normal controls)
First measurement	Positive	<i>n</i> = 99	<i>n</i> = 20
	Negative	<i>n</i> = 9	<i>n</i> = 114
	Negative percentage agreement = 0.917		
	Positive percentage agreement = 0.851		
Overall percentage agreement = 0.880			
Second measurement	Positive	<i>n</i> = 94	<i>n</i> = 15
	Negative	<i>n</i> = 14	<i>n</i> = 119
	Negative percentage agreement = 0.870		
	Positive percentage agreement = 0.888		
Overall percentage agreement = 0.880			
Duplicate measurements	Positive	<i>n</i> = 96	<i>n</i> = 15
	Negative	<i>n</i> = 12	<i>n</i> = 119
	Negative percentage agreement = 0.889		
	Positive percentage agreement = 0.888		
Overall percentage agreement = 0.888			

CONCLUSION

Both the analytical performance and the clinical results demonstrated the reliability of the single-test assay of $A\beta_{1-40}$, $A\beta_{1-42}$ and T-Tau using IMR. The clinical equivalence between a single test and duplicate tests was also demonstrated. The measured plasma $A\beta_{1-42} \times$ T-Tau and $A\beta_{1-42}/A\beta_{1-40}$ in a single test showed > 85% agreement with the clinical diagnosis for assessing aMCI and AD.

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Statistical Analysis and Other Assistance. Prism version 6 software (Graphpad Software Inc., San Diego, CA, USA) was used to calculate the mean, SD, Spearman correlation coefficient and *t* tests of the continuous variables.

Authorship. All of the named authors take responsibility for the integrity of the work as a whole and have given their approval for this version to be published.

Authors' Contributions. Huei-Chun Liu, Chia-Chun Lin and Shieh-Yueh Yang designed the study. Huei-Chun Liu and Shieh-Yueh Yang performed the statistical analysis. Shieh-Yueh Yang prepared the manuscript. Hsin-Hsien Chen and Ming-Jang Chiu performed the IMR measurements. Ming-Jang Chiu analyzed the MRI data. Chia-Shin Ho and Jui-Feng Chang prepared the IMR reagents. Ming-Jang Chiu, Ta-Fu Chen, Chaur-Jong Hu, Sui-Hing Yan and Yu Sun enrolled the subjects and performed the neurological tests for the participants.

Disclosures. Huei-Chun Liu, Hsin-Hsien Chen, Chia-Shin Ho, Jui-Feng Chang, Chia-Chun Lin and Shieh-Yueh Yang are employees of MagQu Co., Ltd. Ming-Jang Chiu, Ta-Fu Chen, Chaur-Jong Hu, Sui-Hing Yan and Yu Sun have nothing to disclose.

Compliance with Ethics Guidelines. All of the subjects and/or their primary caregivers provided written informed consent prior to their participation in this investigation. This study was approved by the ethics committees and institute review boards of the involved hospitals (National Taiwan University Hospital: 201406125DS; En Chu Kong Hospital: 1040102; Renai Branch of Taipei City Hospital: 1031203; Taipei Medical University Shuang-Ho Hospital: 201408011) and was conducted in accordance with the Helsinki Declaration of 1964 and its later amendments.

Data Availability. All of the data generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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