**Original Research Article**

# Long-Term Storage Effects on Stability of Aβ1–40, Aβ1–42, and Total Tau Proteins in Human Plasma Samples Measured with Immunomagnetic Reduction Assays

Ming-Jang Chiu[a–c]    Lih-Fen Lue[d, e]    Marwan N. Sabbagh[f]    Ta-Fu Chen[a]
H.H. Chen[g]    Shieh-Yueh Yang[g]

[a]Department of Neurology, College of Medicine, National Taiwan University Hospital, National Taiwan University, Taipei, Taiwan; [b]Graduate Institute of Brain and Mind Sciences, College of Medicine, National Taiwan University, Taipei, Taiwan; [c]Department of Psychology, National Taiwan University, Taipei, Taiwan; [d]Laboratory of Neuroregeneration, Banner Sun Health Research Institute, Sun City, AZ, USA; [e]Biodesign Institute, Banner Neurodegenerative Disease Research Center, Arizona State University, Tempe, AZ, USA; [f]Lou Ruvo Center for Brain Health Cleveland Clinic, Las Vegas, NV, USA; [g]MagQu Co., Ltd, New Taipei City, Taiwan

## Keywords

Alzheimer's disease · Plasma biomarkers · Immunomagnetic reduction · Storage stability

## Abstract

***Background:*** The stability of Alzheimer's disease (AD) biomarkers in plasma, measured by immunomagnetic reduction (IMR) after long-term storage at $-80°C$, has not been established before. ***Method:*** Ninety-nine human plasma samples from 53 normal controls (NCs), 5 patients with amnestic mild cognitive impairment (aMCI), and 41 AD patients were collected. Each plasma sample was aliquoted and stored as single-use aliquots at $-80°C$. The baseline measurements for Aβ1–40, Aβ1–42, and total Tau protein (T-Tau) concentrations for each sample were done within 3 months of blood draw by IMR. They are referred to as baseline concentrations. A separate aliquot from each sample was assayed with IMR to assess the stability of the measured analytes during storage at $-80°C$ between 1.1 and 5.4 years. This is referred to as a repeated result. ***Results:*** IMR shows that plasma levels of Aβ1–40 and Aβ1–42 exhibit stability over 5-year storage at $-80°C$ and that plasma levels of T-Tau are less stable (approximately 1.5 years). ***Conclusion:*** Although the measured concentrations of T-Tau in human plasma may alter during storage, the diagnostic utility of the results are only slightly affected when the product of Aβ1–42 and T-Tau concentrations are used. The results show that the overall agreement between baseline and repeated measurements in the ability of discriminating NCs from aMCI/AD patients is higher than 80%.

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Shieh-Yueh Yang
3F, No. 12, Lane 538, Zhongzheng Road
Xindian District
New Taipei City 231 (Taiwan)
E-Mail syyang @ magqu.com

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Chiu et al.: Stability of Aβs in Plasma and Tau Protein Using Immunomagnetic Reduction

## Introduction

Availability of biomarkers to be used in clinical diagnosis of Alzheimer's disease (AD) is an important issue. Currently, an imaging biomarker, based on amyloid positron emission tomography (PET), is increasingly used [1–5]. However, due to its high cost and limited accessibility, broad application of amyloid PET in clinics is difficult. Assaying biofluid biomarkers may help. The most promising biofluid biomarkers are β-amyloid (Aβ) and total tau protein (T-Tau) in cerebrospinal fluid (CSF). Reports show that the levels of $Aβ_{1-42}$ are decreased and the levels of T-Tau are increased in CSF samples of patients with AD [6–10]. Although these changes have been demonstrated in clinically diagnosed AD patients, lumbar puncture is not easily accepted in clinical practice in some countries, and there are possible side effects such as back pain, headache, swelling, and bruising. By contrast, obtaining blood samples is less invasive. However, the concentrations of Aβ and T-Tau in plasma are low [11–15].

To meet the challenges of assaying low levels of these AD biomarkers in human blood, several ultrasensitive technologies have been developed, including immunomagnetic reduction (IMR) assays, single-molecule arrays, and single-molecule counting [16–21]. Further validation and comparison of these assays rely on well-characterized and stable samples. Several established biofluid banks play important roles in providing archived samples that have been stored at –80°C for various lengths of time [22–24]. As peptides and proteins could be susceptible to degradation and denaturation during storage, it is crucial to investigate the stability of these types of biomarkers to determine the effects of storage time and conditions when interpreting the results.

In this study, we determined the storage effects on the plasma levels of AD biomarkers that we have previously measured by IMR assays [25, 26]. Previous results have demonstrated that IMR assays detected lower levels of plasma $Aβ_{1-40}$ and higher levels of plasma $Aβ_{1-42}$ and T-Tau in patients with amnesic mild cognitive impairment (aMCI) or AD than in normal controls (NCs) [27, 28]. The overall percentage agreement in discriminating aMCI/AD patients from NCs was higher than 80% when using 455 $pg^2/mL^2$ as a cutoff value for the product of plasma $Aβ_{1-42}$ and T-Tau concentrations [29, 30]. Moreover, the ratio of $Aβ_{1-42}$ to $Aβ_{1-40}$ concentrations in human plasma as detected with IMR was found to be positively correlated with the standardized uptake value ratio probed with ^{11}C-labeled Pittsburgh compound-B PET [31]. All of these published data were gathered from relatively fresh human plasma samples, i.e., within 3 months of storage at –80°C. The stability of AD biomarkers that are measurable by IMR after long-term storage at –80°C is unknown.

In this work, we used human plasma samples collected and assayed previously to evaluate the stability of AD biomarkers during storage. The correlations between baseline and repeated measurement concentrations of $Aβ_{1-40}$, $Aβ_{1-42}$, and T-Tau were analyzed. The recovery rate of each biomarker in every sample was calculated to determine the extent of the biochemical changes in these biomarkers during storage.

## Methods

### Subjects

Ninety-nine human plasma samples were collected at the National Taiwan University Hospital (referred to as the NTUH cohort) during the period from 2011 to 2015 and at the Banner Sun Health Research Institute (referred to as the BSHRI cohort) in the year 2014, following approved protocols by the Institutional Review Boards at NTUH and BSHRI, respectively. Each participant has given their informed consent. According to clinical diagnosis, 53 subjects in the combined cohort were NCs, 5 were patients with aMCI, and 41 were patients

**Table 1.** Demographic information of all enrolled subjects for baseline measurements

| Cohort | Diagnosis | $n$ | Age, years | CDR | FAST | MMSE | Aβ$_{1-40}$, pg/mL | Aβ$_{1-42}$, pg/mL | T-Tau, pg/mL |
|---|---|---|---|---|---|---|---|---|---|
| NTUH | NC | 37 | 66.1±8.3 | 0 | – | 29.1±1.1 | 58.75±10.64 | 14.59±1.49 | 16.61±9.18 |
| | aMCI | 5 | 66.4±6.6 | 0.5 | – | 24.2±0.2 | 44.59±9.80 | 19.26±3.52 | 30.30±8.87 |
| | AD | 25 | 78.1±7.3 | 0.5–2 | – | 20.7±2.2 | 39.54±9.20 | 18.64±1.60 | 43.35±15.14 |
| BSHRI | NC | 16 | 81.9±6.0 | – | 1.17±0.11 | 29.3±0.3 | 54.18±7.17 | 15.33±2.63 | 20.48±4.96 |
| | AD | 16 | 82.5±1.4 | – | 4.22±0.15 | 16.1±1.0 | 47.39±6.60 | 16.80±1.49 | 34.52±15.00 |
| Combined | NC | 53 | 70.9±10.6 | – | – | – | 57.37±9.88 | 14.81±1.91 | 17.78±8.28 |
| | aMCI/AD | 46 | 78.4±8.0* | – | – | – | 42.82±9.05* | 18.07±2.03* | 38.86±15.18* |

Values are given as mean ± standard deviation unless indicated otherwise.

NC, normal cognition; aMCI, amnesic mild cognitive impairment; AD, Alzheimer's disease; CDR, clinical dementia ranking; FAST, functional assessment staging; MMSE; mini-mental state examination.

* $p < 0.001$ between NC and aMCI/AD.

with AD [30, 32]. The age of the enrolled subjects ranged from 49 to 91 years. The scores on the clinical dementia ranking (CDR), functional assessment staging (FAST), and mini-mental state examination (MMSE) for the NC, aMCI, and AD groups in the NTUH and BSHRI cohorts are listed in Table 1.

*Plasma Preparation*

Plasma samples were collected at two institutes for this study. These two institutes used the same protocol for collecting human plasma. A 10-mL K3-EDTA tube was used to draw blood. No fasting was required for blood drawing; however, it could not be done within 2 h after exercise or wake-up in the morning. The tubes were gently inverted 10 times immediately after blood collection, followed by centrifugation at 15–25 °C and 1,500–2,500 $g$ for 15 min using a swing-out (bucket) rotor. The plasma (supernatant) in the tube was divided into units of 1 mL and transferred into a fresh 1.5-mL Eppendorf tube each. In every 10-mL K3-EDTA tube, roughly 4 × 1 mL plasma was collected. All the aliquoted plasma samples were stored at –80 °C before performing IMR assays.

*Sample IMR Assays*

For each plasma sample, the baseline measurements of the levels of Aβ$_{1-40}$, Aβ$_{1-42}$, and T-Tau were done within 3 months after blood draw and are referred to as baseline concentrations. A separate aliquot from each sample was assayed to assess the stability of the measured analytes after storage for 1.1–5.4 years at –80 °C. This is referred to as a repeated concentration. The reagents (MF-AB0-0060, MF-AB2-0060, MF-TAU-0060, and MagQu) used for assaying Aβ$_{1-40}$, Aβ$_{1-42}$, and T-Tau contain magnetic nanoparticles biofunctionalized with antibodies against Aβ$_{1-40}$, Aβ$_{1-42}$, and T-Tau, respectively. For assaying the Aβ$_{1-40}$ and T-Tau levels, 60 μL reagent was mixed with 60 μL plasma. For assaying the Aβ$_{1-42}$ levels, 40 μL reagent was mixed with 80 μL plasma. An IMR reader (XacPro-S, MagQu) was used to detect IMR signals, which were converted to biomarker concentrations via the established relationships between IMR signals and biomarker concentrations. For each biomarker, duplicate measurements were performed.

*Stability Assessment*

To determine the stability of the samples, recovery rates were calculated in each sample and for each storage duration, including baseline, and up to 2, 3, 4, and 5.4 years of storage.

$$\text{Recovery rate } (\%) = \frac{\text{Concentration of repeated measurement}}{\text{Concentration of baseline measurement}} \times 100\%. \tag{1}$$

We also assessed the consistency in identifying aMCI/AD subjects using a cutoff value of 455 $pg^2/mL^2$ between baseline and storage values of the biomarkers. A negative agreement is assigned if the product value of $A\beta_{1-42} \times$ T-Tau is >455 in NC subjects and <455 in subjects with a clinical diagnosis of aMCI or AD. A positive agreement is assigned if the values of the product correctly identified the clinical diagnosis. The rates of positive and negative agreement for each storage duration group were calculated and expressed as positive agreement percentages (PAP) and negative agreement percentages (NAP). These percentages were compared between baseline and storage duration groups to evaluate whether the stability of the biomarkers during storage affects the accuracy of the cutoff values for identifying aMCI/AD subjects.
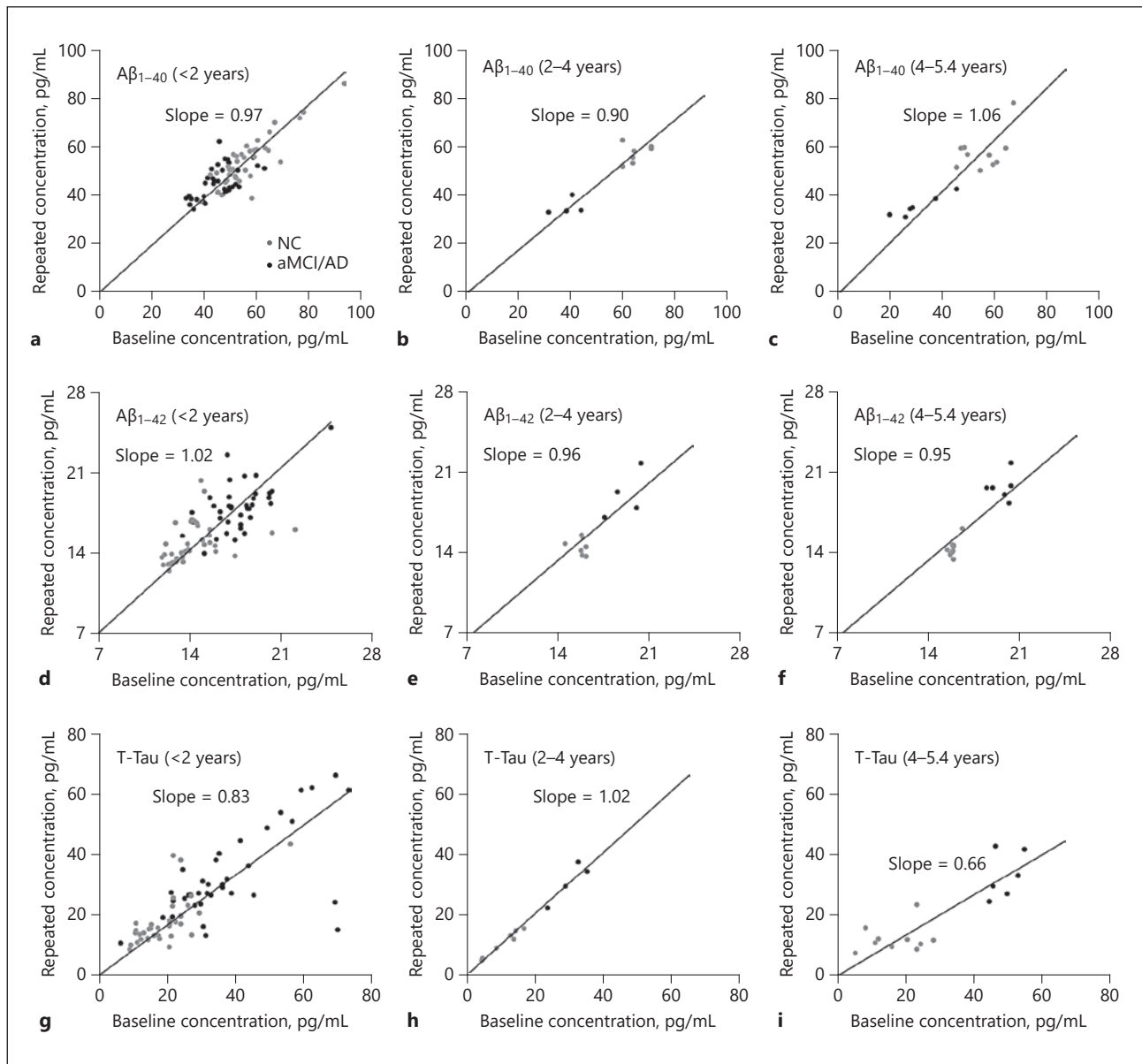
*Statistical Analysis*

Continuous variables for each measurement are presented as mean ± standard deviation. Continuous variables were compared using a *t* test, and the *p* values were determined. Pearson's correlation was done with GraphPad Prism, and *r* was used to describe the correlation between baseline concentrations and repeated concentrations.

## Results

The mean values ± standard deviation for the baseline concentrations of the plasma $A\beta_{1-40}$, $A\beta_{1-42}$, and T-Tau levels from subjects in the NTUH, BSHRI, and combined cohorts are listed in Table 1. It was found that aMCI/AD patients showed lower levels of plasma $A\beta_{1-40}$ ($p < 0.001$) but higher levels of plasma $A\beta_{1-42}$ and T-Tau ($p < 0.001$) than the NC group. These results are consistent with those reported previously [27–30]. After storage for 1.1–5.4 years at –80 °C, repeated measurements of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-Tau in the plasma samples were performed. The repeated concentrations after various periods of storage are plotted versus the baseline concentrations in Figure 1. The repeated concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-Tau with storage periods <2 years are shown in Figure 1a, d, and g, respectively. The repeated concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-Tau with storage periods between 2 and 4 years are shown in Figure 1b, e, and h, respectively. The repeated concentrations of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-Tau with storage periods between 4 and 5.4 years are shown in Figure 1c, f, and i, respectively.

In Figure 1a–c, plasma $A\beta_{1-40}$ baseline and repeated concentrations are shown to be highly correlated for storage periods of <2, 2–4, and 4–5.4 years (<2 years: $r = 0.835$, $p < 0.001$; 2–4 years: $r = 0.932$, $p < 0.0001$; 4–5.4 years: $r = 0.882$, $p < 0.001$). The proportionalities between baseline and repeated concentrations are shown by solid lines. The slopes are found to be 0.97, 0.90, and 1.06 for the various periods of storage. In Figure 1d–f, the Pearson correlation coefficient for the plasma $A\beta_{1-42}$ levels between baseline and repeated concentrations was found to be 0.692 ($p < 0.0001$), 0.883 ($p < 0.01$), and 0.945 ($p < 0.0001$) for storage periods of <2, 2–4, and 4–5.4 years, respectively. The proportionalities between baseline and repeated concentrations of plasma $A\beta_{1-42}$ are shown by solid lines. The slopes are found to be 1.02, 0.96, and 0.95 for the various periods of storage. In Figure 1g–i, a moderate correlation ($r = 0.778$, $p < 0.001$) was found between baseline and repeated concentrations of

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Chiu et al.: Stability of Aβs in Plasma and Tau Protein Using Immunomagnetic Reduction



**Fig. 1.** Correlation between baseline and repeated concentrations of plasma Aβ$_{1-40}$ (**a–c**), Aβ$_{1-42}$ (**d–f**), and T-Tau (**g–i**) using immunomagnetic reduction for various storage periods of <2, 2–4, and 4–4.5 years at –80 °C. The solid line denotes the proportionality between baseline and repeated concentrations. The slope of the proportionality is listed individually.

plasma T-Tau levels for a storage period <2 years. However, a high correlation ($r = 0.988$, $p < 0.0001$) was obtained between baseline and repeated concentrations for a storage period between 2 and 4 years. The correlation between baseline and repeated concentrations for a storage period between 4 and 5.4 years is high ($r = 0.858$, $p < 0.0001$). However, the lower values for the slopes of the proportionality between baseline and repeated concentrations were found for storage periods <2 years (slope = 0.83) and within 4–5.4 years (slope = 0.66).

The results in Figure 1 show that plasma Aβ$_{1-40}$ and Aβ$_{1-42}$ are the most stable among the 3 biomarkers when stored at –80 °C for 5.4 years. Plasma T-Tau levels are lowered by approx-

Chiu et al.: Stability of Aβs in Plasma and Tau Protein Using Immunomagnetic Reduction
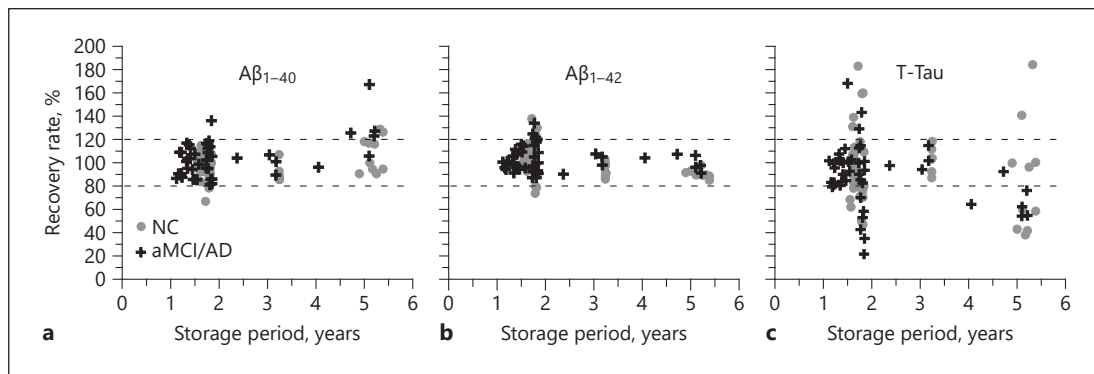


**Fig. 2.** Recovery rate versus storage period for $A\beta_{1-40}$ (**a**), $A\beta_{1-42}$ (**b**), and T-Tau (**c**) in human plasma assayed with immunomagnetic reduction. The dashed lines indicate recovery rates of 120 and 80%.

imately 20% for unknown reasons when it is stored at –80 °C for 2 years, and they are further reduced by 33% when stored for 5.4 years.

We also evaluated the recovery rate of these plasma biomarkers in individual samples using equ. 1, as is shown in Figure 2. All samples showing recovery rates >120% or <80% are considered to have experienced significant changes in biomarker concentration during storage at –80 °C. Such samples are referred as to degraded samples. Recovery rates of 120 and 80% are marked with dashed lines in Figure 2. Each data point corresponds to one individual sample, which was assayed twice: once within 3 months after blood draw and once after a given storage period.

In Figure 2a, no degradation was observed for $A\beta_{1-40}$ samples after 1.5 years of storage. Only 3 plasma samples showed significant changes in $A\beta_{1-40}$ concentrations for storage periods from 1.5 to 5 years, 6 samples showed degradation after storage for 5–5.4 years, accounting for 9.1% of the cases. The samples in which the $A\beta_{1-40}$ concentrations degraded were mainly stored for 5–5.4 years. This result suggests that the stability of plasma $A\beta_{1-40}$ stored at –80 °C is approximately 5 years. Figure 2b shows the storage period-dependent recovery rate for plasma $A\beta_{1-42}$; the detectability of this peptide remained mostly stable up to 1.5 years of storage. Twelve samples stored for 1.5 years showed significant changes in plasma $A\beta_{1-42}$ concentrations. The percentage of $A\beta_{1-42}$ samples that were degraded after 5.4 years was 12.1%. Although there are some degraded samples at 1.5 year, the recovery rate of 2- to 5.4-year-old $A\beta_{1-42}$ samples was within the range of 80–120%. Therefore, $A\beta_{1-42}$ also seems relatively stable until 5.4 years. As shown in Figure 2c, only 1 T-Tau sample was degraded within a 1.5-year storage period at –80 °C. However, 36 T-Tau samples that had been stored for >1.5 years were degraded. The percentage of degraded human plasma T-Tau samples that had been stored for 5.4 years at –80 °C was 37.4%. The results in Figure 2b and c reveal that plasma $A\beta_{1-42}$ and T-Tau at –80 °C are stable for 1.5 years, shorter than $A\beta_{1-40}$.

## Discussion

In this study, we have investigated the effects of storage durations up to 5.4 years on the detection levels of AD biomarkers in plasma by IMR. We have found analyte-specific changes in relation to storage time. Among the 3 analytes that we had evaluated, $A\beta_{1-40}$ and $A\beta_{1-42}$ remained stable throughout the storage duration, while T-Tau was the least stable, showing

Chiu et al.: Stability of Aβs in Plasma and Tau Protein Using Immunomagnetic Reduction

**Table 2.** Analysis of the numbers of NCs and aMCI/AD patients based on clinical diagnosis or the plasma biomarkers $A\beta_{1-42} \times$ T-Tau at baseline and repeated measurements

| Measurement | Storage period, years | $n^{\#}$ | Plasma biomarkers | Clinical diagnosis | |
|---|---|---|---|---|---|
| | | | | NC | aMCI/AD |
| Baseline | ≤0.25 | 99 | NC[a] | 50 | 6 |
| | | | aMCI/AD[b] | 3 | 40 |
| Repeated | ≤2 | 72 | NC[a] | 33 | 11 |
| | | | aMCI/AD[b] | 3 | 25 |
| | ≤3 | 73 | NC[a] | 33 | 11 |
| | | | aMCI/AD[b] | 3 | 26 |
| | ≤4 | 83 | NC[a] | 40 | 11 |
| | | | aMCI/AD[b] | 3 | 29 |
| | ≤5.4 | 99 | NC[a] | 50 | 12 |
| | | | aMCI/AD[b] | 3 | 34 |

The cutoff value of the plasma biomarkers $A\beta_{1-42} \times$ T-Tau is 455 $pg^2/mL^2$ for discriminating aMCI/AD from NC.

NC, normal cognition; aMCI, amnestic mild cognitive impairment; AD, Alzheimer's disease.
[#] Total numbers of subjects in the various storage periods.
[a] NC, $A\beta_{1-42} \times$ T-Tau ≤455 $pg^2/mL^2$; [b] aMCI/AD, $A\beta_{1-42} \times$ T-Tau >455 $pg^2/mL^2$.

discrepant detection values from baseline in a higher percentage of samples for a storage period <2 years. The long-term storage stability of CSF biomarkers for AD has been investigated [33, 34]. In these studies, the storage period of CSF samples at –80°C ranged from 2 to 15 years. The concentrations of CSF biomarkers were measured with enzyme-linked immunosorbent assay (ELISA). It has been reported that the concentrations of $A\beta_{1-42}$ and T-Tau in CSF, stored for years, are reliable. The correlation coefficients for both CSF $A\beta_{1-42}$ and T-Tau concentrations between baseline and repeated measurement were >0.7. However, CSF $A\beta_{1-40}$ showed a lower correlation coefficient ($r = 0.53$), which implies that CSF $A\beta_{1-40}$, in stored samples, is more degradable as compared to $A\beta_{1-42}$ and T-Tau. The studies on long-term storage stability of AD biomarkers, in CSF samples, showed different results from ours in stored plasma samples. At present, the reason for the difference in storage stabilities of $A\beta_{1-40}$, $A\beta_{1-42}$, and T-Tau between CSF and plasma is not clear. It might possibly be due to the biochemical environment of CSF and plasma. In addition, the assay methods used for the detection of the biomarker levels could be another reason.

For each subject, there are two kinds of criteria for the diagnosis of aMCI/AD in this work: clinical diagnosis and plasma biomarkers. The level of agreement in diagnosing aMCI/AD based on these two criteria was investigated. For plasma biomarkers, the levels of $A\beta_{1-42}$, T-Tau, and their combination, measured by IMR technology, carry promising potential to be useful in distinguishing aMCI and AD patients from NCs [27–30]. According to the literature [29, 30], the cutoff values for discriminating NCs from aMCI/AD cases are 16.33 pg/mL, 23.89 pg/mL, and 455 $pg^2/mL^2$ for plasma $A\beta_{1-42}$, T-Tau, and $A\beta_{1-42} \times$ T-Tau, respectively. These cutoff values were applied to divide our subjects into NCs and aMCI/AD patients in this study. Meanwhile, the division into NCs and aMCI/AD based on clinical diagnosis is done for each subject.

The NAP, PAP, and overall agreement percentage (OAP) for discriminating aMCI/AD patients from NCs between clinical diagnosis and plasma biomarkers can be analyzed. An example investigating the agreement between clinical diagnosis and the plasma biomarkers $A\beta_{1-42} \times$ T-Tau is given in Table 2. At baseline, 3 of 53 NCs showed $A\beta_{1-42} \times$ T-Tau levels >455

**Table 3.** NAP, PAP, and OAP for discriminating NCs from aMCI/AD patients based on two different criteria: clinical diagnosis and plasma biomarkers

| Measurement | Storage period, years | Plasma Aβ$_{1-42}$ | | | Plasma T-Tau | | | Plasma Aβ$_{1-42}$ × T-Tau | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | NAP, % | PAP, % | OAP, % | NAP, % | PAP, % | OAP, % | NAP, % | PAP, % | OAP, % |
| Baseline | ≤0.25 | 92.4 | 84.8 | 88.9 | 79.3 | 87.0 | 82.8 | 94.3 | 87.0 | 90.9 |
| Repeated | ≤2 | 77.8 | 83.3 | 80.6 | 86.1 | 77.8 | 81.9 | 91.7 | 69.4 | 80.6 |
| | ≤3 | 77.8 | 83.8 | 80.8 | 86.1 | 78.4 | 82.2 | 91.7 | 70.3 | 80.8 |
| | ≤4 | 81.4 | 85.0 | 83.1 | 88.4 | 77.5 | 83.1 | 93.0 | 72.5 | 84.1 |
| | ≤5.4 | 84.9 | 87.0 | 85.9 | 90.6 | 78.3 | 84.8 | 94.3 | 73.9 | 84.9 |

For plasma biomarkers, the diagnosis of aMCI/AD can be based on the combination of Aβ$_{1-42}$ and T-Tau levels or their individual levels. The cutoff values for discriminating NCs from aMCI/AD are 16.33 pg/mL, 23.89 pg/mL, and 455 pg$^2$/mL$^2$ for plasma Aβ$_{1-42}$, T-Tau, and Aβ$_{1-42}$ × T-Tau, respectively [29].

NAP, negative agreement percentage; PAP, positive agreement percentage; OAP, overall agreement percentage; NCs, normal controls; aMCI, amnestic mild cognitive impairment; AD, Alzheimer's disease.

pg$^2$/mL$^2$. The NAP is 50/53 = 94.3%. Moreover, 6 of 46 patients showed Aβ$_{1-42}$ × T-Tau levels <455 pg$^2$/mL$^2$. The PAP is 40/46 = 87.0%. The OAP is thus (50 + 40)/99 = 90.9%. This reveals that the overall consistency in diagnosing aMCI/AD based on clinical diagnosis and plasma biomarkers is 90%.

The agreement for other baseline and repeated measurements using plasma Aβ$_{1-42}$, T-Tau, and Aβ$_{1-42}$ × T-Tau was analyzed. The results are listed in Table 3. It was found that the OAP varies between 80.6 and 90.9% for baseline and repeated measurements after a storage period of 2–5.4 years at –80°C. Hence, the high consistency (>80%) in discriminating aMCI/AD from NCs between clinical diagnosis and plasma biomarkers occurs regardless of the length of storage time at –80°C. The observed instability in Figure 2 may only matter for the evaluation of historical samples, but it will have no impact on the diagnostic utility of these assays. It is worth noting that the stability results need to be considered if anybody tries to use IMR assays on archived samples from registries, in order for the results to be interpreted correctly depending on the context and goals of the evaluation.

## Conclusion

IMR shows that plasma Aβ$_{1-40}$ and Aβ$_{1-42}$ exhibit long-term stability during 5.4 years of storage at –80°C, but the stability of plasma T-Tau is reduced beyond approximately 1.5 years. Nevertheless, this instability seems not to seriously affect the accuracy of discriminating aMCI/AD patients from NCs, when the product of plasma Aβ$_{1-42}$ and T-Tau concentrations is used as a diagnostic parameter. Hence, both fresh and long-term-stored (≤5.4 years at –80°C) human plasma samples are useful for the diagnosis of AD when plasma Aβ$_{1-42}$ and T-Tau concentrations are assayed using IMR.

## Disclosure Statement

H.H. Chen and S.Y. Yang are employees of MagQu. S.Y. Yang is one of the shareholders of MagQu. M.J. Chiu, L.F. Lue, M.N. Sabbagh, and T.F. Chen do not have any conflicts of interest to declare.

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Chiu et al.: Stability of Aβs in Plasma and Tau Protein Using Immunomagnetic Reduction

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