



Advance in Plasma AD Core Biomarker Development: Current Findings from Immunomagnetic Reduction-Based SQUID Technology

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ABSTRACT

New super-sensitive biomarker assay platforms for measuring Alzheimer's disease (AD) core pathological markers in plasma have recently been developed and tested. Research findings from these technologies offer promising evidence for identifying the earliest stages of AD and correlating them with brain pathological

progression. Here, we review findings using immunomagnetic reduction, one of these ultrasensitive technologies. The principles, technology and assays developed, along with selected published findings will be discussed. The major findings from this technology were significant increases of amyloid beta (A β) 42 and total tau (t-tau) levels in subjects clinically diagnosed with early AD when compared with cognitively normal control (NC) subjects. The composite marker of the product of A β 42 and t-tau discriminated subjects with early AD from NC subjects with high accuracy. The potential of this technology for the purpose of early or preclinical disease stage detection has yet to be explored in subjects who have also been assessed with brain imaging and cerebrospinal fluid AD core biomarker measurements.

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Key Summary Points

Blood-based biomarkers are needed for identifying preclinical and/or high-risk subjects.

Assays based on immunomagnetic reduction (IMR) and superconducting quantum interference device (SQUID) have consistently shown high sensitivity and specificity for discriminating between normal controls and subjects with early to mild Alzheimer's diseases (AD).

IMR-assayed A β 42 in plasma increased in AD, opposite of CSF A β 42 in AD; total tau (t-tau) levels in plasma and CSF are both increased in AD, comparing to levels in NC. Composite markers such as the product of A β 42 and t-tau increased the sensitivity as discriminators.

The potential to use IMR-assayed AD markers as surrogate for amyloid PET imaging to screen potential subjects for clinical trials is promising.

INTRODUCTION

Blood-based Alzheimer's disease (AD) biomarkers could have strategic roles in identifying suitable subjects before they are enrolled for neuroimaging and in clinical trials of disease-modifying therapeutics. Among the candidates as plasma biomarkers, the core AD markers amyloid beta (A β) and tau have been the most investigated. Recently, novel approaches and new technologies that increase the sensitivity and accuracy of the measurements of plasma A β 42, total tau (t-tau), phosphorylated tau (p-tau), and neurofilament light (NFL) have emerged. There could be tremendous value in creating biomarker panels as screening tools for the assessment of degrees of AD pathology, much like hemoglobin A1C (HbA1C) for diabetes or prostate-specific antigen (PSA) for prostate cancer diagnosis.

There is a consensus that the current strategy for treating AD is not effective for slowing disease progression in subjects with clinically diagnosed AD [1]. A better strategy for modifying this disease would be to start treatments prior to the appearance of overt clinical symptoms; this requires methods to identify disease prior to cognitive impairment. As a result of recent failures in costly disease-modifying clinical trials, there is increased attention on lifestyle intervention or preventive approaches to preserve cognition with aging and prevent AD from developing [2–4]. One consistent issue raised with the failed clinical trials is that patients are treated too late in the disease process. Hence, the assessment of the effects of pharmacological or lifestyle approaches will benefit from the availability of routine, reliable, and easy-to-provide tests that measure the progression of disease pathological features as early as possible [5].

Using blood as the source of biomarkers has advantages of high repeatability, convenience, low cost, and minimal invasiveness. Thus, the development of blood-based molecular biomarkers to identify subjects with cognitive decline from those with normal cognition has become a research focus in recent years. If new technologies for blood biomarker measurement could provide accuracy comparable to cerebrospinal fluid (CSF) biomarkers in classifying cognitively normal control (NC), mild cognitive impairment (MCI), and subjects with early AD, the utility of blood-based biomarkers for clinical diagnosis or even preclinical stage detection of AD might become a reality.

NEW ULTRASENSITIVE BIOMARKER TECHNOLOGIES

Innovative approaches and ultrasensitive technologies for blood-based biomarkers have been developed in last few years to address the limitations of the widely used enzyme-linked immunosorbent assay (ELISA) methodology. These technologies include immunomagnetic reduction (IMR), single molecule assay (SIMOA), immuno-infrared sensor technology, multimer detection system (MDS),

immunoprecipitation/mass spectrometry (IP/MS), and liquid chromatography–mass spectrometry (LC–MS/MS) [6–12]. Except the LC–MS/MS analysis, all the technologies employ specific antibodies to target antigens, like ELISA, but incorporate new technologies to enhance sensitivity and accuracy of measurements. The MDS is a platform which uniquely targets A β oligomers. MS-based platforms have the advantages of obtaining both quantitative and qualitative information in plasma samples. This review will focus on IMR technology and results obtained using it, but will compare with findings using SIMOA, the main technology that has been widely used in AD biomarker research studies. We will first discuss some of the challenges in accurately and reliably measuring the AD pathological markers A β and tau in blood; then we review principles, assays, and findings with IMR methods. Lastly, we suggest what might be needed to bring this technology forwards as AD biomarkers in defined context of use.

This article is based on previously conducted studies and does not contain any studies with human participants or animals performed by any of the authors.

CHALLENGES IN MEASURING AD PATHOLOGICAL COMPONENTS A β AND TAU IN BLOOD

As a result of their established association with amyloid plaques and neurofibrillary tangles, the AD brain pathological hallmarks, most biomarker studies in plasma and CSF have focused on measuring levels of A β (1–40) and A β (1–42), t-tau, and p-tau. Several studies have reported on measurements of plasma A β peptides in attempts to validate them as AD biomarkers, including identifying the threshold A β concentrations that correlate with disease stages. However, most of the previous studies based on conventional ELISA on plasma A β yielded conflicting results, and showed very marginal or no differences between patients with AD and NC subjects [13–16]. Problems have been identified with A β measurements in plasma in terms of biological and technical aspects. Firstly, the

pool of plasma A β peptides are not stable, probably because of the presence of multiple sources of A β as well as ongoing production and degradation. Levels of A β peptides in biofluids fluctuated widely over time and varied among individuals [14]. Technically, the most commonly used analytical method ELISA involves specific antigen–antibody interactions, which can be easily affected by molecular hindrance due to high concentrations of proteins naturally present in plasma samples. In addition, levels of A β are also sensitive to methods of blood collection and processing and differences in the types of ELISA [17].

Both brain and peripheral tissues contribute to the pool of A β in the plasma. Soluble forms of A β species in the brain can enter the circulation through low-density lipoprotein receptor-related protein-mediated transport across the blood–brain barrier [18]. More recently, the astrocyte-mediated glymphatic transport system and meningeal lymphatic vessels have been suggested to provide important pathways for brain A β to enter the peripheral circulation [19–21]. Peripheral tissues also produce A β , as amyloid precursor protein (APP) mRNA has been detected in the heart, liver, kidney, pancreas, lymph nodes, spleen, skeletal muscle, aorta, lung, skin, intestines, testis, leukocytes, platelets as well as in the salivary, thyroid, and adrenal glands [22–29]. Among these tissues/cells, A β peptides have been biochemically or immunochemically identified in skeletal muscle, platelets, vascular walls, and liver [14, 26, 30, 31]. Platelets have been suggested to be the primary source of circulating A β . Upon activation, platelets secrete substantial amounts of A β peptides, especially A β 40 [26]. The average level of A β in the serum (after platelet activation) was about 1.6-fold higher than in serum where platelets are not activated [26]. Together, these distinct reservoirs will contribute to plasma A β separately from what is derived from the brain.

Recent studies have found that the amount of A β in the brain correlates with wakefulness [32]. Sleep deprivation acutely increases levels of A β in the interstitial fluid (ISF). Furthermore, the concentration of A β in the brain fluctuates with the sleep–wake cycle [33]. It remains

unclear whether such a diurnal fluctuation of A β concentrations occurs in the blood.

Once A β enters the plasma, it appears to have a short half-life. Using an approach that combined stable isotope labeling kinetics (SILK) with LC–MS/MS analysis, Ovod et al. found that A β isoforms have a mean half-life of approximately 3 h [8]. Interestingly, A β 42 has a faster turnover rate than A β 40.

The accurate measurement of free plasma A β levels can be confounded by its interactions with plasma/serum proteins. A β species are known to interact specifically or non-specifically with a large number of plasma proteins, including albumin, α_2 -macroglobulin, α_1 -antichymotrypsin, amyloid P component, complement proteins, transthyretin, apoferritin, hemoglobin, fibrinogen, apolipoproteins, and lipoproteins [31, 34–37]. In addition to binding to plasma proteins, A β is also known to interact with blood cells, including erythrocytes and macrophages [38]. Binding of A β to plasma proteins can mask the epitopes for specific antibodies used in different assays [36, 39, 40]. It has been reported that to accurately quantify this “sequestered” pool of A β , it is necessary to release A β from binding molecules [41].

Plasma levels of t-tau, p-tau, or a combination of both have also been under development as AD biomarkers. Validating plasma tau as an AD biomarker has encountered different challenges compared to A β . Significant changes in plasma tau between patients with AD and controls were evident in some studies [42–45]. However, the specificity of plasma tau alone as an AD biomarker appears relatively low, possibly because extracellular tau represents evidence of non-specific neuronal injury [46]. Increased plasma tau levels have been associated with several neurodegenerative diseases, not just AD [46–49]. Therefore, it has been generally accepted that plasma tau is not a reliable and specific AD biomarker [49, 50]. Similar observations can also be applied to other neural injury biomarkers present in plasma, such as NFL [51].

INNOVATIVE TECHNOLOGIES: IMMUNOMAGNETIC REDUCTION (IMR)

Magnetic particles have many useful applications in biomedical field: biosensing, separation of cellular components and molecules, imaging, and delivery of therapeutics. The IMR biomarker detection system uses antibody-conjugated magnetic nanoparticles as bioprobes and a high T_C , alternating current (ac) magnetosusceptometer, superconducting quantum interference device (SQUID), to detect the amounts of antigens bound to the bioprobes [52, 53]. Several assays are available from this technology for the quantification of pathological markers involved in neurodegenerative diseases (<http://www.magqu.com>). AD-focused IMR assays and their associated technology are described in the following sections (Fig. 1).

Assay Reagents

IMR assay reagents contain antibody-conjugated magnetic nanoparticles suspended in pH 7.4-phosphate-buffered saline (PBS). The magnetic nanoparticles are 50–60 nm in size and have magnetite (Fe₃O₄) in the core and hydrophilic dextran coating on the surface for antibody conjugation. The concentration of the magnetic particles is 8 mg Fe/ml. For the AD-focused markers A β 40, A β 42, and t-tau, the IMR assay reagents contain anti- β -amyloid (A3981, Sigma-Aldrich, St. Louis, MO, USA), anti- β -amyloid 37–42 (ab34376, Abcam, Cambridge, UK), and anti-tau (T9450, Sigma)-conjugated magnetic nanoparticles. The IMR assay reagents remain homogeneously suspended for 10 months at 2–8 °C storage [43, 52, 54].

Assay Preparation

Plasma samples are obtained from whole blood collected in K₂EDTA tubes after centrifugation at 1500–2500 *g* for 15 min at room temperature. Aliquots of plasma samples need to be stored at –80 °C and assayed within 1 year. During preparation of the assay, both samples and IMR reagents are brought to room temperature and

ImmunoMagnetic Reduction Assays

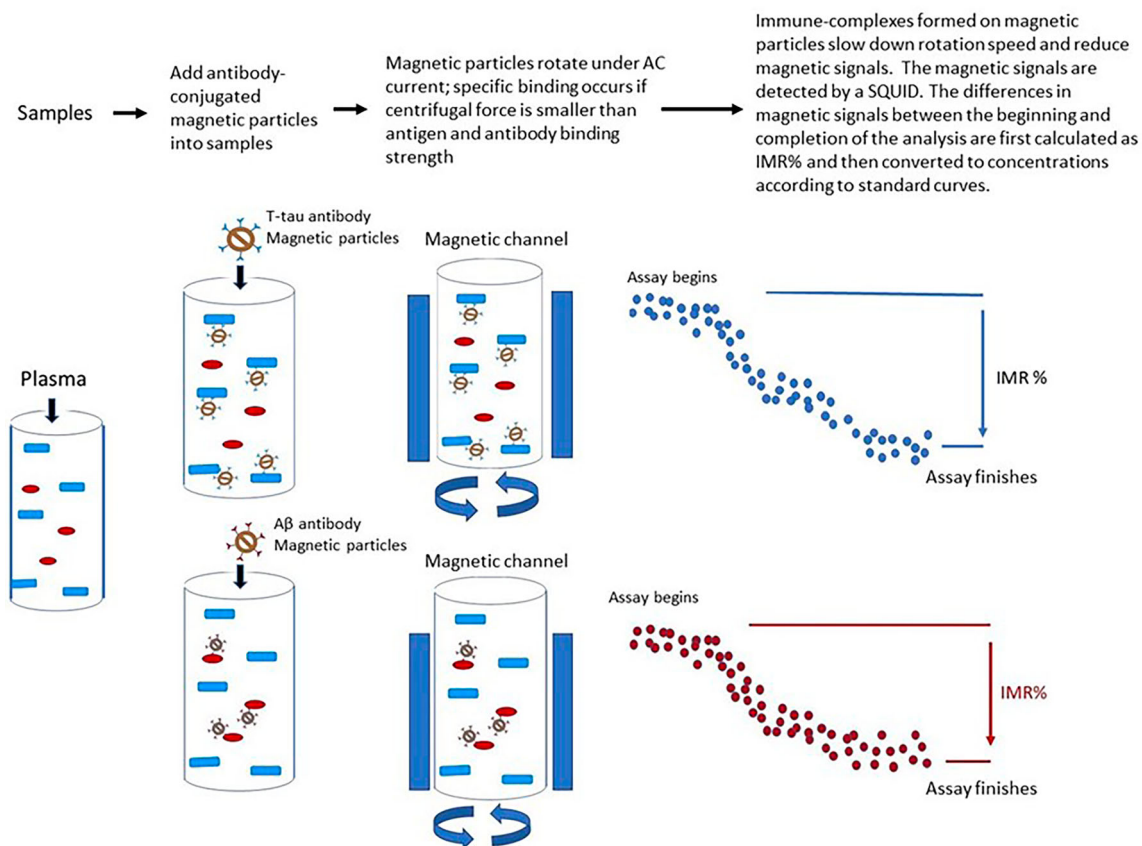


Fig. 1 Illustration of immunomagnetic reduction assays

mixed in differing sample-to-reagent ratios depending on the markers being measured: for example, 40 µl of plasma sample is mixed with 80 µl of IMR reagents for t-tau (MF-TAU-0060, MagQu), p-Tau (MF-PT1-0060), Aβ40 (MF-AB0-0060, MagQu); 60 µl of sample is mixed with 60 µl of reagent (MF-AB2-0060, MagQu) for Aβ42 assay. The total volume of assay reagent and sample mixture is 120 µl. Sample and IMR assay reagent are mixed by vortexing, followed by centrifugation to settle floating particulates and DNA. The tubes are immediately placed into individual holders inside the assay cells of the equipment (Model χ_{ac} Pro-S, MagQu) for immunoreaction.

Magnetic Signal Collection

Magnetic nanoparticles oscillate in a magnetic field generated by external ac. The feature of the

magnetic signals corresponding to oscillation pace and magnitude in mixed frequency ac is termed mixed-frequency ac magnetic susceptibility, χ_{ac} . In the current model of the IMR-SQUID equipment, each sample holder is surrounded by a set of excitation coils and a pickup coil. The excitation coils are driven by ac voltage generators, and magnetic signals emitted from assay mixtures are picked up by an axial gradiometer and transmitted in real time to a flux coupling coil connecting to the SQUID. The SQUID is immersed in an optimal amount of liquid nitrogen and shielded from potential noise-generating interfering environmental magnetic sources.

During sample assay, magnetic nanoparticles become larger as a result of antigen binding with antibody and eventually immune complexes form, which result in reduction of oscillation and changes in magnetic signals. Thus,

by comparing magnetic signals before immune reaction ($\chi_{ac,0}$) and after immune reaction (χ_{ac}), the percentage of IMR% can be calculated with the equation $[(\chi_{ac,0} - \chi_{ac})/\chi_{ac}] \times 100\%$. The real-time magneto-susceptibility during the assay is recorded for 4–5 h [10]. During the initial 100 min, high-pace magnetic signals χ_{ac} are picked up, representing a stage when immune complexes are not yet formed. In the following 100 min or so, χ_{ac} descends gradually during formation of immune complexes. When high-affinity binding forms stable immune complexes, χ_{ac} signals also become stable. A 4- to 5-h recording provides the basis for the IMR% calculation [52, 55, 56].

From IMR% to Concentrations

The concentrations of markers being analyzed can be calculated from IMR% by reference to a standard curve specific for the marker being analyzed. An example of the equation for marker concentration ($\varnothing_{\text{marker}}$) and IMR% is shown below (A , B , γ are fitting parameters):

$$\text{IMR}\% = \left[\frac{A - B}{1 + \left(\frac{\varnothing_{\text{marker}}}{\varnothing_0}\right)^\gamma} + B \right] \times 100\%.$$

A standard curve for each marker is usually established first after the IMR-SQUID analyzer is assembled and set up in a laboratory. Once established, each sample assay run only required inclusion of standard proteins at the highest and lowest concentrations, along with an IMR reagent control. Currently, the IMR-SQUID analyzer is a 36-magnetic channel software-guided system (model χ_{ac} -Pro-S). The equipment can analyze 3–4 different samples in duplicate if three markers, A β 40, A β 42, and t-tau, are to be simultaneously assayed. At completion of an assay, IMR% and corresponding concentrations are calculated automatically by the machine software. The lower detection limits of various IMR assays are in the low pico- to femto-gram per milliliter range according to MagQu company's information (Table 1). These values indicate that IMR-SQUID technology is an ultrasensitive

Table 1 Lower detection limits of IMR assays for neurodegenerative disease markers

IMR assay targets	Low detection limit (LoD), fg/ml
A β 40	170
A β 42	770
t-tau	26
p-tau	19.6
α -Synuclein	1.3
p- α -Synuclein-129	0.072
TDP-43	0.68
Neurofilament light chain (NFL)	3.3

immunoassay platform for the quantification of low-abundance proteins.

Long-Term Sample Storage

The effect of long-term sample storage on IMR assays was evaluated recently in plasma samples collected from 53 NCs and 46 subjects with amnesic mild cognitive impairment (aMCI) or AD [57]. In this study, plasma samples stored at -80°C for at least 3 months after sample collection were compared to measurements of aliquots from the same case after storage for 1.1–5.4 years. The results showed that A β 40 and A β 42 levels were not affected by storage for up to 5.4 years at -80°C , but t-tau levels were reduced by 20% after storage for 2 years at -80°C , and by 33% after storage for 4–5 years. The study also assessed whether storage-related decreases in t-tau measures affected the ability of the composite marker of A β 42 \times t-tau to discriminate between AD and NC but found little effect. It is unclear why t-tau levels are more prone to decline during storage. It remains to be determined whether plasma t-tau is more susceptible to aggregation, degradation, or association with other molecules during or after low temperature storage; these factors could result in loss of epitopes or weaker binding with antibody. A comprehensive study that assessed the effects of various pre-analytical

procedures on plasma AD markers showed that most factors affecting A β also affected t-tau, but decrease in t-tau was particularly noticeable when tubes with half-full samples were examined [17]. There were 12–14% reductions in plasma t-tau levels in half-filled aliquot tubes, which would suggest that sample drying (freezer burn) or oxidation was a factor in t-tau stability.

CURRENT FINDINGS IN AD BIOMARKERS USING IMR ASSAYS

Most of the developed IMR assays for AD pathological markers have focused on A β 40, A β 42, and t-tau and their measurements in clinically diagnosed patients. The IMR assay for p-tau is new with only one publication [54] and will not be featured in this review. Most published findings have assessed the potential of A β 40, A β 42, and t-tau in discriminating clinically diagnosed groups; some have also assessed the relationship between levels of IMR plasma markers and amyloid PET and MRI imaging. A summary of major findings in research using IMR technology is outlined in Table 2 [52, 55, 58–62]. The table is organized from most recent publications to earliest publications. In addition to highlights of the major finding, the number of subjects studied and type of IMR assays used are also indicated.

Levels of IMR Plasma AD Markers

Compiled in Table 3 are the ranges of means for each marker reported in the IMR studies. The ranges of the mean A β 42 levels were 15.3–16.1 for NC and 16.8–34.2 pg/ml AD, while the ranges of mean t-tau levels were 13.5–20.5, 29.7–33.5, and 34.5–47.1 pg/ml for NC, clinically diagnosed MCI, and AD, respectively. A β 40 concentrations detected have higher values than t-tau and A β 42; the ranges of the means were 59.2–65.8, 41.4–48.0, and 36.9–53.2 pg/ml for NC, MCI, and AD, respectively.

As IMR employs a very different technology from frequently used ELISA, the reported range

of AD marker levels can be expected to be different from those reported using ELISA. The best example is illustrated in a systematic review and meta-analysis of plasma t-tau [63]. The plasma t-tau values by ELISA were between 200 and 900 pg/ml. However, the digital ELISA technology, SIMOA (Quanterix, MA, USA), which is gaining popularity for plasma tau measurements, detected t-tau levels in low picogram per milliliter values. The discrepancy in detected values in published studies is most likely related to the antibodies used and assay features of the platforms; however, sample processing, demographics and cognitive stages of the study subjects, and experimental design will also be factors. The comparison of biological markers between different platforms has to be approached with caution because of these many factors involved [64].

How plasma marker levels assayed by IMR compared to CSF levels assayed by ELISA was investigated in a recent study consisting of 43 NC and 63 European Caucasian subjects with AD [65]. The ratios of plasma to CSF A β 42 were 1.6% in NC and 4.1% in patients with AD. In this study, the increase in A β 42 levels in plasma samples from subjects with AD was highly significant ($P < 0.001$), consistent with other IMR findings. A moderately negative correlation between plasma and CSF A β 42 levels ($r = -0.352$) in AD and a weakly positive correlation in NC ($r = 0.186$) were detected. In this study, Teunissen et al. suggested two potential explanations for the inverse correlation in AD [65]. Firstly, it could be due to a disease-associated compensatory mechanism that increases central to peripheral clearance of A β 42 when brain amyloid accumulates. Secondly, the differences could be caused by the abundance of carrier proteins to transport A β .

To elucidate the dynamic mechanisms affecting CSF and plasma A β levels, more research is needed. As the assay platform is a technical factor affecting the outcomes of assays, more valid comparisons between CSF and plasma markers can be made if samples are analyzed with the same assay. In a study using INNO-BIA ELISA for both plasma and CSF samples (Fujirebio Europe NV, formerly Innogenetics, Belgium), the mean plasma A β 42

Table 2 Major findings from studies using IMR assays of plasma AD pathological markers

Study, year	Diagnosis	Imaging	Assays	Major findings
Fan et al. 2018 [60]	39 NC, 25 aMCI, 16 AD	¹¹ C-PiB PET, MRI	Aβ40 Aβ42 t-tau	t-tau at 25.57 pg/ml cutoff identified PiB+ group at 93% sensitivity and 100% specificity; Aβ42 at 16.81 pg/ml cutoff had 87% sensitivity and 82% specificity. As for PiB– group, t-tau at 37.54 pg/ml and Aβ42 at 21.92 pg/ml had 100% sensitivity and specificity
Yang et al. 2018 [54]	23 NC, 29 MCI, and 21 early AD	NA	p-tau ₁₈₁ t-tau	p-tau ₁₈₁ levels were significantly increased in MCI and early AD. To discriminate NC from the diseased subjects, 3.08 pg/ml cutoff achieved 79% sensitivity and 84% specificity
Yang et al. 2017 [43]	66 NC, 24 MCI, 29 AD, 41 PD, 26 FTD, 29 VD	NA	t-tau	t-tau levels were lowest in NC and highest in AD (NC < VD < PD < MCI < FTD < AD); at 17.43 pg/ml cutoff t-tau had 86% sensitivity and 74% specificity for discriminating between NC and diseased subjects
Yang et al. 2017 [55]	68 NC, 24 MCI, 31 early AD	NA	Aβ42 t-tau	Aβ42 × t-tau at 455.5 pg/ml discriminated AD from NC at 95% sensitivity and 97% specificity
Lue et al. 2017 [61]	77 NC, 47 early AD	NA	Aβ42 t-tau	Composite marker Aβ42 × t-tau cutoff at 382.68 (pg/ml) ² distinguished AD from NC at 92% accuracy
Tzen et al. 2014 [62]	20 NC, 25 aMCI and early AD	¹¹ C-PiB PET	Aβ40 Aβ42 t-tau	To discriminate aMCI and AD combined from NC, t-tau 28.27 pg/ml cutoff had 92% sensitivity and 100% specificity; Aβ42/Aβ40 at cutoff 0.3693 had 84% sensitivity and 100% specificity; correlation ($R^2 = 0.326-0.449$, $P < 0.001$) was detected between Aβ42/Aβ40 ratios and PiB-measured brain Aβ deposition
Chiu et al. 2014 [59]	30 NC, 20 MCI, 10 early AD	MRI	t-tau	Significant negative correlation between t-tau and volumes of total gray matter, hippocampus, and amygdala, and functional measures (logical memory, visual reproduction, and verbal fluency)
Chiu et al. 2013 [58]	66 NC, 22 MCI, 45 AD	NA	Aβ40 Aβ42 t-tau	Best discriminator between NC and patients (MCI and AD combined) was the composite marker of Aβ42 × t-tau at 96% sensitivity and 97% specificity

Table 2 continued

Study, year	Diagnosis	Imaging	Assays	Major findings
Chiu et al. 2012 [52]	26 NC, 16 MCI, 18 AD	NA	A β 40 A β 42	To discriminate aMCI and AD from NC, A β 42 at 16.1 pg/ml cutoff had 85% sensitivity and 89% specificity; A β 42/A β 40 ratio at 0.303 cutoff had 85% sensitivity and 96% specificity

AD Alzheimer's disease, *aMCI* amnesic mild cognitive impairment, ¹¹C-*PiB* Pittsburgh compound, *FTD* frontotemporal dementia, *MCI* mild cognitive impairment due to AD, *MRI* magnetic resonance imaging, *NA* not applicable, *NC* normal control, *PET* positron-emission tomography, *PD* Parkinson's disease, *VD* vascular dementia

Table 3 AD core marker levels quantified by IMR assays

Study, year	Group	<i>N</i>	A β 40 Mean (SD)	A β 42 Mean (SD)	t-tau Mean (SD)
Fan et al. 2018 [60]	NC	39	59.2 (11.1)	16.1 (1.8)	14.3 (6.4)
	MCI	25	41.5 (3.9)	17.0 (2.0)	29.7 (8.7)
	AD	16	39.5 (5.8)	19.0 (2.7)	39.4 (5.8)
Teunissen et al. 2018 [65]	NC	43		15.5 (2.1)	
	AD	63		17.9 (4.3)	
Lue et al. 2017, BSHRI cohort [61]	NC	16		15.3 (2.8)	20.5 (4.8)
	AD	16		16.8 (1.6)	34.5 (15.2)
Lue et al. 2017, NTUH cohort [61]	NC	61		15.8 (0.4)	14.9 (3.2)
	AD	31		18.6 (0.8)	47.1 (13.2)
Tzen et al. 2014 [62]	NC	20	60.9 (6.4)	15.9 (0.3)	13.5 (5.5)
	MCI	11	41.4 (1.8)	17.2 (0.3)	33.5 (2.2)
	AD	14	36.9 (1.6)	18.9 (0.3)	46.7 (2.0)
Chiu et al. 2012 [52]	NC	26	65.8 (13.5)	15.8 (0.6)	
	MCI	16	48.0 (16.2)	22.7 (20.8)	
	AD	18	53.2 (34.7)	34.2 (31.6)	

AD Alzheimer's disease, *BSHRI* Banner Sun Health Research Institute, *MCI* mild cognitive impairment due to AD, *NC* normal control, *NTUH* National Taiwan University Hospital, *SD* standard deviation

concentrations were 36.9 ± 11.7 pg/ml from 426 AD samples and 38.2 ± 11.9 pg/ml from 373 subjects with aMCI [66]. The ratios of plasma to CSF A β 42 levels were 5.1% and 4.4%, respectively, for AD and aMCI groups. The study did not detect any correlation between CSF and plasma samples for A β 42 levels.

Although the ELISA-based platform is adequate for measuring AD core markers in CSF samples as CSF contains lower concentrations of potentially interfering proteins, there is an advantage if the same markers in CSF and plasma samples from the same patient could be measured by the same assay platform. This could provide a better assessment of the

interrelationship of these markers between central and peripheral systems.

There have been no published IMR-assayed values for CSF AD markers, but to perform initial assessment of IMR for CSF samples, we carried out a pilot study using CSF samples from nine elderly subjects enrolled at the Banner Sun Health Research Institute (BSHRI, Sun City, AZ, USA). Although undiluted CSF samples were within the dynamic range of IMR assays, a 20-fold dilution was needed to achieve optimal IMR sensitivity. The CSF A β 42 and t-tau concentrations detected were approximately 3- to 4-fold greater than reported for CSF A β 42 levels assayed by multiplexed ELISA platforms, such as the multiplex xMAP Luminex platform (Luminex Corp., Austin, TX, USA) and the INNOBIA AlzBio3 kit (Innogenetics, Ghent, Belgium) (our unpublished data). Further studies to directly compare IMR-assayed CSF and IMR-assayed plasma results from the same subjects could determine if they correlate. The differences in the detected levels between platforms might not be surprising. IMR is an ultrasensitive platform and has best sensitivity in the low picogram per milliliter range. To determine protein concentrations which are abundant, it requires samples to be diluted to ensure the signals are within optimal range of linearity. When the detected results are corrected by the dilution factor, the numbers could represent extrapolated results in some cases.

Plasma AD Marker Levels by Clinical Diagnosis Groups

IMR assays have detected significant or almost significant increases in plasma A β 42 in clinically diagnosed AD cases. A β 42 levels in plasma from subjects with AD measured by IMR assays were 1.1- to 2.2-fold greater than the levels in NC plasma. Similarly, IMR t-tau levels were significantly increased in subjects with MCI and early AD when compared with NC. The t-tau levels in plasma from subjects with AD were 1.7- to 4-fold greater than the levels detected in the NC group [61, 62]. However, in subjects with AD, IMR-measured A β 40 levels changed in the opposite direction to those of A β 42 and t-tau

with A β 40 levels in subjects with AD being 61–80% of levels in NC subjects [59, 60, 62]. In fact, not all IMR-assayed A β 40 levels in plasma showed consistent findings. Chiu et al. detected a broad range of plasma A β 40 levels in subjects with very mild AD or mild-to-severe AD and the results were unable to be used for disease group classification [58]. Another way of analyzing the biomarker utility of these measures was to calculate the ratio of A β 42/A β 40. Significantly higher ratios were obtained in subjects with AD and MCI when compared with NC values [58, 62]. Although A β 40 IMR assay seems to be robust, the exact types of A β peptides being measured in plasma samples require further characterization as the antibody used in the assay was not targeting C-terminal epitopes [67].

For comparison, we present results from studies using the ultrasensitive SIMOA technology. Using samples obtained from the community-based Framingham Heart Study (FHS), and the multi-cohort Memeto study, Pase et al. used SIMOA to assess plasma t-tau for predicting AD in a total of 1852 participants [45]. They found that FHS subjects with plasma t-tau levels greater than the median had a 62% greater risk of dementia and a 76% greater risk of AD dementia. The findings were replicated in the Memeto cohort in the same study. The median baseline levels of plasma t-tau were around 4 pg/ml. Another large study with a total of 1284 participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI) and the BioFinder study to determine whether plasma t-tau could be useful for AD biomarkers also used the SIMOA method to measure plasma t-tau [49]. This study also detected plasma t-tau in a narrow range of low picogram per milliliter values. Notably, there was no significant correlation between CSF and plasma t-tau levels from this study, even though plasma t-tau and CSF t-tau were all significantly increased in the disease groups.

SIMOA was also used to determine the utility of plasma AD markers to screen subjects with subjective memory decline [68]. The presence of abnormal amyloid in the brain was determined by CSF AD core marker levels or ¹¹C-Pittsburgh compound (¹¹C-PiB) PET imaging. They found

that plasma t-tau levels did not correlate with CSF amyloid status, and single A β markers did not perform as well as the ratio of plasma A β species, A β 42/A β 40. It is important to point out that this study also showed low levels of plasma A β 42 and t-tau in PiB amyloid normal and abnormal groups. The plasma A β 42 level was 1.3% of CSF A β 42 level in the amyloid abnormal group and 0.9% in the amyloid normal group, whereas plasma t-tau level was 1.2% of CSF t-tau level in the amyloid normal group and 0.6% in the amyloid abnormal groups. These findings demonstrated the SIMOA platform's ultrasensitivity for measuring both plasma A β and t-tau.

Findings from Receiver Operating Characteristic (ROC) Curve Analysis

The potential clinical utility of IMR-assayed AD markers to identify AD or MCI has been the focus in several studies. In one study consisting of 68 NC, 24 subjects with MCI due to AD, and 31 subjects with early AD, plasma A β 42 and t-tau showed high sensitivity and specificity (greater than 90%) in discriminating between NC and subjects clinically diagnosed with AD [55]. However, these markers lacked the ability to discriminate MCI from early-stage AD as the ROC sensitivity and specificity were lower than 80% [55, 58]. There was a slight improvement of sensitivity and specificity observed by multiplying the concentrations of A β 42 and t-tau markers (the product), but this measure was still not useful for distinguishing between MCI and early AD [55].

Application of the A β 42/A β 40 ratio has been investigated in IMR studies. As IMR-assayed A β 42 increased and A β 40 decreased in subjects with MCI and AD when compared with NC, the increase in the ratios become significant between all pairs of group comparisons [62]. This ratio used for classifying AD and NC outperformed single markers in one earlier IMR study [52]. In a later study, A β 42/A β 40 plasma ratio had significant association with the PiB standardized uptake value ratio (SUVR; $R^2 = 0.326\text{--}0.449$, $P < 0.001$).

The first IMR study outside Taiwan was a study of 16 NC and 16 subjects with AD carried out in a Caucasian population at the BSHRI (Sun City, AZ, USA). These subjects had an older mean age (NC 81.9 ± 6.0 years; AD 82.5 ± 5.6 years) and lower Mini Mental State Examination (MMSE) score (NC 29.3 ± 1.2 ; AD 16.1 ± 4.0) than the Chinese-ethnic subjects studied by IMR in Taiwan [61]. The sensitivity for distinguishing AD and cognitively normal was 56% for A β 42 and 75% for t-tau; the specificity was 81% for A β 42 and 88% for t-tau. Using the product of A β 42 and t-tau increased sensitivity to 88%, but with no improvement in specificity. In the same study, ROC analysis was also performed in the combined BSHRI and National Taiwan University Hospital (NTUH) cohorts. A β 42 and t-tau markers had 89% sensitivity and specificity of at least 90% or greater. However, using their product with a cutoff value of greater than 383 (pg/ml)^2 to identify AD, the sensitivity was improved to 96%, but not the specificity [61]. In an earlier study conducted by Chiu et al., a higher cutoff value of greater than 455 (pg/ml)^2 for the product of two markers achieved 96% sensitivity and 97% specificity for distinguishing between NC and AD, and 80% sensitivity and 82% specificity between MCI and AD groups [58].

IMR AD Markers and Imaging Markers

IMR-assayed plasma AD markers have been assessed for their relationship with brain atrophy, measured by MRI, and amyloid deposition, measured by ^{11}C -PiB SUVR [59, 60, 62]. Chiu et al. reported that plasma t-tau levels could be predicted by a regression model that includes total hippocampus volume (predicting 0.394 of the variance in the plasma tau level) and gray matter density of the superior frontal gyrus (predicting 0.054 of the variance in the plasma tau level) [59]. Plasma t-tau levels did not predict PiB+ SUVR in bilateral frontal, parietal, temporal lobes, and the precuneus [62]. Higher plasma t-tau levels in SIMOA were associated with smaller hippocampi, poorer cognitive function, as well as pathological evidence of microinfarct and neurofibrillary tangles [45].

IMR plasma t-tau levels had a strong correlation with MMSE in a PiB+ group ($r = -0.624$, $P < 0.001$), but not in a PiB- group ($r = -0.295$, $P = 0.072$) [60].

Brain amyloid measured by ^{11}C -PiB PET imaging significantly correlated with the ratios of IMR plasma $\text{A}\beta_{42}/\text{A}\beta_{40}$ ($R^2 = 0.326\text{--}0.449$, $P < 0.01$) [62]. IMR-assayed plasma markers ($\text{A}\beta_{42}$ and t-tau) have also been studied for their ability to distinguish between PiB-positive (PiB+) and PiB-negative (PiB-) subjects [60]. Using the cutoff values for $\text{A}\beta_{42}$ and t-tau of 21.92 and 37.54 pg/ml made it possible to discriminate between PiB- and PiB+ subjects with 100% sensitivity and 100% specificity. To identify the PiB+ group, lower $\text{A}\beta_{42}$ and t-tau cutoff values (16.81 and 25.57 pg/ml) were needed to achieve 86.7% and 93.3% sensitivity and 82% and 100% specificity (respectively for $\text{A}\beta_{42}$ and t-tau). By comparison, using a cortical SUVR 1.2167 cutoff value for PiB imaging achieved 93% sensitivity and 100% specificity could be for identifying the PiB+ group. Thus, the IMR-assayed plasma AD markers had high concordance with PiB positivity, at least as good as CSF core AD markers [69]. The possibility of using IMR-assayed AD markers as surrogates for amyloid PET imaging to screen potential subjects for clinical trials shows promise.

CLINICAL POTENTIALS OF IMR-ASSAYED PLASMA AD MARKERS

Validated blood biomarkers for detecting pre-clinical stages of AD, identifying subjects at risk of AD, and pre-screening subjects in clinical trials prior to expensive amyloid or tau imaging would have significant use in research and clinical practice. To achieve this will require routine, reliable assays that can sensitively and accurately detect the earliest biochemical changes that distinguish ongoing pathological abnormality from normal aging in the brain. This still poses great challenges at both technical and knowledge levels. The former relies on innovation in the technology, and the latter is dependent on further understanding of the early-stage events that precipitate AD pathology. IMR-SQUID technology has demonstrated

consistent ultrasensitivity to detect $\text{A}\beta_{42}$ and t-tau levels in plasma from clinically diagnosed groups. On the basis of the published findings, it would be beneficial to incorporate IMR-based blood tests in initial and follow-up diagnostic procedures in clinics. However, it remains to be determined whether IMR-assayed plasma markers could be used as prediction markers. Further vigorous research in well-designed multicenter longitudinal studies is needed to determine the potential of using IMR assays for plasma biomarkers to identify at-risk, preclinical subjects, and subjects with MCI in those who also have undergone PiB imaging and CSF core AD marker testing.

Blood-based biomarkers could be the quickest and most straightforward way of detecting the pathological and biochemical changes associated with clinical symptoms as in most community-based clinical practice in the USA, CSF samples are not usually collected. On the basis of combined use of CSF and imaging biomarker findings, Jack et al. proposed that alterations in $\text{A}\beta$ levels in CSF could reflect the earliest pathological events, which precede the amyloid deposition observed by PET imaging [70]. $\text{A}\beta$ in CSF of subjects with no cognitive impairment results from normal metabolism of brain-derived APP. As mentioned previously, this material is rapidly degraded. Now, it is believed that $\text{A}\beta$ levels in CSF start to decrease as this material is incorporated into brain or cerebrovascular plaques. The $\text{A}\beta$ levels present in plasma is affected by more complicated factors. Nevertheless, if the more complex sequence of changes of biomarkers in plasma compared to CSF and brain imaging could be established, it would help determine feasibility of plasma changes as a substitute for CSF markers. Additionally, it could also help assess how the periphery might be involved in the pathological changes in CSF and brain. As plasma AD biomarkers have potential utility in monitoring the effects of drugs in clinical trials, whether these markers could be used to assess the effects of current acetylcholinesterase inhibitor treatment in patients with MCI and AD will also be a relevant research area to be investigated.

CONCLUSIONS

The IMR-SQUID technology has demonstrated consistent findings in its ability to distinguish cognitively normal subjects from those with very early AD and MCI, as well as to predict brain atrophy, amyloid positivity determined by ^{11}C -PiB PET, and cognitive decline in subjects with MCI. This situates IMR assays among those promising blood-based biomarker platforms for diagnosis of AD at early stages. The research findings also provide strong rationales for further validation of IMR technology in large-cohort longitudinal study to confirm the accuracy for predicting conversion to AD in subjects with MCI and identifying A β amyloid PET positive subjects. The National Institute on Aging and Alzheimer's Association (NIA-AA) diagnostic criteria for AD had recently proposed preclinical diagnosis to include imaging and CSF amyloid/tau/neurodegeneration (A/T/N) biomarkers [71–73]. New technologies available have significantly improved the accuracy of detecting AD plasma markers. This opens the opportunity of exploring the potentials of A/T/N biomarkers in plasma. As IMR technology has assays for detecting A β , t-tau, p-tau and NFL, future study could determine whether an IMR-based plasma panel of A/T/N biomarkers could have the sensitivity to characterize the stages of AD pathological development as CSF biomarkers have shown.

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