Neurol Ther (2017) 6 (Suppl 1):S25–S36
DOI 10.1007/s40120-017-0074-8

REVIEW

Amyloid Beta and Tau as Alzheimer's Disease Blood Biomarkers: Promise From New Technologies

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Received: February 9, 2017

ABSTRACT

The utility of the levels of amyloid beta (Aβ) peptide and tau in blood for diagnosis, drug development, and assessment of clinical trials for Alzheimer's disease (AD) has not been established. The lack of availability of ultra-sensitive assays is one critical issue that has impeded progress. The levels of Aβ species and tau in plasma and serum are much lower than levels in cerebrospinal fluid. Furthermore, plasma or serum contain high levels of assay-interfering factors, resulting in difficulties in the commonly used singulex or multiplex ELISA platforms. In this review, we focus on two modern immune-complex-based technologies that show promise to advance this field. These innovative technologies are immunomagnetic reduction technology and single molecule array technology. We describe the technologies and discuss the published studies using these technologies. Currently, the potential of utilizing these technologies to advance Aβ and tau as blood-based biomarkers for AD requires further validation using already collected large sets of samples, as well as new cohorts and population-based longitudinal studies.

Keywords: Alzheimer's disease; Amyloid beta; Blood biomarkers; Plasma; Tau; Ultra-sensitive technology

INTRODUCTION

Alzheimer's disease (AD) core pathological components, amyloid beta (Aβ) peptide 42 (Aβ42), Aβ40, tau, and tau phosphorylated at threonine-181 (Thr181P), have been targets for biomarker development for two decades [1–5]. The underlying rationale of using these molecules as biomarkers of AD is that definitive diagnosis of this condition relies on confirmation from neuropathological hallmarks containing these components, and the ability of tracking or measuring these components in brains or biofluids of living subjects could provide evidence of ongoing pathophysiology. There has been tremendous progress made towards achieving this goal. Ligands have been developed for visualizing amyloid or tau pathologies in the brain by positron emission

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This review contains part of the presentation in the first International Symposium on Biomarkers for Alzheimer's Disease and Related Diseases held on 25 October 2016 in Taipei, Taiwan.

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tomography (PET) [6, 7]. The utility of amyloid imaging and cerebrospinal fluid (CSF) biomarkers of Aβ and tau for clinical diagnosis has been validated in large cohorts and population studies, as well as in neuropathologically confirmed cases [8–15]. The use of the ratios of CSF Aβ42 to tau or Thr181P has been established as a means for identifying clinically diagnosed AD [2, 9, 16–22]. The inclusion of AD core biomarkers in the criteria for the diagnosis of probable and possible AD has been recommended by the National Institute on Aging–Alzheimer's Association workgroups [23–26]. The strategy of combining amyloid visualization by PET and CSF core markers has been demonstrated to improve the accuracy of predicting the pre-clinical stage of AD [25, 26]. As PET primarily visualizes fibrillar amyloid deposits, CSF Aβ measures could be more sensitive in detecting changes in Aβ levels at the pre-clinical stage or even earlier [27–29]. By contrast, the development of assays for use in measuring AD core pathological molecules in blood as disease biomarkers has fallen behind [30, 31]. Several factors could have hampered the development. One of the major challenges in developing AD core pathological components such as blood biomarkers has been the lack of sensitive assays. Analyses using singulex or multiplex enzyme-linked immunosorbent assay (ELISA) platforms for determining the levels of Aβ and tau in plasma or serum have led to conflicting findings—with levels either unchanged, decreased, or increased from the normal controls—suggesting limitations due to the assay platforms [32–41].

This article is based on previously conducted studies and does not involve any new studies of human or animal subjects performed by any of the authors.

FACTORS AFFECTING ASSAY RESULTS OF BLOOD AD CORE BIOMARKERS

The discrepancy in the levels of AD core markers in blood samples could be due to a wide range of causes: biological nature, assay sensitivity, platform, sample processing, storage condition, clinical criteria, and/or demographic features of the participants, as discussed in a previous review [42]. From a biological point of view, the concentrations of Aβ and tau in the circulation are much lower than those in the CSF because these molecules present in the brain do not directly enter the circulation due to the presence of the blood–brain barrier (BBB). Some Aβ molecules are cleared at the BBB through receptor-mediated mechanisms, but others are cleared from the CSF through the lymphatic drainage system [43–45].

The assay platforms for measuring CSF Aβ, tau, and Thr181P, namely traditional singulex ELISA and luminex-based multiplex ELISA, are well-established [19, 46–48]. It has been consistently shown that compared to normal controls Aβ42 levels in the CSF are lower in patients with AD, while both tau and phosphorylated tau (p-tau) levels are higher in patients with AD (for example, see [48]). Combining CSF tau, p-tau, or Aβ40 levels as ratios with Aβ42 is more effective than standalone markers in predicting brain Aβ deposition detected by PET imaging in patients with AD or in the preclinical stage of AD [49–51].

Figueski et al. measured Aβ42 and Aβ40 levels in plasma samples using similar ELISA platforms and found that the plasma levels were one-fifth to one-tenth the levels found in the CSF [33]. Using an ultra-sensitivity platform, Janelidze et al. found even wider differences in detection levels between plasma and CSF samples [36]. Plasma Aβ levels tend to be near the lower limits of detection of current ELISA assays and close to the low-end of the linear range of a calibration curve. Under these conditions, the ELISA assays lose their sensitivity for detecting narrow differences between biological samples. The performance of immunoassays also depends on the epitopes and affinity of the antibodies selected for capturing antigen contained in the samples. Moreover, plasma and serum contain high concentrations of albumin and immunoglobulins, which are known Aβ-binding proteins that can interfere with accurate detection of the free forms of Aβ [52, 53]. Endogenous immunoglobulins, autoantibodies, and heterophilic antibodies can also interfere with the performance of ELISAs.

This has been discussed in depth previously [54].

In addition to the above-mentioned factors, improper sample handling can affect assay accuracy. Most of the lessons in this regard were learned during the process of establishing these AD core markers for CSF. As plasma contains much higher concentrations of proteins, including degradative enzymes, as well as more complex components than CSF, additional problems of these sorts are to be expected. These include the need for rigorous procedures of sample collection, volume of sample aliquots, type of tubes for storing aliquots, number of freeze–thaw cycles, type of calibration proteins, batch-to-batch reagent variability, and site-to-site operations [55].

ULTRA-SENSITIVE TECHNOLOGIES

To overcome the challenges of detection encountered using traditional ELISA platforms, new approaches and technologies are emerging with the potential to provide superior sensitivity and specificity for measuring Aβ and tau in blood samples [56–58]. For example, a new approach that used immunoprecipitation to pull down various Aβ fragments in plasma samples followed by mass-spectrophotometry analysis has led to discovery of a new marker, APP669–711, whose ratio to Aβ1-42 demonstrated 93% sensitivity and 96% specificity to discriminate Pittsburgh compound B (PIB)-positive subjects from PIB-negative subjects [58]. New immunoaffinity-based assays have also been applied to AD core marker analysis in biofluids, including immunomagnetic reduction (IMR) and single molecule array (SIMOA) for the analysis of plasma samples and the assay by Meso Scale Discovery (Rockville, MD) for CSF samples [59]. Here, we limit the scope of this review to the IMR and SIMOA assays, which have been used to quantify plasma Aβ and tau in studies involving medium to large numbers of subjects.

The IMR technology was developed by MagQu Company, Ltd. (New Taipei City, Taiwan), and the SIMOA technology was developed by Quanterix (Lexington, MA). Quantification with both platforms is based on immunoreactivity between specific antibodies and analytes or protein standards. However, the principle and design of the two detection systems are quite different. IMR technology detects alternating-current magnetic susceptibility by a superconducting quantum interference device (SQUID), while SIMOA technology detects the presence of antigen by fluorescence imaging of single enzyme-labeled immunocomplexes reacting with the fluorogenic substrate, resorufin β-D-galactopyranoside [57, 60]. The technical features of the two platforms are summarized in Table 1.

IMR Technology

Immunomagnetic reduction assays quantify the concentrations of analytes in a sample by measuring the percentage magnetic signal reduction after immunocomplex formation at the surface of magnetic nanobeads, with the magnetic signals being detected by SQUID [57]. The binding of antibody with analytes changes the oscillation speed of the magnetic nanoparticles under a mixed frequency alternating current. Thus, the magnitude of reduction in the oscillation speed corresponds to the amount of the analytes bound to the antibodies. Sample analyte concentration is calculated according to the established relationship of protein standard concentrations and associated percentage IMR [57, 61].

The IMR reagents manufactured by MagQu Company contain capture antibody-conjugated magnetic nanobeads (diameter 50–60 nm) at a concentration of 10^9 beads per milliliter. Current IMR reagents use a monoclonal antibody to tau that recognizes six isoforms: a rabbit polyclonal antibody to Aβ37–42 for the Aβ42 assay and a mouse monoclonal antibody to the N-terminal of Aβ as Aβ40 capture antibody. Although it has been shown that spiking with Aβ42 did not increase the measured levels of Aβ40 in an IMR Aβ40 assay, the possibility of measuring both Aβ species by the IMR Aβ40 assay using the current antibody remains to be clarified [57].

Table 1 Summary of the immunomagnetic reduction and single molecule array technologies

Assay characteristics	IMR[a]	SIMOA[a]
Assay principles	The IMR assay measures the change in magnetic susceptibility over time caused by the association of antigen with antibody-coated paramagnetic nanobeads	Digital ELISA counts antibody coated paramagnetic microbeads that have undergone a procedure similar to conventional ELISA techniques
Diameter of magnetic beads	50–60 nm	2.7 μm
Capture antibodies	Tau: Anti-tau (Sigma, St. Louis, MO; T9450) Aβ42: Anti-β amyloid 37–42 (ABCAM, Cambridge, UK; ab34376) Aβ40: Anti-β amyloid (Sigma; A3981) [61]	Tau: Tau5 targeting a linear epitope in the mid-region of all tau isoforms Aβ42/Aβ40: Antibodies targeting N-terminus of Aβ
Detection antibodies	None	Tau: HT7 and BT2 targeting linear epitopes in the N-terminal region of T-tau Aβ42/Aβ40: biotinylated C-terminal-specific antibodies
Washing steps	None	Two 3-step washes, and one 8-step wash with 5× phosphate buffered saline + 0.1% Tween-20
Type of signals for detection	Magnetic susceptibility detected by SQUID magnetometer	Digital counting of enzyme-labeled and unlabeled microbeads via presence and absence of fluorescent substrate
Equipment capacity	36 Wells (XacPro-S)	96-well plate (four 24-array discs) (Simoa HD-1) [63]
Low limit of detection	Tau: 0.002 pg/ml Aβ42: 7.53 pg/ml Aβ40: 4.91 pg/ml	Tau: 0.019 pg/ml Aβ42: 0.044 pg/ml Aβ40: 0.522 pg/ml
Low limit of quantification	Information not available	Tau: 0.061 pg/ml Aβ42: 0.137 pg/ml Aβ40: 1.23 pg/ml
Assay range	Tau: 0.002–2500 pg/ml Aβ42: 7.53–50,000 pg/ml Aβ40: 4.91–500 pg/ml	Tau: 0–360 pg/ml Aβ42: 0–400 pg/ml Aβ40: 0–800 pg/ml
Sample volume (plasma)	Tau: 40 μl Aβ42: 60 μl Aβ40: 40 μl	Tau: 45.5 μl Aβ42: 32.5 μl Aβ40: 32.5 μl

Table 1 continued

Assay characteristics	IMR[a]	SIMOA[a]
Dilution factor (plasma)	Tau: threefold dilution Aβ42: twofold dilution Aβ40: threefold dilution	Fourfold dilution for all analytes in an automatic procedure

IMR, Immunomagnetic reduction assay; SIMOA, single molecule array assay; SQUID, superconducting quantum interference device; ELISA, enzyme-linked immunosorbent assay; T-tau, total tau

[a] Information in this table was obtained from the websites www.magqu.com and www.quanterix.com, and in the published studies which used these technologies, as shown in the table

The IMR procedure requires no washing steps. The antibody-containing IMR reagent is mixed with samples at a defined volume ratio. Plasma samples are not pre-diluted, and the total volume for each assay is 120 μl, with the detection of reaction being measured over a 5-h period. The company has developed a 36-channel SQUID-based immunomagnetic analyzer (Model *X*ac-Pro-S). Additional information for the IMR assays is listed in Table 1.

SIMOA Technology

The SIMOA assay detects the presence of antigen at the single molecule level using digital counting technology [60, 62]. An assay-specific capture antibody is attached to 2.7-μm paramagnetic microbeads that contain 250,000 antibody attachment sites per bead. The assay procedure involves formation of antigen–antibody immune complexes at the surface of the microbeads, followed by interaction with first a biotinylated-detection antibody and then streptavidin-beta galactosidase. The microbeads are allowed to settle into individual femtoliter-sized wells containing fluorogenic enzyme substrate. Those wells containing fluorescent signals generated by the beta-galactosidase reaction with the substrate are detected and counted by a fluorescence analyzer. The calculation of antigen concentration in the sample is based on the ratio of the number of the wells containing an enzyme-labeled bead to the total number of wells containing a bead [measuring unit is average enzymes per bead (AEB)]. The Quanterix company has developed a high-capacity, fully automated SIMOA HD-1 Analyzer that can handle triplex analysis (assays for cytokines: tumor necrosis factor-alpha, interleukin (IL)-6 and -10) [63]. The details of two-plex and three-plex assays that analyze Aβ and tau levels are also available at the company's website (www.quanterix.com). The overall instrument throughput is 68 samples/h at steady-state usage, while it takes 2 h to assay a 96-well plate [63]. Additional information on SIMOA assays is shown in Table 1.

MEASUREMENT OF AB AND TAU IN HUMAN PLASMA USING SIMOA AND IMR TECHNOLOGIES

The IMR and SIMOA assays were developed to increase the detection sensitivity of immunoassays, and they have been used to analyze plasma levels of Aβ and tau in human subject studies.

Studies using IMR assays have mainly been conducted in Taiwanese cohorts [57, 61, 64–66], and studies involving other ethnic groups are ongoing (personal communication by authors). The IMR assays performed to date in Taiwanese subjects have revealed elevated Aβ42 levels, reduced or no change in Aβ40 levels, and increased tau levels in patients with AD when compared to normal controls [61, 64–66]. Receiver operating characteristics curve analyses

showed 96% sensitivity and 97% specificity for distinguishing healthy controls from a heterogeneous group of study subjects consisting of those with mild cognitive impairment (MCI) due to AD and those with mild to severe AD (Clinical Dementia Rating scores 0.5–3), whereas an 80% sensitivity and 82% specificity was obtained for discriminating patients with AD from those with MCI [61]. When amyloid PET imaging was used to stratify the study subjects, there was 84% sensitivity and 100% specificity to predict the results of amyloid detected by PET when the ratio of Aβ42 to Aβ40 was used [66]. The excellent AD diagnostic performance indicated by sensitivity and specificity in these studies using the product of Aβ42 and tau has yet to be compared in independent studies from other sites.

The SIMOA platform has been shown to detect increases in tau level in patients with AD from tau levels in patients with MCI and normal controls, and for Aβ and for tau in studies of AD [36, 39, 56, 67–69]. The results of plasma tau studies have shown significant increases in tau in patients with AD, and increases or no changes in those with MCI compared with normal controls, while higher plasma tau levels have also been associated with reduced memory performance [39, 68]. The authors of these studies drew the same conclusion that due to substantial overlap between clinically diagnosed groups plasma tau concentration cannot be used as a prognostic or diagnostic marker. When plasma Aβ42 and Aβ40 levels were assayed by SIMOA, significant decreases were detected between patients with AD and those with MCI, between those with AD and subjects with subjective cognitive decline (SCD), and between those with AD and normal controls [36]. The detection levels of plasma Aβ1–42 [mean ± standard deviation (SD); normal controls: 19.6 ± 5.2 pg/ml; AD: 13.2 ± 7.3 pg/ml) were less than 10% of those of plasma Aβ1–40 (mean ± SD; normal controls: 276.7 ± 66.1 pg/ml; AD: 244.3 ± 105.8 pg/ml). In this study, the CSF Aβ levels were not assayed by the SIMOA assay, but by the Euroimmun immunoassay (EUROIMMUN AG, Lübeck, Germany) [49, 51]. CSF Aβ1–42 levels in patients with AD were significantly decreased compared with other groups ($P < 0.0001$), while Aβ1–40 levels in patients with AD were only significantly lower than those of SCD subjects ($P = 0.003$). The detected values of CSF Aβ42 were 554.0 ± 195.1 pg/ml in normal controls and 289.5 ± 103.8 pg/ml in patients with AD ($P < 0.0001$), whereas the values of Aβ40 were 4688.5 ± 1650.0 pg/ml in normal controls and 4387.2 ± 1761.6 pg/ml in patients with AD (no statistical significance). Although different platforms were used to assay CSF and plasma samples, the results showed that CSF and plasma Aβ42 and Aβ40 levels were significantly positively correlated (Pearson's correlation analyses in all participants: $r = 0.274$, $P < 0.001$ for Aβ42; $r = 0.136$, $P = 0.001$ for Aβ40).

CONCLUSION

Strategies for the development and utility of blood-based biomarkers for AD have been discussed in detail recently in several review articles [30, 70–72]. In this article, we focused on two new ultra-sensitive immunoaffinity-based technologies that offer promise for establishing Aβ and tau as blood biomarkers for AD. Currently, these two platforms are uniquely situated for further assessment, especially in large population studies. However, the advance could be limited by the cost of the instruments, the lack of high-throughput capacity, and single suppliers of assay reagents. The availability of a throughput automated instrument, such as the SIMOA HD-1 analyzer, will certainly appeal to pharmaceutical companies when considering biomarkers to assess the progress of clinical trials in large numbers of subjects, in which plasma Aβ and tau measurements might have potential utilities. Nevertheless, recent studies on plasma tau have not confirmed its feasibility as a diagnostic or prognostic biomarker due to large overlap between AD and MCI, and between AD and normal controls, regardless of the presence of differences in disease-associated expression. The SIMOA assay for plasma Aβ had shown preliminary potential for utility of diagnosis. In these regards, the IMR technology seems to make more progress, evident from a series of cohort studies that showed good

sensitivity and specificity and promising correlations with PET imaging of amyloid and tau. However, the IMR technology will need to be assessed vigorously in cohorts of different ethnicity, and in longitudinal study of those subjects stratified by amyloid or tau imaging, or by CSF Aβ and tau profiles. Although both platforms are consistent in showing increases in plasma tau levels in patients with AD, the Aβ42 findings were opposite. It has been cautioned that comparing findings between different platforms could be problematic [73, 74]. However, future studies are needed to replicate the differences in findings between platforms before the issue of whether plasma Aβ42 levels are increased or decreased in AD can be resolved.

In summary, ultrasensitive platforms are necessary for establishing whether plasma AD core markers can be valid blood-based biomarkers. As pointed out recently by O'Bryant and colleagues, significant breakthrough in establishing blood-based biomarkers could be achieved when the context of use can be defined at the beginning of biomarker development and if approaches from academic research and industry can be integrated during the process [71]. Preliminary assessment of published findings support that both IMR and SIMOA technologies warrant multicenter cross-validation study.

ACKNOWLEDGMENTS

No special funding or sponsorship was received for the study and publication of this article. All named authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work as a whole, and have given final approval for the version to be published. The authors thank Dr. Eric Reiman for supporting blood-based biomarker research and a previous funding from the Arizona Alzheimer's Consortium through a block Grant from the Arizona Department of Health Services for the study of new biomarker platforms in Alzheimer's disease.

Disclosures. Lih-Fen Lue receives research funding from MagQu Company Ltd, for a biomarker discovery study; however, MagQu Company Ltd has had no influence on the content of this review. Douglas G. Walker and Andre Guerra have nothing to disclose.

Compliance with Ethics Guidelines. This article is based on previously conducted studies and does not involve any new studies of human or animal subjects performed by any of the authors.

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