Blood biomarkers for Alzheimer's disease in clinical practice and trials

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ABSTRACT [was 156 words – should be ≤150 words]

Blood-based biomarkers hold great promise to revolutionize the diagnostic and prognostic work-up of Alzheimer's disease (AD) in clinical practice. This is very timely, considering the recent development of anti-Aβ immunotherapies. Several assays for measuring phosphorylated tau (p-tau) in plasma exhibit a high diagnostic accuracy in distinguishing AD from all other neurodegenerative diseases in patients with cognitive impairment. Prognostic models based on plasma p-tau levels can also predict future development of AD dementia in patients with mild cognitive complaints. The use of such high-performing plasma p-tau assays in the clinical practice of specialist memory clinics would reduce the need for more costly investigations involving cerebrospinal fluid samples or positron emission tomography. Indeed, blood-based biomarkers already facilitate identification of individuals with pre-symptomatic AD in the context of clinical trials. Longitudinal measurements of such biomarkers will improve the detection of relevant disease-modifying effects of novel drugs or life-style interventions.

Main text 7155 words – after edits main text is ~6600 words

Guidelines: Up to 6000 words, 4–6 display items (figures, tables or boxes), 150 references.

Introduction

A neuropathological diagnosis of Alzheimer's disease (AD) is based on the presence of widespread cortical plaques containing amyloid-β (Aβ) fibrils in combination with neuronal neurofibrillary tangles and neuropil threads containing hyperphosphorylated tau (p-tau).[1] Tau-containing tangles restricted to the medial temporal lobe are found in most people older than 60 years. In AD, Aβ plaques start to accumulate 10-30 years before dementia onset, and these changes are thought to facilitate the spread of pathological tau species from the medial temporal lobe throughout the neocortex.[2] The mechanism by which Aβ aggregates drive tau spread and accumulation is not yet known but could involve increased tau phosphorylation in neurons**[Au:OK?]** and secretion of soluble tau forms.[3] Even though tau pathology affects different cortical regions in a rather stereotypic order,[2] there is evidence that spreading of tau might occur along four main trajectories, resulting in four main tau patterns that are associated with somewhat different clinical syndromes and prognosis.[4]

Au: the review should start with a short introduction (1-2 paragraphs). The introduction should provide a quick background to the topic — the text above could be used for that. It should then explain the rationale for the review, i.e. why this is an important topic and why is now a good time to review it. It should end with a brief overview of what the review will cover (e.g. 'In this Review, we will first discuss X, then Y and finally Z'.). You could consider including adding an introductory figure that summarizes the basic pathophysiological process in AD involving Aβ and tau for those readers who are less familiar with this field.

Current imaging- and CSF-based biomarkers for Alzheimer's disease

Imaging-based biomarkers

There are several positron emission tomography (PET) tracers that can detect Aβ fibril load in the brain. Three Aβ-PET tracers (flutemetamol, florbetapir and florbetaben) are approved for clinical use, and several large-scale studies have shown high concordance between the *in vivo* uptake of these PET tracers and the density of Aβ plaques as determined post-mortem.[2] A normal Aβ-PET scan result rules out AD as the underlying etiology in a patient with cognitive symptoms; an abnormal Aβ-PET scan is indicative of AD in a younger patient with cognitive symptoms, but in an older patient such a result should be interpreted with caution considering that about 40% of individuals aged 90 have Aβ plaques in the brain.[5]

Several PET tracers can detect the load of insoluble tau aggregates in the brain.[2] One tau-PET tracer (flortaucipir) is approved for clinical use in the USA. This tracer has been validated against neuropathology, and it can reliably detect the density of both neurofibrillary tangles and neuropil threads,[6,7] although it lacks the sensitivity to detect the earliest tau stages (restricted to the medial temporal lobe).[7] Tau-PET has shown excellent diagnostic accuracy for distinguishing AD dementia from most other neurodegenerative diseases,[8] and it has been suggested that this method can be used to rule in AD in patients with cognitive impairment even at older ages, considering the high specificity of neocortical tau-PET retention for patients with AD.[9] In a recent study, cognitively unimpaired individuals with both positive Aβ-PET and positive tau-PET had a 20x and 40x increased probabilities of developing mild cognitive impairment (MCI) and dementia, respectively, compared to those with normal PET scans.[10] Cognitively normal individuals with positive Aβ-PET but negative tau-PET had very minor risk of developing cognitive impairment.**[Au: ref 10?]** Together, these results support the NIA-AA research framework for AD, which states that individuals with both Aβ (A) and tau (T) pathology should be labeled as AD independent of cognitive status, i.e., including cognitively unimpaired individuals.[11]

CSF-based biomarkers

Aβ and tau can also be measured in cerebrospinal fluid (CSF).[2] CSF Aβ42 levels **[Au:OK?]**, and especially the ratios of Aβ42/Aβ40 or Aβ42/p-tau, correlate strongly **[Au:OK?]** with Aβ-PET status[12,13] and AD neuropathology.[14] Several CSF Aβ and p-tau assays on high-performing, fully automated platforms are currently used in clinical practice.[13,15] Given the

high agreement between Aβ-PET and CSF Aβ, there is usually no need to perform both investigations in the same patient.[16]

Tau can be phosphorylated at more than 40 different positions. Tau phosphorylation at threonine 181 (p-tau181) is increased **[Au: in CSF?]** in AD but not in other neurodegenerative diseases, including other tauopathies.[17] Other p-tau isoforms have also been investigated extensively in CSF, and there is converging evidence that p-tau217 levels exhibit stronger associations with both tau-tangle and Aβ plaque load than levels of p-tau181 and p-tau231,[18,19] although some results indicate that the assay set-up may be more important than the phosphorylation site as such.[20,21] Furthermore, CSF p-tau217 levels might distinguish AD dementia from other dementias with even higher accuracy than other p-tau isoforms and this has improved prognostic utility.[18,19,22,23]

Combining PET- and CSF-based measures **[Au: suggested subheading OK?]**

According to the NIA-AA research framework for AD, Aβ pathology (A) can be determined using either Aβ-PET or CSF Aβ.[11] This is likely to be correct in most situations,[24] but there are subtle differences between these two measures. First, levels of CSF Aβ42 **[Au:OK?]**, and potentially also Aβ42/40, change earlier than Aβ-PET signals; this is also the case for Aβ42/40 levels in blood samples.[25-27] Also, the Aβ-PET signal increases with disease progression **[Au:OK?]** as it measures insoluble Aβ laden plaques, whereas in CSF and blood, the Aβ42/40 ratio decreases with development of pathology. Using tau-PET and CSF p-tau interchangeably for tau pathology (T) seems to be even more complex; for example, in cognitively unimpaired populations, more individuals are identified as T-positive when using CSF p-tau vs tau-PET.[24] This is because p-tau levels in CSF and plasma start to increase much earlier than the tau-PET signal reaches the threshold for detection during the preclinical stages of AD.[28,29] In fact, Aβ-positive individuals who are positive for CSF p-tau but still negative for tau-PET might represent an early AD population just about to start accumulating tau aggregates in the neocortex.[30] It has therefore been suggested that the NIA-AA research framework be updated to include p-tau and tau-PET as separate biomarker entities, i.e. using “APT” instead of “AT” where P stands for P-tau (measuring the levels of soluble hyperphosphorylated tau) and T for Tau-PET (measuring the density of insoluble tau fibrils).[30]

Finally, according to the NIA-AA research framework, markers of neurodegeneration (N) provide additional information about disease status.[11] Hippocampal volume and/or the cortical thickness of temporoparietal regions can be determined using structural MRI and reflect the disease stage of AD. Furthermore, several fluid biomarkers of neurodegeneration have emerged. For example, CSF levels of total tau (t-tau) reflect axonal degeneration and injury. Disorders with rapid neurodegeneration, such as Creutzfeldt-Jakob disease and autoimmune encephalitis, are characterized by normal CSF p-tau but a more pronounced increase in t-tau[31,32] than that found in AD (which has a slower clinical course). Similarly, in acute neuronal injury such as stroke and acute brain trauma, CSF t-tau shows a temporary increase associated with severity of the neuronal damage and long-term clinical outcome, while p-tau remains normal.[33,34] Another promising neurodegeneration biomarker is neurofilament light (NfL), which reflects axonal degeneration and injury of the longer myelinated axons of the brain and spinal cord structures, irrespective of cause. Nfl levels in CSF **[Au: correct?]** are especially increased in amyotrophic lateral sclerosis, frontotemporal dementia and atypical parkinsonian disorders, but also in AD.[35] Importantly, in most neurodegenerative disorders, higher levels of NfL are associated with faster disease progression and higher brain atrophy rates.[35,36] NfL can therefore be regarded as a measure of the intensity of ongoing neurodegeneration.

Blood-based markers (BBMs) for AD and related disorders [Au:OK?]

As in CSF, plasma levels of Aβ42/Aβ40 are associated with the presence of Aβ plaques in the brain as determined by neuropathology.[37] In many studies across several platforms, including different immunoassays and mass spectrometry-based assays, plasma Aβ42/40 ratio is lower in Aβ-positive than in Aβ-negative groups when either CSF or PET is used as the reference standard, regardless of cognitive status of the cohort.[38-43] However, the performance of different plasma Aβ42/40 assays varies substantially, and a recent head-to-head comparison showed that certain mass-spectrometry based assays could detect Aβ-pathology with areas under the receiver operating characteristic curves (AUCs) of 0.84–0.87,

whereas many commonly used immunoassays performed much worse (AUCs 0.64–0.69).[44] Adding *APOE* genotype to plasma Aβ42/Aβ40-based prediction models increases the AUCs by ~10%.[41,43,44] The assays with better diagnostic performances are characterized by superior control of measurement error. Still, these relatively high-performing Aβ42/Aβ40 assays exhibit only modest correlations between the levels in plasma and CSF (r_s of 0.56–0.65),[44] likely because much of the Aβ in plasma is derived from peripheral sources.

Several high-sensitivity assays have recently been developed that can reliably detect different p-tau isoforms in plasma, including p-tau181,[45-48] p-tau217,[49] and p-tau231.[50] These assays performed well in detecting AD as defined using neuropathology.[45-50] A few head-to-head comparisons of these assays using plasma from patients with cognitive complaints showed that assays quantifying plasma p-tau217 are somewhat better at detecting AD pathology and predicting future development of AD dementia.[51-54] The best performing p-tau217 assay showed a high correlation between plasma and CSF levels, with a correlation coefficients of 0.89[43], whereas plasma p-tau231 starts increasing at very low Aβ plaque levels.[50,55,56] These results are congruent with a recent study showing that plasma p-tau231 is associated with Aβ plaque load, but not tau tangle load.[37] By contrast, p-tau181 and p-tau217 were associated with both Aβ plaques and tau tangles, with p-tau217 showing stronger correlations.[37] There is currently no tangle-specific tau plasma marker, but recent developments in CSF markers hold great promise.[57]

Like CSF NfL, plasma NfL is a measure of active neurodegeneration in several neurodegenerative disorders.[58] Plasma NfL levels generally correlate well with the levels in CSF.[59] Nfl levels are associated with neurodegeneration in AD, but the effect size is smaller

for plasma than for CSF, as is the case in other neurodegenerative diseases, e.g., Huntington's disease.[60]

Glial fibrillary acidic protein (GFAP), which likely reflects reactive astrocytes, can be reliably measured in both blood and CSF. Plasma levels of GFAP are increased in individuals with early Aβ-pathology[61-63] and can predict subsequent cognitive decline and conversion to AD dementia in cognitively unimpaired subjects[64] and in MCI patients.[65] Plasma GFAP levels are also increased in other neurodegenerative diseases, including FTD associated with progranulin mutations.[66] It is currently unclear whether plasma GFAP levels correlate with the number of reactive astrocytes as determined postmortem using immunohistochemistry.

BBMs for diagnosis and prognosis of cognitively impaired patients

BBMs as diagnostic biomarkers

Once anti-Aβ therapies (e.g. lecanemab) can be used in patients with MCI or mild dementia, it will be crucial that a highly accurate yet time- and cost-effective diagnostic workflow for AD is in place. Blood-based biomarkers (BBMs) hold great promise in this respect (Figure 1). **[Au:OK?]** In clinics without access to Aβ-PET or CSF AD biomarkers, implementation of accurate AD BBMs will improve the diagnostic work-up quite substantially compared to the care-as-usual of today. In specialist clinics with access to CSF and/or PET, BBMs will speed up the diagnostic process and substantially reduce the costs. BBMs will likely be sufficient to support or reject an AD diagnosis in most patients with MCI or dementia; only those patients with uncertain BBMs outcomes are likely to need confirmatory testing with Aβ-PET or CSF AD biomarkers (Figure 2)**[Au: do you mean Figure 1?]**. Indeed, a recent study showed that a diagnostic algorithm based on plasma p-tau217 resulted in an accurate AD diagnosis

in ~80% of patients with MCI, whereas ~20% had uncertain blood biomarker results and needed further confirmatory testing with CSF AD biomarkers.[67] A newly developed, highly accurate mass-spectrometry assay for p-tau217 might result in fewer patients with uncertain biomarker outcomes, reducing the need for CSF and PET even further.[51]

An important question is which plasma biomarkers for AD should be implemented in the assessment of patients with MCI and dementia. Although plasma GFAP and NfL levels are increased in patients with MCI or dementia due to AD, they are unlikely to contribute significantly to accurate detection of AD pathology when combined with high-performing plasma p-tau and Aβ42/40 assays.[68,69] Several different p-tau variants, including p-tau181, p-tau217 and p-tau231, are increased in the plasma of patients with MCI or dementia due to AD, and these can be used to distinguish AD from other neurodegenerative diseases with high diagnostic accuracy, often on par with PET and CSF AD biomarkers (for reviews see e.g.[2,70-72]). Plasma p-tau217 is the tau variant that shows the largest fold increase in individuals with symptomatic AD, with increases of about 300-700% compared to both healthy individuals and patients with other neurodegenerative diseases.[49] Therefore, the clinical performance of this biomarker is less susceptible to test–retest variability when compared to many other plasma biomarkers[73], and the effects of comorbidities (e.g. kidney dysfunction) on plasma p-tau217 levels are minor (see below).[74] The latter is especially true when the p-tau217/t-tau217 ratio is used as quantified using mass spectrometry.[75] Together, these characteristics of plasma p-tau217 result in a robust clinical performance of this biomarker for detection of AD in patients with MCI or dementia. However, plasma levels of p-tau217 are very low in healthy individuals and it might therefore be challenging to establish this biomarker on many of the fully-automated platforms used in clinical practice today, as has been the case for the Roche Elecsys platform.[69] Although plasma p-tau217 is

currently the best-performing diagnostic biomarker for symptomatic AD, there are also high-performing assays for plasma p-tau181,[51,52] and plasma levels of p-tau181 are generally higher than p-tau217 **[Au:OK?]** and therefore easier to detect with fully-automated platforms.[69]

When it comes to plasma Aβ42/Aβ40 levels, the very modest drop of 8-15% in symptomatic AD[2] means this biomarker has low performance and robustness in routine clinical settings, even if analytical variability and systematic bias are kept at a minimum[76] — and few current Aβ42/Aβ40 assays fulfil this requirement, resulting in large variability in the clinical performance of different plasma Aβ assays.[44] Nevertheless, high-performing plasma Aβ42/Aβ40 assays might contribute to plasma p-tau-based diagnostic algorithms that are designed to detect AD pathology in patients with MCI.[68]

A recent paper proposed that high-performing BBMs can already be used in specialist clinics to facilitate detection of AD pathology in patients with MCI or dementia.[77] Importantly, BBMs should be combined with a thorough clinical assessment, including psychiatric and neurological examinations, cognitive testing and structural brain imaging. BBMs should never replace such investigations, and they should only be used in patients with cognitive impairment for whom AD is a possible diagnosis and where such a diagnosis will likely change the management of the patient.[77] These recommendations are primarily based on the risk that false-positive results could lead to anxiety, depression or rash behavior; even a 5% false-positive rate would mean thousands of people would be inappropriately diagnosed with AD if the tests were used in broad screening.

BBMs as prognostic biomarkers

Information about individual-level *prognosis* is of key interest for patients with mild cognitive complaints as well as for their care partners and responsible physicians.[78] Higher baseline plasma p-tau217 and p-tau181 levels in patients with mild cognitive complaints are associated with subsequent progression to AD dementia.[46,79-81] Combining plasma p-tau217 (or p-tau181) levels with a few brief cognitive tests — an easy-to-use prognostic tool — outperforms predictions made by dementia experts and performs similar to CSF-based prognostic models when predicting development of AD dementia within 2–6 years in patients with mild cognitive complaints.[80] Neither plasma NfL nor Aβ42/Aβ40 contributed much in this particular context.[80,81] However, plasma NfL might have a value when predicting future decline of global cognition in patients with MCI or dementia: prognostic models based on plasma p-tau and NfL can predict changes in global cognition (MMSE and CDR-SB) in patients with MCI, with performances similar to models based on CSF biomarkers.[81] A recent study similarly **[Au:OK?]** showed that tau-PET imaging may have value for predicting global cognitive decline in patients with MCI or dementia, and that plasma NfL is the only plasma biomarker that provides any additional prognostic information.[82] However, tau-PET is a costly and currently not widely available in clinical practice.

BBMs as prescreening biomarkers **[Au:OK?]**

Plasma AD biomarkers will also facilitate recruitment of patients with MCI or dementia due to AD for *clinical trials*. About 40-60% of patients with MCI and 20-30% of those with clinically diagnosed AD dementia do not have brain Aβ pathology [2]. Thus, when recruiting patients with prodromal AD or mild AD dementia for clinical trials, prescreening individuals

for, e.g. plasma p-tau217 would reduce the need for confirmatory investigations involving Aβ-PET or CSF AD biomarkers (Figure 3). Such prescreening with high-performing BBMs is likely to be more cost-effective in patients with MCI than patients with dementia, considering the lower prevalence of Aβ-positivity in MCI. In certain interventional AD trials, such as trials evaluating life-style interventions, a high-performing plasma biomarker might be enough to confirm AD pathology, removing the need for CSF and PET altogether, which would substantially reduce the costs and increase the scalability of such trials.

BBMs for diagnosis and prognosis of cognitively unimpaired individuals

High-performing assays for plasma p-tau181, p-tau217, p-tau231, Aβ42/Aβ40 and GFAP, but not NfL, can detect AD-related pathological changes in cognitively normal individuals and in patients with subjective cognitive decline (for reviews see e.g.[2,70,71]). A recent study analyzed all these plasma biomarkers in preclinical AD and showed that plasma p-tau231 and Aβ42/Aβ40 could be used to detect the earliest AD brain changes.[56] Indeed, a combination of plasma p-tau and Aβ42/Aβ40 was found to be the best biomarker combination for detection of amyloid pathology in cognitively unimpaired individuals, and high-performing Aβ42/Aβ40 assays might contribute more to the diagnostic work-up in this very early disease stage compared to later disease stages.[68] Although there is currently no obvious clinical need to detect AD in cognitively normal individuals, this might change when phase 3 trials evaluating anti-amyloid therapies, like lecanemab (NCT04468659) and donanemab (NCT05026866), will read out in 2027/2028. Use of BBMs might be considered in certain patients with subjective cognitive decline, where cognitive test results are still

normal but the patient history indicates a gradual cognitive deterioration. Such patients could be investigated similar to patients with MCI (see above).

Even if AD BBMs should not be widely used for cognitively normal individuals in clinical practice in the foreseeable future, they will be a gamechanger for clinical trials conducted in patients with preclinical AD. As only 10-30% of individuals aged 60-80 years are amyloid-PET or CSF Aβ positive[5], a large number of PET (or CSF) examinations is currently needed to identify a sufficient number of individuals for phase 3 trials focusing on preclinical AD. For the A4 (Anti-Amyloid Treatment in Asymptomatic Alzheimer's) trial — the first phase 3 trial in preclinical AD — it took 3.5 years and more than 4000 amyloid PET scans to identify 1169 participants eligible for the study. As shown in Figure 2, a pre-screening step with high-performing BBMs could greatly reduce the number of PET (or CSF) investigations. Using high-performing plasma Aβ42/Aβ40 assays in this way indeed resulted in substantial cost- and time-savings.[42,83,84] This was particularly evident if the plasma test was incorporated early in the enrollment process, even before the screening visit.[83] As mentioned above, combining plasma Aβ42/Aβ40 with p-tau231[56] (or p-tau217[68]) levels might result in even more efficient detection of preclinical AD. Several large-scale phase 3 anti-Aβ trials already use plasma Aβ42/Aβ40 (NCT05026866) or p-tau217 (NCT04468659) to identify individuals with a high probability of having preclinical AD.

As shown in Figure 2, efficient clinical trials also need to enrich the preclinical AD population for those that will likely worsen in the primary outcome over a reasonable time period (3–5 years). This is because many subjects with preclinical AD do not deteriorate over 5-10 years or even during their lifespan[85-87], and without enrichment for vulnerable individuals, very large and extended trials would be needed. Power calculations indicate

that if only amyloid-positivity is included as a requirement, about 2000 participants are needed per group to detect a treatment effect of 25% over 4 years using a cognitive composite measure optimized for preclinical AD as a primary endpoint.[85] In two independent cohorts, plasma p-tau217 levels could accurately predict future cognitive decline in preclinical AD[88]; in this setting plasma p-tau217 performed better than other plasma and CSF biomarkers (p-tau231, p-tau181, GFAP and NfL) or amyloid PET. Importantly, power calculations revealed that using plasma p-tau217 levels to enrich for cognitively normal individuals likely to show cognitive decline resulted in large reductions in required sample sizes.[88] Tau pathology has consistently been shown to be more strongly associated with clinical deterioration than Aβ pathology, even in cognitively unimpaired individuals.[10,89] Therefore, future phase 2 trials might use accumulation of tau pathology over time (as measured with longitudinal tau-PET) as a more precise primary outcome than cognitive measures, which exhibits high intra-individual variation. Of note, the increase in tau-PET signal over time in amyloid-positive AD populations are modest. However, plasma p-tau217 was recently shown to accurately predict future accumulation of tau aggregates in the brain, and a combination of p-tau217 and tau-PET at baseline could be used to substantially reduce the needed sample sizes with >40% when using longitudinal tau-PET as the primary outcome in preclinical AD trials.[90]

Potential use of BBMs in primary care settings

Most patients with cognitive symptoms are managed in primary care rather than specialist clinics. Although few studies in primary care settings have systematically evaluated the accuracy of AD diagnoses against a valid reference standard (e.g., dementia expert

diagnoses supported by CSF or PET), it seems that ~50-70% of patients with cognitive impairment are currently not recognized or correctly diagnosed in primary care, due to lack of easily accessible, time- and cost-effective, and accurate diagnostic tools.[91] The problem is even worse in early stages of the disease, i.e., in patients with subjective cognitive decline (SCD)**[Au:OK?]** or MCI, because there are no accurate methods for personalized prognosis of AD in primary care. This leads to patients not receiving appropriate **[Au:OK?]** diagnostic and prognostic information, and also results in suboptimal treatment strategies and care. Misdiagnosis can also lead to unnecessary care-seeking and costly investigations due to diagnostic uncertainty. Considering that CSF and PET cannot be used in primary care, AD BBMs have the potential to enable primary care physicians to provide patients with an accurate diagnostic and prognostic work-up.

Several prospective studies are currently evaluating AD BBMs in primary care. For example, a study in Sweden that includes 800 patients with cognitive symptoms at primary care centers evaluates whether AD BBMs can be analyzed prospectively in primary care using pre-defined cutoffs in a diverse population where many patients have several comorbidities; whether the diagnosis and treatment of the patients improves by adding AD BBMs to the "care-as-usual"; and whether BBMs can be used to predict future development of AD dementia in non-demented individuals with cognitive complaints in primary care. Regulatory authorities in many countries will likely require such studies before AD BBMs biomarkers can be widely implemented in primary care settings, which is why the Alzheimer's Association appropriate-use recommendations does not yet endorse use of AD BBMs in primary care.[77] Once BBMs for AD have been validated in primary care, education packages regarding when to use the biomarkers, what they represent, how to interpret the

results, and what to do with the results must be developed in close collaboration between primary care physicians, dementia experts, and patient representatives.[77]

BBMs for monitoring disease progression

Fluid biomarkers and brain-imaging methods are increasingly being used as outcome measures in clinical trials evaluating disease-modifying therapies for AD and other neurodegenerative disorders. The use of such surrogate endpoints will be especially important in preclinical AD trials, where very large and long-term studies are needed when using a clinical outcome such as cognitive function[85]. Aβ-PET, but not yet any AD-related fluid biomarkers, are deemed by the US Food and Drug Administration (FDA) to be a *reasonably likely surrogate endpoint*, which means that it is "supported by strong mechanistic and/or epidemiologic rationale, but the amount of clinical data available is not sufficient to show that they are a validated surrogate endpoint".[92] Such a biomarker can be used to support FDA's Accelerated Approval program. However, only *validated surrogate endpoints* can be used as a primary endpoint in pivotal trials, and no AD biomarker currently meets this definition.

Many of the fluid tau and neurodegeneration biomarkers discussed above are more or less directly related to disease progression. The best-established biomarker for general neurodegeneration is NfL.[58,93] The magnitude of Nfl increases in CSF and/or plasma reflects the intensity of the neurodegenerative process and predicts imaging and clinical evidence of disease progression.[94,95] In AD, high **[Au:OK?]** NFl levels are associated with longitudinal neurodegeneration as determined by MRI; however, this is only obvious at more advanced dementia stages.[95] Such associations are clearer in other neurodegenerative diseases such

as multiple sclerosis[96], amyotrophic lateral sclerosis[97] and frontotemporal dementia[98], in which Nfl levels are generally much higher than in AD.[58] Interestingly, disease-modifying treatment in, *e.g.*, multiple sclerosis and spinal muscular atrophy, reduce NfL levels, and the reductions correlate **[Au:OK?]** with the clinical efficacy of the intervention.[99,100] In anti-Aβ antibody trials in AD, attenuated increases of CSF NfL have been reported,[101,102] but no such results have been obtained thus far for plasma NfL.[103] NfL may be a better surrogate marker for neurodegenerative disease other than AD, considering the modest increases in plasma NfL in AD and considering the fact that many elderly individuals have other brain pathologies (e.g. TDP-43) that are more related to increased NfL levels.

Early studies showed that people with clearly increased CSF tau levels had a faster AD progression, suggesting that this marker also reflects the intensity of the neurodegenerative process,[104,105] but (unlike Nfl) in an AD-specific rather than general neurodegeneration-reflecting manner. **[Au:OK?]** Similarly, studies with novel blood tests for P-tau forms[2,70-72] showed that longitudinal changes in plasma P-tau levels are associated with both brain atrophy and cognitive decline in AD populations.[106-108] Importantly, promising anti-Aβ antibody trials have shown treatment-induced reductions in plasma P-tau markers associated with less clinical deterioration, supporting disease modification and a slowing of the neurodegenerative process.[103,109] In clinical practice, it is possible that certain plasma P-tau forms will be used to assess the effect of anti-Aβ antibody treatments for both treatment evaluation and disease monitoring purposes. One could even envision yearly plasma P-tau testing to detect reoccurrence of disease activity, if and when treatment with anti-Aβ antibodies for 1-2 years eventually becomes a reality.

In addition to P-tau and NfL, other markers of disease intensity markers that predict AD progression and have shown promising results in clinical trials include plasma GFAP. Plasma GFAP levels increase over time in AD[65] and clear reductions are observed after efficient removal of Aβ plaques by anti-Aβ immunotherapy.[103] Furthermore, CSF and plasma Aβ42/40 has been suggested to detect target engagement of anti-Aβ antibodies. However, the therapeutic antibodies may change the half-life of the biomarkers, making data interpretation difficult[110] , as has been reported for biofluid-based tau biomarkers in anti-tau antibody trials.[111]

Few longitudinal studies have performed head-to-head comparisons of different plasma AD biomarkers. Recently we reported that plasma P-tau217 increases more clearly over 4-6 years in preclinical and prodromal AD compared to Aβ42/Aβ40, P-tau181, P-tau231, GFAP and NfL; P-tau217 also had the strongest associations with brain atrophy and cognitive decline in two independent cohorts.[56] If replicated in other studies, this might indicate that plasma P-tau217 could be a key biomarker for detecting disease-modifying effects in drug trials and other interventional studies (e.g. involving physical activity) targeting preclinical and/or prodromal AD stages.

Standardization, robustness and clinical cutoffs of BBMs

Standardization

Before biomarker-based diagnostic tests can be introduced into routine clinical practice, biomarker standardization and the development of consensus-based standards and guidelines are essential to assure high quality of laboratory test results (and thereby patient care and safety), specifically the accuracy of diagnostic classifications.

For the core AD CSF biomarkers, a Working Group under the International Federation of Clinical Chemistry and Laboratory Medicine has led standardization efforts.[112] These have resulted in mass spectrometry methods for CSF Aβ42 that have been approved by the Joint Committee for Traceability in Laboratory Medicine as Reference Measurement Procedures. They have also resulted in three Certified Reference Materials (low, medium, and high Aβ42 levels) intended to be used to calibrate commercially available immunoassay, thereby harmonizing levels across assays.[113] Similar standardization efforts have been initiated for AD BBMs. A first round-robin study (which aims to verify a new method and compare results across methods and laboratories) on Aβ methods showed disappointingly poor correlations across plasma Aβ42 assays (r= 0.41–0.54), including mass spectrometry methods, whereas correlations for Aβ40 assays were better (r= 0.59-0.79).[114] Using the Aβ42/Aβ40 ratio did not improve correlations [114], and another study obtained similar results.[44] When the same immunoassays are applied for CSF samples, correlations are generally very high (r= 0.94-0.99).[115] In contrast to plasma Aβ42, correlations between different high-performing plasma p-tau assays are tight.[51,54]

A widespread launch and implementation of the AD blood biomarkers for clinical use will require not only analytical standardization, but also ensuring that blood biomarkers can be measured **[Au:OK?]** on the type of laboratory analyzers available in non-specialized, smaller hospital laboratories. Methods for the measurement of these AD BBMs on high-precision, fully automated instruments have been published,[69] and other assay formats have been released as laboratory-developed tests (LDT) for potential clinical implementation.

Standardization of sample collection procedures are also crucial for clinical implementation. Pre-analytical sample handling procedures has been examined extensively

for CSF biomarkers, since such factors may affect biomarker values.[116] For blood biomarkers, the same type of sampling tubes (EDTA plasma) should be used for all the biomarkers, such that all the blood biomarkers can withstand up to three freeze-thaw cycles.[117,118] In contrast to CSF Aβ, plasma Aβ is not sensitive to collection tubes made of glass, and tubes with gel separator can be used. Importantly, both Aβ42 and Aβ40 are unstable in whole blood, with levels decreasing already after 2 hours; samples should therefore be centrifigued early (optimally within 1 hour) and plasma separated, after which it can be stored at +4°C for up to 6 hours before freezing.[117]

Robustness

Robustness describes a biomarker's ability to classify patients with high consistency and high clinical accuracy.[72,73,76] For a biomarker to be suitable for clinical use, its levels should be higher (or lower) in AD samples compared to all relevant differential diagnostic groups. The effect size, e.g. percentage or fold change **[Au:OK?]**, can be used to compare several biomarkers. This effect size should be much larger than the total measurement variability of the BBM, the latter being caused by biological variability, variability induced by variations in preanalytical handling of blood samples, and total error in the analytical measurements (Figure 3). In other words, a robust biomarker can withstand the variability and bias across measurements that occurs in clinical routine. Factors that may affect biomarker measurements are shown in Figure 3. Factors that contribute to biological variation can influence classification accuracy and may need consideration when establishing cut-offs (see below). In addition, both pre-analytical (e.g., time to centrifugation) and analytical (assay

imprecision) factors and drifts or bias in values across rounds of measurements will also add to the total measurement variability (Figure 3).

Blood biomarkers such as the plasma Aβ42/40 ratio that have a fold-change of 8–15% reduction in amyloid-positive cases[119] may be problematic even if the total measurement variability is lower than 5-10%. This small effect size, combined with the total error in the plasma Aβ42 assays mean this biomarker has low robustness.**[Au:OK?]** This may complicate the introduction of the Aβ42/Aβ40 ratio in clinical routine. By contrast, plasma p-tau217 levels are increased 500–700% in symptomatic AD. [49]

Biomarker robustness can be tested through simulations that test the influence of increasing analytical total error of blood biomarker measurements on clinical classifications. Such simulations have shown that increases in total error strongly affect the performance of the plasma Aβ42/40 ratio to identify brain Aβ pathology but not that of other blood biomarkers (NFL, GFAP and p-tau181).[120] A second study found a large effect of introducing a 10% bias on performance of plasma Aβ42/40 ratio but not CSF P-tau/Aβ42 ratio as a biomarker for amyloid positivity.[76]**[Au:OK?]** A third study showed that even though plasma Aβ42/40 has lower test–retest variability than plasma p-tau217, NfL and GFAP, plasma p-tau217 was least affected by this test–retest variability, with a change in diagnostic accuracy of <1%.[73] This robustness is due to p-tau217 having a substantially higher effect size than the other BBMs.[73] Consequently, plasma p-tau217 and p-tau181 seem to be robust AD BBMs.[73,120] Of note, the robustness might depend on disease stage: the effect size increases with severity of pathology, because there is a gradual increase in fold change from preclinical AD to prodromal AD, with the highest levels in AD dementia.[45,46,48,49] Thus, even if these biomarkers are very robust in symptomatic AD, they might be less robust in detecting

preclinical AD, which may have implications for pre-screening in preclinical AD trials (see above).

Clinical cut-offs

Regarding the clinical diagnostic performance of the biomarkers, it should be noted that in principle all data published so far come from retrospective studies, in which all samples were analyzed in batch, after which the optimal cut-off was identified and descriptive data on the performance calculated (AUC, sensitivity, specificity). To generate data on the 'real-life' diagnostic performance, *prospective* studies are needed, with fixed biomarker cut-offs set before the start of the study, and biomarkers analyzed on a routine (daily or weekly) basis, allowing influence from the true total measurement error.

For use in clinical practice, biomarkers need well-defined and widely accepted clinical cut-offs. Ideally, each biomarker should have a cut-off value established based on the discrimination between clinical groups (or established proxies for neuropathology), or alternatively (and commonly used in laboratory medicine), based on the 95th percentile of values in a well-characterized control group.[121]

Baseline physiological levels of brain proteins in blood depend on various non-disease-associated factors. For example, blood NfL levels are strongly age-dependent[122]. Studies assessing sex differences in blood biomarkers have shown inconsistent results. Although it is common to have age or sex specific normative ranges, most comorbidities are typically left to add to the biological heterogeneity that is observed in the measurements and taken into account when setting the normative range. Indeed, several comorbidities (e.g., chronic kidney disease and obesity) are associated with increases in plasma P-tau[74]

and plasma Aβ40, Aβ42, NFL and GFAP[123], even though Aβ42/Aβ40[124] and p-tau217/t-tau217[75] ratios seem to be unaffected. Of note, although associations between blood biomarkers and co-morbidities may be statistically significant in large clinical or population-based cohorts, it is important to describe the magnitude of such effects, especially the effect size,[125] and whether it is of clinical relevance. Indeed, in two large clinical cohorts, plasma NfL and GFAP and, to a lesser degree, p-tau were associated with kidney dysfunction and BMI, but these potential confounders had no clinically meaningful effects on either prediction of brain pathophysiology or future cognitive change.[124] In line with these results, chronic kidney disease, obesity and other comorbidities affect the reference ranges for the AD blood biomarkers only slightly.[74,124] In general, biomarker cut-offs in laboratory medicine are not routinely adjusted for comorbid disorders, but it is often useful to understand their influence on the biomarker results, as they might confound the interpretation at an individual patient level (e.g. in a patient with severe kidney disease and obesity).

Although common laboratory tests (e.g., hemoglobin, platelet count, gamma-glutamyl transferase) show differences across racial/ethnic groups, [126] reference intervals for normality are usually developed predominantly with Caucasian populations, and not separately for different subpopulations. Possible differences in blood biomarker levels across racial/ethnicity groups have also been discussed, but recent large studies on the BBMs Aβ42, Aβ40, t-tau, p-tau and NfL found levels were similar across white, Black and Spanish-speaking Americans.[127,128] These results suggest that the same cut-off for AD BMMs can be used across racial/ethnicity groups. However, further studies are needed to assess possible physiological differences in blood biomarker levels across ethnic groups, also adjusting for socioeconomic status and comorbidities linked to this.

When BBM levels are close to the established cut-offs, the interpretation is more uncertain **[Au:OK?]**. Patients with such uncertain results could be referred for confirmatory CSF or PET testing (Figure 1). Indeed, categorization of individuals into a low probability ("non-AD"), high probability ("AD") and intermediate probability ("gray zone") groups have been suggested for the most common AD biomarkers, and a combined model using several markers resulted in fewer patients in the intermediate probability ("gray zone") group[73]. A similar classification system is used for a test available for clinical use in the US — a probability score based on combining *APOE* genotype, age, and plasma Aβ42/40 ratio.[119] The use as a screening test along with the intermediate category reduces the risks associated with AD BBM tests. As mentioned above, a p-tau217 based diagnostic algorithm could classify about 80% of patients with MCI correctly as having or not having AD, with 20% ending up in the intermediate probability ("gray zone") group.[67]

Future directions

AD is a common disease for which promising drugs are now emerging that may slow or even stop Aβ-triggered breakdown of neuronal networks. Disease-modifying drugs with different targets (e.g., anti-tau therapeutics and synapse stabilisers) are also underway. The emerging availability of this broader range of potentially disease-modifying drug candidates directed against distinct pathogenic mechanisms in the AD process resembles recent developments in, *e.g.*, rheumatology, where effective targeted treatments started to become available 20 years ago and have now been implemented in clinical practice in close collaboration between primary healthcare physicians and specialists using biomarker-supported

personalised medicine approaches. We envision similar developments in AD in the next few years and the recently developed BBM will play a very important role in this process.

We envision that individuals presenting to primary care physicians with cognitive concerns will be first examined according to standard clinical procedures, starting with an evaluation of the patient's medical history, present comorbidities, duration of cognitive symptoms, basic neurological examination, and brief cognitive testing. The clinician can subsequently make a request for BBM testing after having discussed its potential implications with the patient and his/her relatives. Elevated levels of plasma P-tau would suggest that AD pathology is responsible for the observed cognitive impairment, whereas normal plasma P-tau levels would indicate non-AD causes. If P-tau is normal, increased blood NfL concentration could suggest the presence of non-AD neurodegeneration. We must stress, however, that BBMs might help the clinician in decision-making but should in no case substitute a proper neurological assessment. Indeed, confirmatory diagnosis in specialist care settings will continue to be important for some time for many patient populations, but in the future, it will probably be possible to accurately diagnose and treat many of these patients in primary care only.

The ability of plasma P-tau measurements to identify AD pathophysiology in individuals with symptomatic disease demonstrates the potential of this marker for identifying and recruiting Aβ-positive symptomatic participants for clinical trials. In addition, we expect that blood P-tau will be important for the recruitment of pre-symptomatic Aβ-positive cohorts, which will result in reduced rates of negative PET scans and substantial cost- and time-savings. Plasma P-tau biomarkers will also be useful to evaluate effects of

therapeutic intervention: significant decreases in plasma P-tau concentration, or a reduction in the rate of increase over time, could indicate beneficial effects of anti-Aβ treatments.

The discussions above point to a revolution in the next 2-4 years, in which widespread and routine analyses of blood P-tau become routine practice in clinical assessments and research studies, likely combined with i) high-performing assays of plasma Aβ42/40 ratio for preclinical AD (Figure 4, ii) with brief digital cognitive testing for prognosis (Figure 4), and iii) with plasma NfL when suspecting non-AD neurodegenerative diseases. However, several outstanding challenges must be addressed. We need to obtain analytical standardization and quality control to provide a framework in which biotechnical companies and clinical laboratories can ascertain they produce valid biomarker results. We need to demonstrate biomarker validity in diverse cohorts. Finally, we need to perform studies to prospectively generate real-world clinical data on the performance of blood-based AD biomarkers, especially in primary-care settings. We do not yet know how observations from such cohorts will translate to the setting of routine memory clinics, which see patients with greater heterogeneity in demographics, disease presentation, co-morbidities. Therefore, whether blood P-tau can be used as a single marker or replace CSF biomarkers that have been tested in larger varieties of disease conditions remains unclear. Realistically, we might need to exercise caution in projecting immediate diagnostic use of blood P-tau levels as a CSF substitute until large-scale clinical characterization studies have been performed. Finally, we want to stress that we need to develop blood biomarkers for non-AD brain pathologies, especially for pathological changes in TDP-43, 3R tau, 4R tau, α-synuclein, cerebrovascular changes, as well as synaptic dysfunction.

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KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper.

HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Acumen, Alector, Alzinova, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pinteon Therapeutics, Prothena, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work).

JLD is an inventor on patents or patent applications of Eli Lilly and Company relating to the assays, methods, reagents and / or compositions of matter related to measurement of P-tau217. JLD has served as a consultant for Abbvie, Genotix Biotechnologies Inc, Gates Ventures, Karuna Therapeutics, AlzPath Inc, Cognito Therapeutics, Inc., and received research support from ADx Neurosciences, Fujirebio, AlzPath, Roche Diagnostics and Eli Lilly

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FIGURE LEGENDS

Figure 1. Suggested BBM-based workflow for Alzheimer's disease diagnostics.

Patients with cognitive complaints undergo blood sampling as part of the standard diagnostic work-up. High-performing blood AD biomarkers (e.g., p-tau217) are used to determine the individual-level probability of having Alzheimer's disease. For patients deemed to have a low probability based on BBMs, another cause of the symptomatology should be sought. For patients deemed to have a high probability based on BBMs, appropriate treatments might be initiated. Patients with an intermediate probability, whose BBM results lie in an uncertain "gray zone", might be referred for confirmatory testing with either CSF or PET AD biomarkers. The percentage of individuals in such a "gray zone" will depend on the accuracy of the blood-based diagnostic algorithm (very high-performing BBM assays will have few results ending up in the "gray zone").

Figure 2. Suggested workflow for inclusion of study participants into preclinical Alzheimer trials.

In the 'pre-screening' step, a *diagnostic algorithm* based on blood-based biomarkers for AD identify cognitively normal as being at low risk or high risk of having pre-symptomatic (preclinical) AD.[Au:OK?] In the 'screening' step, individuals deemed high-risk will undergo further tests, involving Aβ-PET or CSF AD biomarkers, to confirm or rule out the presence of AD pathology. In the 'enrichment' step, a *prognostic algorithm* can be used to identify individuals who are likely to exhibit more severe spread of tau pathology and cognitive decline, so that the population to be included in the trial is enriched for such individuals.

[Au: Please add a sentence explaining the 'predictive models', explaining how they related

to the diagnostic and prognostic algorithms, and how they contribute to 'low cost' and 'high accuracy'.]

Figure 3. Robustness defines a clinically useful biomarker that gives a reproducible classification of patients.

[Au: this display item contains text but not any illustration or schematic. I don't think it would work as a table either because the left vs right half are not equivalent or a compare-and-contrast. I therefore suggest turning this into a text box.

I also think the link is not clear between the factors in the pink boxes on the left and the sentence in the main text that says total measurement variability is determined by (1) biological variability, (2) variability induced by variations in preanalytical handling of blood samples, and (3) total error in the analytical measurements. It would be best to use the same terms in text and display item (i.e. match up the bold terms in the pink boxes to the terms in the text. Please also explain how 'bias' fits in here.]

To the left, the figure summarizes factors causing measurement variability. The total variability of a given biomarker must be clearly lower than the relative change observed in the same biomarker when comparing Alzheimer's disease to all relevant differential diagnostic groups. If this is the case, the biomarker will exhibit a high clinical performance.

Figure 4. Summary of key diagnostic and prognostic markers for Alzheimer's disease

The figure depicts key biomarkers for diagnosis and prognosis of preclinical AD, prodromal AD and AD dementia, respectively. **[Au: please explain the meaning of the dotted curve and how it relates to the straight line/triangle and the graded colours within it. If they all mean the same thing (i.e. declining cognitive function with increasing clinical stage) please adjust the figure so that it includes only of the three. I would suggest keeping the curve and divide it into three sections (with vertical dotted lines or colours). This would simplify and clarify the figure.**

I also suggest distinguishing clearly between blood-based, CSF-based and imaging-based biomarkers, considering that the review is focused on blood-based biomarkers.

Furthermore, it may be helpful to explain in the legend why the indicated markers are the 'key' markers, considering that in the review you also discuss other markers — why are those not 'key'?]