Blood-based high sensitivity measurements of amyloid beta and phosphorylated tau as biomarkers of Alzheimer's disease: A focused review on recent advances

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ABSTRACT

Discovery and development of clinically useful biomarkers for Alzheimer's disease (AD) and related dementias have been the focus of recent research efforts. While cerebrospinal fluid (CSF) and positron emission tomography (PET)- or magnetic resonance imaging (MRI)-based neuroimaging markers have made the *in vivo* detection of AD pathology and its consequences possible, their high cost or their invasiveness have limited their widespread use in the clinical setting. On the other hand, advances in potentially more accessible blood-based biomarkers had been impeded by lack of sensitivity in detecting changes in markers of the hallmarks of AD, including amyloid-β (Aβ) peptides and phosphorylated tau (P-tau). More recently, however, emerging technologies with superior sensitivity and specificity for measuring Aβ and P-tau have reported high concordances with AD severity. In this focused review, we describe several emerging technologies, including immunoprecipitation-mass spectrometry (IP-MS), Single molecule array (Simoa) and Meso Scale Discovery (MSD) immunoassay platforms, and appraise the current literature arising from their use to identify plaques, tangles and AD. Whilst there is potential clinical utility in adopting these technologies, we also highlight that further studies are needed to establish Aβ and P-tau as blood-based biomarkers for AD, including validation with existing large sample sets, new independent cohorts from diverse backgrounds as well as population-based longitudinal studies. In conclusion, the availability of sensitive and reliable measurements of Aβ peptides and P-tau species in blood holds promise for the diagnosis, prognosis, and outcome assessments in clinical trials for AD.

(242 words)

INTRODUCTION

There are over 50 million people worldwide suffering from dementia, a number which is projected to triple by 2050 [1]. The high prevalence of dementia is accompanied by a massive social and economic burden; the current annual cost of dementia is estimated at US $1 trillion and is set to double by 2030 [1]. The most common cause of dementia is Alzheimer's disease (AD), which accounts for approximately 60% to 80% of all dementia cases [2]. AD is characterized by progressive memory deficits as well as non-amnestic cognitive symptoms, including impairments in language, visuospatial and executive function that severely impact activities of daily living. Neuropathologically, AD is characterized by progressive synaptic dysfunction and neuronal loss in brain regions that are essential for higher cognitive functions [3], culminating in brain atrophy and clinical symptoms in patients. The hallmarks of AD include cortical extracellular amyloid-β (Aβ) plaques composed of highly aggregated, fibrillar 40- to 42-amino-acid Aβ peptides (Aβ40 and Aβ42), as well as intracellular neurofibrillary tangles (NFTs) and dystrophic neurites surrounding the plaques, both composed of paired helical filament-forming, abnormally hyperphosphorylated tau protein aggregates [2]. As neurodegeneration is thought to be well underway by the time patients exhibit clinical symptoms [4], these core pathophysiological features have been targets for AD biomarker development – a key area in dementia research that holds promise for early detection of the disease which in turn helps improve the drug development process for AD (see below). Whilst *postmortem* neuropathologic evaluation remains the gold standard for confirmatory diagnosis of AD pathology, the core AD diagnostic biomarkers in living individuals with positron emission tomography

(PET assessment of *in vivo* Aβ and tau cortical burden) or cerebrospinal fluid (CSF measurements of Aβ42, tau, and P-tau) analyses have gained acceptance [5] but remain relatively inaccessible.

APPLICATIONS AND CHALLENGES OF BLOOD-BASED AD BIOMARKERS

The shift towards a biological definition of AD

Historically, AD was conceived of as a clinical-pathological construct, such that cognitive symptoms defined the presence of AD [6]. However, approximately 30% of individuals clinically diagnosed as AD do not display significant AD neuropathological changes at autopsy or by *in-vivo* imaging [7]. The NIA-AA research framework has recently suggested that AD should be defined as a biological construct using biomarkers that are characteristic of AD pathophysiology, such as Aβ and phosphorylated tau [5, 7]. This biological definition of AD is potentially useful in both research and clinical care settings. In the former, it facilitates efforts to understand the sequence of events leading to cognitive impairment that is associated with AD neuropathology. In the latter, it allows clinicians to confirm the etiological diagnosis with greater certainty and provide biomarker-guided targeted therapies when they are available [6]. Furthermore, it improves early identification (screening) of older people at risk of developing AD, providing access to important patient cohorts crucial for the clinical evaluation of promising therapeutic strategies of those with significant burden of pathologic proteins.

Recruitment of appropriate trial subjects for disease-modifying therapies

Given that current AD treatments provide only symptomatic relief, there has been a focus on clinical trials of disease-modifying therapies (DMT), targeting specific AD pathologies, in particular amyloidosis but recently also tau pathology. In DMT trials, it is imperative to show that an intervention modifies the target pathophysiological process and improves clinical symptoms [7]. Disappointingly, past DMT trials have largely reported no or minimal cognitive benefits. A possible explanation is the inclusion of participants lacking the target pathology since recruitment was based mainly on clinical assessment. Another possibility is the inclusion of individuals too advanced in the disease process, with a clinical course which may be difficult to modify regardless of the efficacy of the drug candidates. Thus, it is necessary to conduct biomarker-driven subject selection in DMT trials to facilitate target engagement and reliably assess the efficacy of interventions [5]. An example of such a trial is a phase II clinical study of BAN2401 (also called lecanemab) in early AD subjects with positive biomarkers for brain amyloid pathology. BAN2401 is a humanized monoclonal antibody that selectively binds to, neutralizes and eliminates soluble toxic Aβ aggregates. BAN2401 showed a significant, dose-dependent reduction in PET Aβ such that 81% of participants demonstrated reduced Aβ aggregate densities and were reclassified from Aβ positive to negative at 18 months with the highest tested dose. Importantly, participants also showed slower cognitive decline [8, 9]. Given these promising results, a global multi-center phase III clinical study of BAN2401 (Clarity AD) has been initiated. Other examples include the Phase III trials, ENGAGE and EMERGE, which aimed to assess the cognitive and functional impacts of the aggregated Aβ-binding monoclonal antibody

drug aducanumab in individuals with mild cognitive impairment (MCI) due to early AD as determined by a positive PET Aβ scan. In a report at the end of 2019, it was noted that at the highest dose, participants in EMERGE had a significant reduction in the rate of cognitive decline using standardized neuropsychological test (Clinical Dementia Rating – Sum of Boxes, CDR-SB) [10]. Similarly, in ENGAGE, a subgroup of participants who received the highest dose at least ten times declined more slowly on the CDR-SB. In sub-studies, aducanumab caused a dose-dependent reduction in brain amyloid burden. Overall, these clinical trials point to the importance of trial recruitment with supporting biomarker evidence to ensure that the appropriate participants are enrolled and to provide evidence of the efficacy of the tested drugs in targeting AD pathology.

Current challenges in the use of AD biomarkers

While AD biomarkers measured by PET or CSF are highly indicative of AD pathophysiology, the challenges involved in their usage, including invasiveness of procedures, high cost, limited accessibility to scanners and cyclotrons, and limited utility as a screening tool, have greatly impeded their application in the clinical and research settings [3, 6]. In contrast, blood-based biomarkers would be a more widely accepted and practical approach if they had comparable sensitivity and specificity to the neuroimaging and CSF markers [3, 5]. However, most early studies on blood Aβ42, Aβ40 and Aβ42/40 ratio using conventional ELISA or multi-analyte Luminex immunoassays reported little or no difference between AD and control groups [11]. Furthermore, the majority of these early studies were based on comparing blood Aβ in clinically diagnosed AD patients with cognitively unimpaired controls which, given the

uncertain value of clinical assessments in assessing pathological burden, might have underestimated and confounded the diagnostic value of plasma Aβ [5, 11]. The specific limitations of conventional blood Aβ measurements are outlined below.

Low abundance of Aβ in peripheral blood

Due to filtering effects of the blood-brain barrier, dilution in the large plasma volume, rapid metabolism and clearance and adhesion to other plasma proteins leading to epitope masking and analytical interference, concentrations of Aβ in the blood are much lower than those in CSF [3, 5, 6]. This low abundance impeded the reliable, quantitative measurement of peripheral Aβ in earlier studies using conventional immunoassays with their inherent limits in sensitivity.

Measurement of soluble Aβ in the peripheral blood may not be reflective of brain amyloid plaque burden, as indicated by the poor correlation between blood and CSF Aβ levels (REF). Furthermore, early studies used assays that measured only soluble Aβ in the blood and could not recognize aggregated isoforms, leading to under-detection of Aβ oligomers that are widely postulated to be the major neurotoxic forms of Aβ [12-14]. The ability to measure specific isoforms of Aβ isoforms such as Aβ oligomers could therefore be potentially more closely linked to brain amyloid pathological burden.

Emerging technologies for blood-based amyloid measurements

In recent years, there has been a growing impetus for the development of new platforms and immunoassays to measure peripheral Aβ. These developments helped address the major challenges listed above by having improved sensitivity compared with conventional assays, and/or the ability to measure Aβ variants that may be more relevant to AD pathology [5]. The following section aims to summarize these emerging techniques or technologies, with brief descriptions of the assay principles followed by appraisals of their ability to identify individuals with significant brain Aβ burden (Aβ+) compared to those without (Aβ-, as confirmed by brain PET or CSF analyses). To assess the diagnostic performance of blood Aβ in detecting elevated Aβ, areas under the receiver operating characteristic curves (AUC) were given where reported. For associations between blood Aβ and continuous variables such as the standardized uptake value ratio (SUVR) and CSF Aβ levels, correlation or regression results was considered. A summary of the results is provided in **Table 1** (with detailed cohort information given in **Supplementary Table S1**). While the use of amyloid PET or CSF analyses to classify participants into amyloid positive or negative, as compared to classifications based on clinical AD and cognitively unimpaired elderly, improves performance, an inherent issue with blood Aβ as a biomarker is that the fold change is much lower (10-15%) in plasma as compared with 40-50% in CSF, probably related to that a large proportion of Aβ comes from peripheral expression of the peptide.

Next generation enzyme-linked immunosorbent assays (ELISAs)

As mentioned, early generation ELISAs were largely unable to accurately detect and measure AD-associated changes in blood amyloid. However, significant

improvements have been made in newer ELISAs to enhance their performance. For instance, the EUROIMMUN ELISA study [15] employed C- and N-terminal antibodies, while previous ELISAs did not, and consequently detected different Aβ fragments. Furthermore, improvements in assay design and conjugation method may also have resulted in increased sensitivities, thus enabling the detection of more subtle variations in biomarker profiles than attainable with early generation ELISAs. These improvements likely led to similar performance between the EUROIMMUN ELISA and the ultrasensitive Simoa ("Amyblood") assay (see below) in a head-to-head comparison, where the accuracy of detecting PET Aβ+ in non-demented participants through measurements of plasma Aβ42/Aβ40 ratio was similar, and plasma Aβ42/Aβ40 as well as Aβ42/t-tau ratios measured by both methods correlated to the same extent with amyloid PET and CSF Aβ42/t-tau [15]. Nevertheless, more head-to-head comparisons of the different available ELISAs are needed.

Single molecule array (Simoa) immunoassay

The Simoa immunoassay is a digital ELISA, allowing concentrations to be determined digitally rather than by measurement of the total analog signal (Quanterix, USA). Briefly, after formation of the immunocomplex on paramagnetic beads, the beads are transferred to hundreds of thousands of femtoliter-sized wells, each sized to hold only one bead. By confining the fluorophores generated by individual enzymes to extremely small volumes (approximately 40 fL), a high local concentration of fluorescent products is achieved. Therefore, a readable signal is detected even if only a single sandwich complex is present on the bead. The analyzer counts the number of wells

containing an enzyme-labelled bead and wells containing a bead. The ratio between the counts provides the average enzyme per bead (AEB) number, and the concentration of the target analyte in the sample is derived from a standard curve.

The first version of the Simoa assay for blood Aβ was published in 2011. Using this assay, two large, independent studies, Janelidze et al. [16] and Verberk et al. [17], reported no or weak correlations between the plasma Aβ biomarkers and established measurements such as SUVR, CSF Aβ42 or CSF Aβ42/Aβ40 ratio (rho=0.16 to 0.38). In distinguishing between PET Aβ+ and Aβ- subjects, Janelidze et al. reported a modest AUC of 0.60 to 0.62 for the plasma Aβ biomarkers, while Verberk et al. reported numerically higher AUCs of 0.66 to 0.79. The varying reported ranges may be due to differences in the cohorts used, adjustments of AUCs (for age, gender and APOE genotype) or the combination of detector-capture antibody pairs used. Simren et al. did, however, show that longitudinal change in Simoa Aβ42/Aβ40 measures were sensitive to grey matter loss in cognitively normal unimpaired individuals. Janelidze et al. used the "first-generation" singleplex from Quanterix which employed N-terminal antibody 6E10 as the capture antibody and isoform-specific C-terminal antibodies, namely H31L21 and 2G3 for Aβ42 and Aβ40 respectively, as the detector antibodies. However, such antibody pair combination may result in lower assay specificity [15]. In contrast, Verberk et al. used the Neurology 3-plex which switched the antibodies that are used to capture and detect plasma Aβ [17]. Matching the antibody orientation of Verberk et al., a small-scale study by Thijssen et al. [18] chose the C-terminal antibodies 21F12 and 2G3 as capture antibodies for Aβ42 and Aβ40 respectively, and N-terminal antibody 3D6 that binds to the first five residues of the Aβ peptide, as the detector antibody. This differed

from the commercially available Quanterix assay, which used an N-terminal antibody to capture both peptides, followed by 21F12 or 2G3 for detection of Aβ42 and Aβ40 respectively. When compared with the commercially available assay, the prototype assay performed better in discriminating between Aβ+ and Aβ- subjects (AUC 0.95 vs 0.85) and produced a better correlation between plasma Aβ42/Aβ40 ratio and CSF Aβ42 (rho=0.71 vs rho=0.53). Overall, these studies indicated that whilst Simoa provides superior sensitivity, the antibody orientation should be carefully considered to maximize assay performance.

A major advantage of the Simoa immunoassay is its increased sensitivity when compared with conventional immunoassays, measuring proteins at femtomolar concentration and allowing detection of low abundance targets such as peripheral Aβ. Moreover, the assay may be performed in a fully automated setup, increasing efficiency and output, and minimizing variability of results common in a manual setup. The scalability of this method implies that it is highly adaptable to clinical care and clinical trial settings. However, as discussed above, further optimization to the assays to confirm its sensitivity, specificity and consistency is warranted.

Immunoprecipitation-mass spectrometry (IP-MS)

This technique involves the isolation and enrichment of Aβ peptides from plasma by immunoprecipitation (IP) using a specific antibody, followed by identification and relative quantification of the individual Aβ isoforms including Aβ42, Aβ40, Aβ38 or APP669-711 with highly sensitive mass spectrometry (MS) [19-22]. While the principle underlying this

approach is similar for the four studies listed below and in **Table 1**, there are key differences in methodologies. Kaneko et al. [19] and Nakamura et al. [20] employed matrix-assisted laser desorption/ionization-time-of-flight MS (MALDI-TOF/MS), whereas Ovod et al. [21] used liquid chromatography tandem mass spectrometry MS (LC-MS/MS). Pannee et al. [22] used MALDI-TOF/MS Aβ profiling followed by LC-MS/MS to confirm the identities of the Aβ peptides. In comparison with LC-MS/MS which generates multiple mass spectra for each fragmented peptide and compares these spectra to a database for protein identification, in MALDI-TOF/MS, only one MS spectrum is generated, compiling the masses of all peptides. As such, MALDI-TOF/MS provides a quicker analysis and higher throughput, making it more feasible for large-scale studies. However, MALDI-TOF/MS is less accurate and reliable in protein identification due to the lack of true sequence dependence of data since only a mass spectrum is generated for all peptides.

Next, the studies have selected different antibodies and a number of rounds of IP, which may affect the overall specificity and sensitivity of the assay. In addition to Aβ42 and Aβ40, Kaneko et al. and Nakamura et al. measured APP669-711 (Aβ-3–40) while Pannee et al. and Ovod et al. measured Aβ38 (APP672-709). The purpose of these measurements was to correct for inter-individual variability in the overall metabolic production of Aβ [23]. Similar to Aβ40, both APP669-711 and Aβ38 are thought to increase due to higher overall Aβ production, but are unaffected by AD pathology [23]. Thus, the ratio of each individual isoforms and Aβ42 may serve as a better predictor of brain amyloid burden than Aβ42 alone. For instance, CSF Aβ42/Aβ38 ratio was

reported to be better than CSF Aβ42 alone at predicting PET Aβ positivity, with a performance that is comparable to CSF Aβ42/Aβ40 ratio [24].

The published AUC results suggested that plasma APP669-711/Aβ42 ratio (AUC ≥ 0.86), Aβ42/Aβ40 ratio (AUC ≥ 0.80) or Aβ40/Aβ42 ratio (AUC ≥ 0.87) was able to discriminate between Aβ+ and Aβ- subjects. In their study, Nakamura et al. evaluated the classification ability (Aβ+ vs Aβ-) of a composite biomarker – an average of the normalized values of APP669-711/Aβ42 ratio and Aβ40/Aβ42 ratio. Although the composite biomarker (AUC=0.91) showed the highest classification ability among the Aβ biomarkers, there was reservation regarding the interpretation of the data. A composite biomarker should be derived from two independent markers but APP669-711/Aβ42 ratio and Aβ40/Aβ42 ratio are not independent – Aβ42 was accounted for twice in the formula and being the most impactful on the analyses, it could potentially skew the overall results and conclusion.

A major advantage of IP-MS is the consistency of the ROC results across different studies, despite differences in methodology. All three studies [19-21] reported high AUCs for the combined ratios of Aβ42 and Aβ40 or APP669-711, indicating the superior ability of these ratios to detect elevated brain Aβ. Furthermore, this approach allows for the detection of multiple Aβ-related peptides in human plasma such as APP669-711, for which there is currently no commercially available antibody-based assay kits. Nonetheless, there are limitations to IP-MS which may reduce feasibility for widespread clinical use, such as complexity of the procedures, long processing times and inconsistency in analytical procedures across different diagnostic labs.

Development of an automated assay system to standardize the analytic factors and increase throughput is needed to support its usage in a larger setting.

Elecsys immunoassays

The Elecsys immunoassay is an electrochemiluminescence (ECL) method using a sandwich principle performed in a fully automated setup (Roche Diagnostics). First, samples are mixed with biotinylated antibodies specific to the target Aβ isoforms and ruthenium-labelled detection antibodies, resulting in the formation of a sandwich complex. Next, streptavidin-coated magnetic beads are added which bind to the biotinylated antibodies. For measurement, the reaction mixture is aspirated into a measuring cell where the beads are magnetically captured onto the surface of an electrode. To start the reaction, voltage is applied to the electrode, leading to emission of photons from the ruthenium complex, which is measured by a photomultiplier. The analyte concentration is determined via a calibration curve.

Using CSF Aβ42/Aβ40 ratio as a marker of Aβ status against which plasma Aβ was compared, Palmqvist et al. reported a decrease in Elecsys-measured plasma Aβ42/Aβ40 ratio in Aβ+ subjects [25], and there was also a marked decrease in plasma Aβ42 and Aβ40, especially in AD dementia subjects [25]. The plasma Aβ42, Aβ40 and Aβ42/Aβ40 ratio predicted Aβ positivity with an AUC of 0.71, 0.54 and 0.77 respectively. When using plasma Aβ42 and Aβ40 as separate predictors in a logistic regression, the AUC improved slightly to 0.80, and further increased to 0.85 after the addition of *APOE* genotype to the model. As *APOE4* allele carriers have significantly lower CSF Aβ42

levels, it is suggested that the combination of APOE genotype and blood-based biomarkers could further support the diagnosis of AD [26]. In the study, a moderate positive correlation (r=0.48) was reported between CSF and plasma Aβ42/Aβ40 ratios. While the performance for the individual biomarkers was not satisfactory, the inclusion of *APOE* genotype in a combined model significantly improved the AUC, suggesting that future biomarkers studies may consider the inclusion of AD risk factors (e.g. age, gender, *APOE*) to improve prediction [17, 20].

The Meso Scale Discovery (MSD) platform

Similar to the Elecsys immunoassay, the MSD platform is an ECL-based immunoassay (Meso Scale Diagnostics). However, unlike Elecsys, the MSD platform allows multiplexing. The MSD plate has a working electrode surface where specific capture antibodies are immobilized on independent and well-defined spots. Depending on the capture antibody immobilized on each spot, multiple target analytes may be captured from the sample. Furthermore, both techniques utilize different ECL labels and antibody pairs, which may impact on their sensitivity and specificity. Plates containing samples, capture antibody and detection antibody conjugated with MSD SULFO-TAG™ labels are loaded into the MSD instrument where a voltage is applied to the plate electrodes, causing the captured labels to emit light. Multiple excitation cycles amplify signals to increase the sensitivity of the assay and analyte concentrations are determined via a calibration curve.

Using PET for Aβ status, Vogelgsang et al. reported no significant difference in plasma Aβ42 and Aβ42/40 ratio between Aβ+ and Aβ- groups [27]. The study also showed a moderate but positive correlation between CSF and plasma Aβ42/Aβ40 ratios (rho=0.43), comparable to results reported for Elecys (see above). This may not be surprising given the similarity of the underlying assay principles. Currently, there is little evidence supporting the use of MSD platform in the measurement of plasma Aβ as a blood-based biomarker. Since the study cohort was small (n=41), future studies using a larger cohort may provide more indication on the potential use of this platform.

Immunomagnetic Reduction (IMR)

IMR quantifies the concentrations of target proteins/peptides in a sample by measuring the percentage reduction in the alternating current magnetic susceptibility of the IMR reagent caused by the binding of the antibody-coated magnetic nanobeads with the targets (MagQu Co.Ltd., Taiwan). When the IMR reagent which contains the antibody-coated magnetic beads is mixed with the sample, the target analytes bind with the antibodies and part of the magnetic beads become enlarged, leading to a reduction in the oscillation speed and magnetic signal of the beads. The reduction percentage is referred to as the IMR signal. Sample analyte concentration is then calculated based on the established relationship between protein standard concentration and IMR signal.

In contrast to other immunoassays, IMR-measured plasma Aβ42 or Aβ42/Aβ40 ratio was increased in Aβ+ subjects [28, 29]. A possible explanation is that other immunoassays are mostly based on sandwich ELISA which relies on the binding of both

capture and detection antibodies to measure plasma Aβ. Since Aβ is frequently bound to plasma proteins, this may induce a potential stereoscopic obstacle for two antibodies to associate with one Aβ molecule simultaneously, leading to partial loss of signal. In comparison, the IMR method uses a single antibody to capture Aβ molecule in the plasma. Therefore, the IMR method has a higher possibility of capturing and detecting the target Aβ molecules in various conformations, such as isolated, complex or oligomeric forms.

To date, two IMR-based studies have reported contradictory results, with Tzen et al. [28] showing a significant association between IMR-measured plasma Aβ42/Aβ40 ratio and PET SUVR (β=0.652) while Teunissen et al. [29] reported a negative correlation between plasma Aβ42 and CSF Aβ42 (rho=-0.352). Future studies may need to compare AUC performances between PET and CSF markers, as well as their associations with IMR within the same cohort. Based on the current results, there is a lack of consistent findings to support diagnostic utility for this approach.

Amplified Plasmonic Exosome (APEX) platform

Whilst the above-mentioned techniques and platforms mainly addressed Aβ sensitivity issues, APEX and the subsequently described methods have the potential to specifically measure different Aβ aggregation species. APEX is based on the finding that exosomes can bind to extracellular Aβ42 proteins via glycoproteins or glycolipids on the exosomal plasma membrane. In their study, Lim et al. established that exosomes have a higher binding affinity to larger Aβ42 aggregates which also have a stronger

propensity to form aggregated, fibrillar structures [30]. Briefly, after binding of the target to the APEX nanosensor via the capture antibody, insoluble optical deposits are formed on the sensor through *in-situ* enzymatic amplification. This deposition enhances the surface plasmon resonance (SPR) signal, as represented by a greater spectral shift. Through modifications in the sensor's design and fabrication (e.g., patterned silicon nitride membrane, double-layered nanostructure, back illumination), APEX is designed to achieve nanoscale detection. To measure exosome-bound Aβ42 from plasma, Aβ42 is directly enriched from plasma via the Aβ42 capture antibody on the APEX nanosensor, and the relative amount of CD63, an established exosomal marker associated with the captured Aβ42, is measured via the CD63 detection antibody. Measurements are made relative to a sample-matched negative control, where the same sample was incubated over a control sensor functionalized with IgG isotype control antibody.

In their small cohort of 72 subjects, Lim et al. reported a strong positive correlation between the level of plasma exosome-bound Aβ42 and SUVR (rho=0.95). Using the same cohort, Tanaka et al. [31] performed the first head-to-head comparison study between APEX and Simoa and showed that plasma exosome-bound Aβ42 (AUC=0.995) outperformed Simoa-measured Aβ42/Aβ40 ratio (AUC=0.816) and Aβ42 alone (AUC=0.776) in distinguishing between PET Aβ+ and Aβ- subjects. These preliminary findings are promising but further validation in larger independent cohorts is needed to establish APEX as a biomarker platform.

Multimer Detection System (MDS)

MDS is a sandwich ELISA that preferentially detected oligomers over monomers [32]. A unique epitope exists in the Aβ monomer, with multiple copies of this epitope found in the multimer. Thus, if antibodies targeting the unique/overlapping epitope were used for both capturing and detecting antibodies, the monomer would only be occupied by one of the antibodies and no signal would be produced. In contrast, multiple copies of the unique epitope in a multimer would allow binding of both the capturing and detecting antibodies to produce detectable signals.

In an earlier study, An et al. [33] reported that MDS was unable to discriminate crude Aβ oligomer (AβO) levels in the plasma of AD patients from controls due to low concentrations. However, spiking the plasma samples with synthetic Aβ42 resulted in significant increases in Aβ oligomers (AβO) levels in the AD patients, but not in controls [33]. A possible explanation is that oligomerization of Aβ is influenced by potential factors in plasma of AD patients, which may be absent or in lower concentrations in controls. Adopting a similar concept to measure plasma AβOs by MDS, Wang et al. spiked synthetic Aβ42 peptide and used epitope-overlapping Aβ antibodies at the N-terminus 3-4 of Aβ [32]. They also reported a moderate correlation between plasma AβO levels and PIB PET SUVR (rho=0.43) or CSF Aβ42 levels (rho=-0.44). The present study samples were small (n=50). Furthermore, the current long incubation time (144 hours) needs to be reduced to be feasible for clinical use.

Interdigitated Microelectrode (IME) Sensor system

Kim et al. introduced the use of an IME sensor system, together with 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (EPPS) – a molecule which aids in converting aggregated Aβ into its monomeric form; for the measurement of plasma Aβ in the heterogenous and monomerized states [34]. Briefly, the chip's sensing zone was coated with anti-Aβ antibody to capture the Aβ in plasma. Two microchannels were incorporated on top of the chip to simultaneously load two different plasma samples – the EPPS treated and nontreated samples. The interaction between plasma Aβ and anti-Aβ antibodies was measured using an impedance measurement system, which was constructed to cancel noise signals from parasitic capacitance of IME and subsequently amplify the low-level signals. The impedance change increases as the concentration of Aβ increases. To mitigate inter- and intraindividual variations in Aβ levels, a self-standard ratio was calculated by dividing the concentration of homogenous Aβ monomers (EPPS treated) by that of heterogenous Aβ (nontreated).

Using blood collected from two clinical institutes, Kim et al reported a positive correlation between the self-standard ratio and PET SUVR in both cohorts (rho=0.551 and 0.414 respectively). Similar to the majority of the other studies, more results, including AUC, are needed to establish the usefulness of this method.

Blood-derived phosphorylated tau: A promising marker of AD pathophysiology

Besides Aβ, there has been concerted efforts to accurately measure another established CSF biomarker, namely phosphorylated tau (P-tau), in blood. These attempts are driven by the development of ultrasensitive technologies enhanced by

automation and improved throughput, such as the Simoa platform (see above). Following initial analytical challenges that hampered successful application of blood P-tau assays, recent advances in the understanding of tau biochemical processing in the brain as well as the subsequent release of soluble P-tau into biofluids have enabled unprecedented rapid development of P-tau biomarkers in blood. Firstly, a blood alternative of the established CSF marker of tau pathology, P-tau181, was developed by focusing on fragments containing the N-terminal portion of the protein that appears to be released into blood more abundantly than mid-region forms that the conventional CSF assays focus on. Blood P-tau181 assessment methods from different sources, mainly the University of Gothenburg and Eli Lilly (using Simoa and MSD technologies, respectively), have shown excellent analytical and diagnostic performances in several landmark publications (**Table 2**, with detailed cohort information given in **Supplementary Table S2**). Next to be developed were P-tau biomarkers focusing on other phosphorylated sites, e.g., P-tau217 [35] and P-tau231 [36] which have shown comparable diagnostic utility and, in some cases, superior predictive abilities particularly in the preclinical stages of AD (**Table 2**). Furthermore, IP-MS technology-based multiplexed assays have been developed that can measure multiple P-tau biomarkers in the same plasma samples simultaneously. These include P-tau181, P-tau217, P-tau199, P-tau202, P-tau205 and P-tau231 (any citations?).

Summary of the recent blood P-tau181 findings

Most current studies employed Simoa or MSD for the measurement of blood P-tau181, with results broadly similar across the two platforms. Several studies showed

that blood P-tau181 concentrations were increased in MCI due to AD and AD dementia, but not in non-AD dementia including FTD, PD and VaD [37-44]. Specifically, blood P-tau181 was consistently elevated in the Aβ+ subjects (e.g. Aβ+ MCI and Aβ+ AD) [37, 40, 42, 44, 45]. Plasma P-tau181 was also shown to be able to differentiate AD from non-AD neurodegenerative diseases (AUCs=0.82 to 0.94) [37, 42-44]. Furthermore, plasma P-tau181 detected elevated brain Aβ (Aβ+) in combined NCI, MCI and dementia subjects (all subjects; AUCs= 0.79 to 0.91) [37, 40, 42, 43], as well as in subgroups of non-demented (NCI+MCI; AUCs=0.75 to 0.81) [40, 42], CU (AUCs= 0.70 to 0.86) [37, 39, 40, 43] and MCI subjects (AUCs= 0.74 to 0.94) [37, 40, 43, 45]. Some of the above-mentioned studies further demonstrated P-tau181 associations with neurofibrillary tangle burden [37, 39, 40, 42-44], grey matter atrophy (50, simren), hippocampal atrophy [37, 39, 44] and cognitive impairment [37, 39, 44].

Comparison of biomarker performance between P-tau species

A few papers have evaluated P-tau181 and P-tau217 in the very early phases of MCI/dementia. A study examining familial AD found significantly higher P-tau217 concentrations 20 years before the expected year of onset (EYO) of symptoms in individuals diagnosed with MCI [35], while another reported significant increases in P-tau181, 16 years before EYO in symptomatic FAD mutation carriers (both MCI and AD) [46]. More recently, head-to-head comparisons of N-terminal-directed P-tau181 and P-tau217 biomarkers in CSF [47, 48] showed similar performances; however, both were superior to conventional P-tau181 measured on mid-region fragments. These findings support the idea that both the measured plasma P-tau biomarkers are increased in the

preclinical phase of FAD. Further, studies evaluating these P-tau biomarkers in later stages of disease showed tight associations between biomarker levels in plasma samples taken during life and AD neuropathology assessed postmortem, with AUC values for differentiating AD from non-AD neurodegenerative disorders at 0.89 for P-tau217 [35], 0.97 for P-tau181 [38], and 0.997 for P-tau231 (REF= Ashton, no. 36). These findings further support the hypothesis that increased P-tau specifically reflect AD pathology. However, further studies directly comparing these tau biomarkers in plasma using the same assay technology in the same cohort are needed to ascertain whether P-tau181, P-tau217 and P-tau231 substantially differ in diagnostic utility in different phases of the AD continuum. In asymptomatic AD, P-tau231 shows increases in individuals with subthreshold levels of PET-measured Aβ burden in the first quartile, while CSF P-tau217 and plasma P-tau181 were increased in the third or fourth quartile [36]. Lastly, a few studies have demonstrated that longitudinal increases of blood P-tau levels were associated with longitudinal brain atrophy and cognitive decline, particularly in the AD patients, and may differentiate MCI converters from non-converters [36, 37, 49, 50], suggesting potential utility in longer term monitoring in therapeutic trials.

A multi-marker approach towards AD biomarkers

Since AD is pathologically characterized by amyloid plaques, neurofibrillary tangles as well as neurodegeneration, a comprehensive ATN (amyloid, tau, neurodegeneration) classification system has been advanced to better account for the complex pathophysiological processes central to AD pathogenesis [7]. To the extent that blood-based biomarkers accurately reflects brain changes, a corresponding

combination of biomarkers may yield superior utility as well. For example, using data from an Asian cohort of AD patients with concomitant cerebrovascular diseases (CeVD), it has been reported that combining Simoa P-tau181 with Aβ42 measures yielded better AUCs for amyloid positivity and hippocampal atrophy than any single marker evaluated (P-tau181, total tau, Aβ40 and Aβ42)[51]. An earlier study reported similar improved performance of combining IMR measurements of Aβ and tau in identifying AD in both prodromal and dementia phases [52]. However, as various P-tau species seem to be associated with both amyloid and tau pathologies (any citations?), they could potentially be used together as biomarkers to detect A and T pathophysiology, especially in settings where IP-MS plasma Aβ measurements are not feasible, or when plasma Aβ measures cannot distinguish A+ and A- cases. Nonetheless, the fact that different P-tau markers appear to be altered at different stages of AD pathology could be a source of complications. Finally, amyloid and tau pathologies are also associated with various pathophysiological conditions, including synaptic dysfunction, apoptotic cell death, neuroinflammation and oxidative stress [53]; as well as with concomitant CeVDs which may be relatively frequent in Asian and other less-studied populations and interact additively or synergistically with AD in worsening cognitive functions [54, 55]. It would therefore be of interest to investigate if combining Aβ and tau markers with those for synaptic, apoptotic, inflammatory, oxidative stress and endothelial / vascular injury in a multi-marker panel may yield improved clinical utility and further insights into disease mechanisms.

CONCLUSIONS

Recent advances in the development of ultrasensitive, high throughput analytical technologies and platforms have enabled the discovery and potential clinical application of promising biomarkers that reflect AD brain pathology in blood samples. These biomarkers will be critical in both diagnostic and prognostic assessments for AD as well as in longitudinal monitoring and therapeutic trials. However, there is a need for further research to 1) directly compare the performance of different biomarker platforms; 2) validate initial findings in larger cohorts from diverse backgrounds; and 3) evaluate the utility of multi-marker panels, in order to fully realize the potential of blood-based biomarkers for AD and associated conditions.

CONTRIBUTORS

JRC, NJA, TKK, TT, MS, HZ, KB, MKPL and CPC contributed to the conception and structure of the review, drafted the text and prepared the tables.

COMPETING INTERESTS

HZ has served at scientific advisory boards for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies and CogRx, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam,

Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Prothena,

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Program.

(Please add to this section if relevant)

PATIENT CONSENT FOR PUBLICATION

Not required.

SUPPLEMENTARY MATERIAL

Supplementary Tables S1 and S2.

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