



# An Update on Blood-Based Markers of Alzheimer's Disease Using the SiMoA Platform

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## ABSTRACT

The development of blood-based biomarkers of Alzheimer's disease (AD) pathology as tools for screening the general population, and as the first step in a multistep process to determine which non-demented individuals are at greatest risk of developing AD dementia, is essential. Proteins that are reflective of AD

pathology, such as amyloid beta 42 ( $A\beta_{42}$ ), tau proteins [total tau (T-tau) and phosphorylated tau (P-tau)], and neurofilament light chain (NfL), are detectable in the blood. However, a major challenge in measuring these blood-based proteins is that their concentrations are much lower in plasma or serum than in the cerebrospinal fluid. Single molecule array (SiMoA) is an ultrasensitive technology that can detect proteins in blood at sub-femtomolar concentrations (i.e.,  $10^{-16}$  M). In this review, we focus on the utility of SiMoA assays for the measurement of plasma or serum  $A\beta_{42}$ , P-tau, T-tau, and NfL levels and discuss future directions.

**Keywords:** Alzheimer's disease; Amyloid-beta; Blood biomarkers; Neurofilament; Single molecule array technology; Tau

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### Key Summary Points

A major challenge in measuring blood-based proteins of Alzheimer's Disease (AD) pathology is that their concentrations are much lower in plasma or serum than in cerebrospinal fluid.

Single molecule array (SiMoA) is the most established ultrasensitive technology in the field of blood-based biomarkers of AD pathology from a research perspective. Kits to measure plasma or serum amyloid-beta ( $A\beta_{42}$ ), phosphorylated tau (p-Tau), total tau (T-tau), and neurofilament light chain (NfL) are available.

Initial studies of plasma  $A\beta_{40}$  and  $A\beta_{42}$  support a potential role in screening for amyloid at the population level, but additional research is needed to ascertain the degree to which vascular factors affect plasma levels.

Little research to date has examined blood phosphorylated tau using SiMoA.

Blood neurofilament light chain and total-tau are promising non-specific markers of neurodegeneration and have shown potential as prognostic markers or as surrogate endpoints of neurodegeneration in clinical trials.

## INTRODUCTION

Within the context of developing disease-modifying therapies for Alzheimer's disease (AD), there is an urgent need to identify and validate biomarkers of AD pathology, such as amyloid-beta ( $A\beta$ ), tau, and neurodegeneration. Based on the current state of science, the AT(N) scheme [ $\beta$  amyloid (A), pathological tau (T), and neurodegeneration (N)] of the new National Institute on Aging-Alzheimer's Association (NIA-AA) Research Framework suggests cerebrospinal fluid (CSF), positron emission tomography (PET), and magnetic resonance imaging (MRI) biomarkers for  $A\beta$ , tau, and neurodegeneration

[1, 2]. Biomarkers of  $A\beta$  include amyloid PET imaging or MR  $A\beta_{42}$  imaging. Biomarkers of paired helical filament tau include CSF phosphorylated tau (P-tau) or PET tau imaging. Lastly, biomarkers of neurodegeneration include CSF total tau (T-tau), fluorodeoxyglucose (FDG)-PET hypometabolism, or atrophy in specific brain regions based on structural MRI. However, from the perspective of healthcare policy and economics, it is not feasible to quantify AD pathology at the population level using neuroimaging modalities (e.g., amyloid PET, tau PET, and MRI) nor is it feasible to conduct a lumbar puncture for the collection of CSF due to cost and resource availability. Thus, the development of blood-based biomarkers of AD pathology are essential for screening the general population, as well as for representing the first step in a multistep process to determine which non-demented individuals are at greatest risk of AD dementia [3]. It would also be more feasible to obtain serial measures of blood than to perform PET or CSF punctures when the goal is to assess the rate of disease progression and to determine the effect modification of potential therapies on the disease process.

The NIA-AA Research Framework left open the possibility of new markers of A, T, and N as new technologies are developed and new research shows the utility of the markers [1, 2]. Notably, proteins that are reflective of AD pathology, such as  $A\beta_{42}$ , tau proteins (T-tau and P-tau), and neurofilament light chain (NfL), are detectable in blood. However, a major challenge in measuring these blood-based proteins is that their concentrations are much lower in plasma or serum than in the CSF. For example, the concentrations of  $A\beta_{42}$  [4] and tau [5] in plasma are approximately 30- and 100-fold lower, respectively, than those in the CSF. A second challenge is that plasma and serum have a higher total protein concentration (i.e., 50–70 g/L for an adult) and a more complex protein matrix than does the CSF. The binding of blood  $A\beta_{42}$  to many proteins in plasma or serum (e.g., albumin, lipoproteins,  $A\beta$  autoantibodies, fibrinogen, immunoglobulin, apolipoprotein J, apolipoprotein E, transthyretin,  $\alpha$ -2-macroglobulin, serum amyloid p component, plasminogen, and amylin) [6–9] can further reduce the concentration of blood  $A\beta_{42}$  available for

measurement. Current mainstream immunoassays typically measure proteins in blood at concentrations  $> 10^{-12}$  M [10]. Thus, more sensitive technologies are needed to quantify markers of AD pathology at blood concentrations in the range of  $10^{-15}$  to  $10^{-12}$  M.

Over the past few years, technologies such as single molecule array (SiMoA) [10], immunoprecipitation mass spectrometry (IP-MS) [11, 12], immunomagnetic reduction–superconducting quantum interference (MagQu) [13], and the interdigitated microelectrode sensor system [14] have emerged. These ultrasensitive technologies provide new opportunities for the development of blood-based biomarkers of AD pathology.

In this review, we focused on SiMoA assays for the measurement of AD-related blood-based biomarkers. Given the large and increasing number of articles reporting the use of SiMoA for the measurement of AD biomarkers, we did not conduct a comprehensive review of all articles but highlighted studies with the largest sample sizes or those that specifically examine certain aspects of the assays (i.e., stability over multiple freeze–thaw cycles). We first briefly review the measurement principle of SiMoA and then discuss published evidence on plasma or serum SiMoA assays ( $A\beta_{42}$  or  $A\beta_{42}/A\beta_{40}$ , T-tau, P-tau, and NfL) in the context of AD screening, diagnosis, and prognosis. We have attempted to distinguish between commercially available and home-brewed SiMoA assays when possible because home-brewed assays can perform differently and there may be a difference between blood plasma assay and serum assay results.

### 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.

## MEASUREMENT PRINCIPLE OF THE SIMOA

Single molecule array is an ultrasensitive technology that can detect proteins in blood at sub-

femtomolar concentrations (i.e.,  $10^{-16}$  M) [10]. Traditional immunoassays are conducted in a relative large reaction volume of 50–100  $\mu$ L (e.g., wells in 96-well plates), which results in a diluted and diffuse signal molecule and limited sensitivity to the picomolar range (i.e.,  $10^{-12}$  M). In contrast, SiMoA restricts this diffusion by confining a single magnetic bead coupled with enzyme and substrate to femtoliter-sized wells. When the enzyme label catalyzes substrate conversion to a fluorescent product, the resulting fluorophores are confined to the well, creating a measurable fluorescence signal within a short period of time. The field of view of the camera encompasses hundreds of thousands of microwells; thus, thousands of single-molecule signals in the array can be counted simultaneously. The counting of active and inactive wells constitutes a digital signal corresponding to the presence or absence of single enzyme molecules. This extreme sensitivity permits the use of low quantities of labeling reagent, which in turn lowers non-specific interactions and dramatically increases signal–background ratios [15]. To date, kits have been developed for measurement of  $A\beta_{40}$ ,  $A\beta_{42}$ , T-tau, P-tau, and NfL in either the serum or plasma. In addition, a 3-plex of  $A\beta_{40}$ ,  $A\beta_{42}$ , and T-tau and a 2-plex of  $A\beta_{42}$  and T-tau are currently available.

## BLOOD-BASED SIMOA $AB_{40}$ AND $AB_{42}$ ASSAYS

Amyloid-beta peptides of various lengths are found in the plasma or serum [16]. It has been estimated that 30–50% of the  $A\beta$  peptide pool in the central nervous system is transported to the blood for clearance [17]. The most commonly measured  $A\beta$  peptides examined in the context of AD are  $A\beta_{40}$  and  $A\beta_{42}$ , which exist as both monomers and aggregates (i.e., oligomers) [14]. The SiMoA  $A\beta_{40}$  and  $A\beta_{42}$  assays utilize the same capture antibody targeting the N-terminus of  $A\beta_{40}$  and  $A\beta_{42}$  but different C-terminus detection antibodies, namely, 2G3 and 21F12, respectively [18]. The peptide standards for  $A\beta_{40}$  and  $A\beta_{42}$  are also made by different manufacturers: the SiMoA  $A\beta_{40}$  assay uses the  $A\beta_{1-40}$

peptide from AnaSpec (Fremont, CA, USA) and the SiMoA  $A\beta_{42}$  assay uses the  $A\beta_{1-42}$  peptide from Covance Inc. (Princeton, NJ, USA) [4]. Presumably, the SiMoA  $A\beta_{40}$  and  $A\beta_{42}$  assays measure monomeric forms of  $A\beta_{40}$  and  $A\beta_{42}$ . A study examining the effect of up to four freeze–thaw cycles on  $A\beta_{40}$  and  $A\beta_{42}$  concentrations found no significant difference in plasma  $A\beta_{42}$  levels after four cycles [19], but there was a small reduction in plasma  $A\beta_{40}$  after the third freeze–thaw cycle and a further reduction after the fourth.

Some studies of the plasma  $AB_{40}$  and  $AB_{42}$  examine the  $AB_{42}/AB_{40}$  ratio whereas other studies examine the  $AB_{40}/AB_{42}$  ratio. The results are often similar but the direction is in the opposite direction (for example a high  $AB_{40}/AB_{42}$  ratio vs. a low  $AB_{42}/AB_{40}$  ratio). Studies of participants with subjective memory concerns have reported lower plasma  $A\beta_{42}$  levels and higher  $A\beta_{40}/A\beta_{42}$  ratios compared to cognitively unimpaired controls without memory complaints [20, 21]. A number of studies have also examined the predictive ability of plasma  $A\beta_{42}$  level for brain amyloid. The best diagnostic cut-points for plasma  $A\beta_{42}$  had a sensitivity of 52% and a specificity of 78% for detecting elevated brain amyloid via amyloid PET or CSF amyloid; the sensitivity for the plasma  $A\beta_{40}/A\beta_{42}$  ratio ranged from 76 to 78% and the specificity from 75 to 76%. Further, the plasma  $A\beta_{40}/A\beta_{42}$  ratio slightly improved models to predict brain amyloid deposition after age was taken into consideration, 10-word delayed recall or the Mini-Mental State Examination score, and the apolipoprotein E gene (*APOE*) [22]. Taken together, this evidence suggests that the plasma  $A\beta_{42}/A\beta_{40}$  ratio as determined by SiMoA may have clinical utility for screening for elevated brain amyloid. Of note, however, studies have shown that vascular disease conditions, such as white matter lesions, cerebral microbleeds, hypertension, diabetes, and ischemic heart disease, can increase plasma  $A\beta_{42}$  and  $A\beta_{42}/A\beta_{40}$  levels measured by the SiMoA [4]. Therefore, additional research is needed to determine the degree to which vascular factors and other comorbidities affect plasma amyloid levels. The SiMoA HD-1 analyzer (Quanterix Corp., Billerica, MA, USA) has a throughput of 66 tests per

hour, thus supporting a potential role in screening for amyloid at the population level [15].

Although the plasma level of  $A\beta_{42}$  and the  $A\beta_{42}/A\beta_{40}$  ratio measured by SiMoA have been shown to be lower in older adults with AD dementia than in cognitively unimpaired individuals [4], consistent with results using IP-MS methods [11, 12], a study using MagQu reported the opposite, namely, lower levels in AD dementia patients than in cognitively unimpaired individuals [13]. These contrasting plasma  $A\beta$  levels may be due to methodological differences between the assays. A major difference between the SiMoA and MagQu assays is that SiMoA resembles a traditional enzyme-linked immunosorbent assay (ELISA) and uses monoclonal capture and detection antibodies, whereas MagQu uses polyclonal antibodies that specifically capture the C-terminal (amino acids 37–42) of  $A\beta_{42}$  [23]. The design of the MagQu assay is such that it has a higher probability of detecting  $A\beta_{42}$  in isolated, complex, or oligomeric forms [23]. Despite the differences in these methods, they are all ultrasensitive, and the results obtained suggest that measurement of the plasma  $A\beta_{42}$  level or the  $A\beta_{42}/A\beta_{40}$  ratio may have potential clinical utility for screening general populations for elevated brain amyloid deposition.

## PLASMA SIMOA P-TAU ASSAY

Although both CSF P-tau and T-tau are elevated in patients with prodromal AD or AD dementia, T-tau is also elevated in patients with stroke, traumatic brain injury, and Creutzfeldt-Jakob disease whereas P-tau is not [24–29]. Thus, P-tau is thought to be more specific to the pathophysiological state associated with the accumulation of AD-type tau pathology and is considered to be the ‘T’ in the AT(N) scheme of the NIA-AA Research Framework [1, 2].

Blood P-tau levels, however, have been difficult to measure due to their low levels. To date, only a few studies using the SiMoA platform have been published. Tatebe et al. [30] reported higher plasma P-tau 181 (pTau181) levels in 20 AD dementia patients compared to

15 age-matched controls (all purchased samples) and higher levels in 20 patients with Down syndrome compared to 22 age-matched controls. However, the authors did not use the pTau181 SiMoA kit for their assays; instead, they used the SiMoA-HD1 hTau kit and modified the detector reagent to use the AT270 mAb specific for pTau181. They also used a standard from a different kit (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) without providing sufficient sensitivity and specificity for the “new” assay [30]. Thus, interpretation of the results regarding their measurements of blood P-tau level is difficult. Another study using the pTau181 SiMoA kit reported that plasma pTau181 correlated with the severity of Braak tau staging, defined using tau PET AV-1451, among a group of 76 participants [52 cognitively normal, 9 with mild cognitive impairment (MCI), and 15 with AD dementia] [31]. A limitation of this study was that plasma pTau181 was only quantifiable in 51 of the 76 participants, which is in contrast to plasma T-tau which was quantifiable in 75 of the 76 participants. The reasons for the lack of quantification of pTau181 in one-third of the participants were not discussed by the authors, but this limitation is concerning. A third study of 172 cognitively unimpaired participants, 57 participants with MCI, and 40 AD dementia patients reported that pTau181 levels were higher in AD dementia patients than in those who were cognitively unimpaired [32]. In addition, plasma pTau181 was more strongly associated with both amyloid and tau PET, compared to plasma T-tau, was a more sensitive and specific predictor of elevated brain amyloid than plasma T-tau, and was as good as, or better than, the combination of age and *APOE*. Although plasma T-tau in this study was measured using a SiMoA kit, measurement of pTau181 was performed on a streptavidin small spot plate using the Meso Scale Discovery (MSD) platform (Meso Scale Diagnostics, Rockville, MD, USA) with different antibodies compared to the SiMoA kit. However, development of a pTau181 SiMoA kit with these different antibodies is currently being investigated. In addition to kits for measuring pTau181, a SiMoA kit for the measurement of P-tau 231 is available,

but little research has been published with this kit to date.

## PLASMA SIMOA T-TAU ASSAY

The commercially available SiMoA T-tau assay measures mid regions of tau protein isoforms, hence the term total tau (T-tau). Tau protein isoforms in the blood are different from those in the CSF in that full-length tau is the dominant isoform in plasma but not in the CSF. This difference suggests that the majority of plasma tau comes from peripheral sources rather than from the brain [33]. Using the SiMoA assay, levels of T-tau have been found to be stable up to four freeze–thaw cycles [19]. Indeed, plasma T-tau has been found to have weak correlations with CSF T-tau or P-tau and with Tau PET AV-1451 [32, 34, 35]. Elevated plasma T-tau and CSF T-tau have also been associated with differential brain atrophy patterns in gray matter density [36]. In one small study, however, the plasma T-tau/ $A\beta_{42}$  ratio was predictive of brain tau deposition [31].

Cross-sectional studies of the SiMoA total tau assay have shown elevated plasma T-tau levels in AD dementia patients compared to cognitively normal controls, as well as associations with lower cortical thickness [32, 34, 36, 37]. Higher levels have also been found in those with MCI compared to cognitively unimpaired controls, but there was considerable overlap in levels between diagnostic groups. In longitudinal studies, higher plasma T-tau levels been associated with cognitive decline and risk of MCI, and this association was independent of brain amyloid levels [38]. A more recent study reported, in two independent samples, that higher plasma T-tau levels were associated with an increased risk of all-cause dementia and AD dementia as well as hippocampal volume loss [35]. However, pathological confirmation and amyloid PET data were not available to confirm the diagnosis of AD dementia, which was solely based on a clinical diagnosis.

Together, these results suggest that plasma T-tau, as measured by SiMoA, will not be a useful stand-alone biomarker for the diagnosis of preclinical or prodromal AD. Moreover, given

that the association of plasma T-tau with cognition and MCI has been found to be independent of elevated brain amyloid, this marker is not specific to the pathophysiological process of AD. However, plasma T-tau could be useful as a prognostic marker of non-specific cognitive decline and neurodegeneration and fits into the AT(N) scheme of the new NIA-AA Research Framework as a potential blood-based measure of neuronal injury and neurodegeneration ('N') [1]. Additional research is therefore needed to elucidate what plasma T-tau is indicative of in terms of brain structure and function and which aspect of neurodegeneration. Plasma T-tau has been reported to be higher in those with a history of diabetes, hypertension, atrial fibrillation, and myocardial infarction, suggesting the possibility that plasma T-tau could also be indicative of cerebrovascular pathology [37].

## PLASMA SIMOA NEUROFILAMENT LIGHT ASSAY

Neurofilament light chain is a recognized biomarker of subcortical large-caliber axonal degeneration [39, 40]. Unlike T-tau, multiple studies have reported modest, yet similar, correlations between plasma and CSF NfL, ranging from 0.569 in the The Alzheimer's Disease Neuroimaging Initiative to 0.590 in the Mayo Clinic Study of Aging [41, 42]. Moreover, plasma NfL has been shown to have similar effect sizes to CSF NfL in terms of short-term change in cognition and imaging measures of neurodegeneration [42].

Historically, plasma NfL has been measured using ELISA technology and electrochemiluminescence (ECL) assay technology. However, the sensitivity of ELISA for quantifying plasma NfL has been found to be insufficient, and ECL is not sufficiently sensitive to detect the lowest concentrations of plasma NfL. Therefore, studies now utilize SiMoA technology for measuring blood NfL due to its higher sensitivity [43, 44]; in contrast, CSF NfL can be measured with other technologies because of the relatively higher concentration of NfL in the CSF. The levels of NfL are higher in serum than in plasma, but the majority of studies have utilized plasma to

quantify NfL. A study utilizing serum NfL reported that concentrations were stable up to four freeze–thaw cycles [19]. In contrast, a study examining plasma NfL reported little change after one freeze–thaw cycle, but found that subsequent cycles were associated with slight but significant increases [45]. Whether the difference in the two studies is due to using plasma versus serum to quantify NfL is not known, but additional research is needed to clarify the effect of multiple freeze–thaw cycles on NfL measurements. In other analyses assessing the stability of plasma NfL, samples kept for 5 days at room temperature or stored in a refrigerator had higher plasma NfL levels than those which were subjected to rapid freezing [45].

Elevated plasma NfL levels have been found in multiple neurodegenerative conditions associated with neuronal axonal damage, including frontotemporal dementia [46], multiple sclerosis [47], traumatic brain injury [48], atypical parkinsonian disorders [49], and AD dementia [32, 50]. Thus, NfL is hypothesized to be a non-specific marker of neurodegeneration and, like T-tau, fits into the AT(N) scheme of the new NIA-AA Research Framework as a potential fluid-based measure of neuronal injury and neurodegeneration ('N'). Indeed, among participants who are cognitively unimpaired or have MCI, the relationship between plasma or CSF NfL and longitudinal changes in cognition or brain imaging measures of neurodegeneration are independent of elevated brain amyloid [32, 50].

Cross-sectional studies have shown that blood NfL increases with increasing symptom severity across the clinical AD spectrum and as familial AD mutation carriers age comes closer to the estimated age of onset [51]. However, although significant differences in NfL levels are often shown in comparisons of AD dementia patients and cognitively normal individuals, with excellent accuracy based on the area under the curve, reports of significant differences between those with MCI and cognitively normal individuals have been mixed (e.g., [50, 52]). Individuals with more rapidly progressing neurodegenerative diseases (e.g., frontotemporal dementia, HIV-associated dementia) have higher levels of plasma NfL that do AD

dementia patients because of the greater rate of axonal degeneration in the former diseases, but plasma NfL levels again have been found to overlap across diagnostic groups [53]. Thus, given the lack of disease specificity of blood NfL, or even CSF NfL, it is unlikely that NfL can be used as a diagnostic tool for AD [44].

In longitudinal studies, plasma NfL has been found to predict short- (15 months) and longer-term (up to 4 years) change in cognition among cognitively unimpaired individuals and those with a diagnosis of MCI or AD dementia [42, 52]. Rates of change in plasma NfL have also been found to correlate with rates of change in cognition, CSF  $A\beta_{42}$  and P-tau markers, hippocampal volume loss, and FDG-PET hypometabolism [42, 52]. These results suggest that plasma NfL may be a useful as a prognostic marker of cognitive decline and neurodegeneration and to track longitudinal neurodegeneration in AD. This marker could also be useful as a surrogate endpoint of neurodegeneration in clinical trials.

## CONCLUSION

The technological advances in the last decade have led to increasing opportunities to measure blood-based biomarkers of AD pathology, including  $A\beta_{42}$ ,  $A\beta_{40}$ , P-tau, T-tau, and NfL. The development of ultrasensitive technologies has been particularly important in moving the field forward. SiMoA is the most established ultrasensitive technology in the field of blood-based biomarkers of AD pathology from the research perspective. However, it is still uncertain whether the current ultrasensitive technologies, including SiMoA, will be readily available in clinical laboratories for screening for AD pathology and longitudinally tracking neurodegeneration in AD patients. The translation of blood-based AD biomarkers into diagnostic biomarkers for routine use in patient care will involve many steps, including well-defined intended use, validation of analytical and clinical performance in the context of intended use, and regulatory approval (e.g., United States Food and Drug Administration). Even though regulatory approval may not be required for a

biomarker to be used clinically [e.g., available as a laboratory-developed test through Clinical Laboratory Improvement Amendments (CLIA) by CLIA-certified clinical laboratories], the wide adoption of a clinical test is mainly driven by clinical needs. Clinical needs of blood-based biomarkers of AD pathology will most likely be driven by the availability of disease-modifying treatments and precision medicine (e.g., the right drug for the right patient). It remains to be seen whether SiMoA assays will be used in the clinical setting for patient care. Nevertheless, the exciting scientific evidence generated by SiMoA assays to date and in the future will continue to enhance and influence our understanding of AD.

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