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REVIEW

Validating blood tests as a possible routine diagnostic assay of Alzheimer's disease

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ABSTRACT

Introduction: In recent years, exciting developments in disease modifying treatments for Alzheimer's disease (AD) have made accurate and timely diagnosis of this disease a priority. Blood biomarkers (BBMs) for amyloid pathology using improved immunoassay and mass spectrometry techniques have been an area of intense research for the last 10 years and are coming to the fore, as a real prospect to be used in the clinical diagnostics of the disease.

Areas covered: The following review will update and discuss blood biomarkers that will be most useful in diagnosing AD and the context necessary for their implementation.

Expert Opinion: It is clear we now have BBMs, and technology to measure them, that are capable of detecting amyloid pathology in AD. The challenge is to validate them across platforms and populations to incorporate them into clinical practice. It is important that implementation comes with education, we need to give clinicians the tools for appropriate use and interpretation. It is feasible that BBMs will be used to screen populations, initially for clinical trial entry but also therapeutic intervention in the foreseeable future. We now need to focus BBM research on other pathologies to ensure we accelerate the development of therapeutics for all neurodegenerative diseases

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and diagnosing it during life has presented many challenges in the years since it was first described. For many years, the only diagnosis of an ante-mortem was via clinical means [1], patient symptoms and cognitive tests providing a diagnosis for a disease which we now know was developing many years prior to symptom onset.

The pathophysiological hallmarks of the disease, amyloid plaques, neurofibrillary tangles formed of hyperphosphorylated tau, and neurodegeneration [2], have in more recent years been detected by cerebrospinal fluid (CSF) biomarkers for amyloid beta 40 and 42 (Aβ40/42), total and phosphorylated tau (tTau and pTau181) and neurofilament light chain (NfL) or imaging biomarkers such as amyloid-positron emission tomography (PET) and tau-PET, which have traditionally been seen as the gold standard and used for clinical trial selection [3] Use of these biomarkers has been somewhat formalized by the National Institute on Aging and Alzheimer's Association (NIA-AA) framework, first proposed in 2016 [4] and updated in 2018 [5], describing the amyloid, tau (neurodegeneration) (AT(N)) system to define AD biologically. This system is currently undergoing an update, with the revised framework expected to be released during 2024.

The advent of disease-modifying therapies such as lecanemab [6], which has been given full FDA approval in July 2023, aducanumab [7], and donanemab, which will likely be approved given the recently reported phase 3 trial results [8], which were reported at the Alzheimer's Association International Conference in July 2023, makes it critical that diagnosis becomes more streamlined, cost-effective and available to all.

Traditionally, CSF has been the fluid of choice to research and diagnose neurodegenerative disease, and this makes sense due to its proximity to the brain as the CSF surrounds the central nervous system (CNS) and has indeed been included in clinical practice in certain settings [9,10]. However, collection of CSF requires specialist personnel and settings, and is perhaps most importantly viewed as an unpleasant experience by participants. Blood would be a much easier fluid to collect and work with, and for this reason efforts to measure brain-derived proteins in blood have been an intense area of work over the last 10 years or so. Progress was slow at first due to the fact that the concentrations of proteins of interest in blood are incredibly low and exquisite assay sensitivity would be required – the technology was just not available. Then in 2010, Rissin et al. [11] published a paper describing an immunoassay using

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Article highlights

- Alzheimer's disease (AD) is a progressive neurodegenerative disorder and until recently there were no disease modifying therapies available.
- There is now an urgent need for fast, accurate, and inexpensive diagnostic tests.
- Fluid biomarkers used for the diagnosis of possible AD have been measured in cerebrospinal fluid for a number of years.
- The use of super-sensitive immunoassay technology and mass-spectrometry methods are now allowing us to measure these biomarkers in blood.
- In order to use blood biomarkers we need to fully validate them with respect to pre-analytical processing and assay methods suitable for clinical labs.
- It is feasible that within the next few years we will have the materials and knowledge to effectively screen populations for AD and see more cost-effective and equitable access to therapeutics.

a single-molecule counting method which enabled assay sensitivity up to a thousand-fold greater. This technology relies on compartmentalizing the enzyme reaction to a single well containing a single bead enabling digital detection and crucially for serum and plasma measurements, dilution of the sample, thereby decreasing matrix effects that can make blood measurements difficult. Single-molecule array (Simoa) technology in the form of the Quanterix HD platforms were subsequently brought to market and the field for ultra-sensitive immunoassay [12] platforms and the capabilities also from other companies, has been growing ever since [13].

2. Biomarkers for Alzheimer's disease

As mentioned previously, a biological diagnosis of Alzheimer's pathology was proposed, first as an unbiased descriptive classification of the biomarkers used in aging research [4], and was only concerned with CSF fluid and imaging biomarkers, which gave us the amyloid, tau (neurodegeneration)(AT(N)) classification. This original classification system proposed seven major biomarkers divided into three binary classes – 'A' refers to an amyloid biomarker (amyloid PET or CSF Aβ42); 'T' a tau pathology biomarker (CSF p-tau or tau PET) and 'N' a quantitative or topographic biomarker of neurodegeneration or neuronal injury (CSF t-tau, Fludeoxyglucose (FDG)-PET, or structural MRI). This framework, along with clinical information can confirm a diagnosis of AD or be used to support a diagnosis of non-AD dementia. With the advent of new blood biomarker possibilities, it is possible to update this system to include blood markers in all these categories as reviewed by Alawode and colleagues [14] (Table 1 adapted from 10).

2.1. Amyloid peptides

Aβ-related pathology is the hallmark of AD, and two different peptides, namely 40 and 42 amino acid-long Aβ peptides (Aβ40 and Aβ42, as well as their ratio Aβ42/40) are of interest as blood biomarkers in AD; these two differ in two amino acids, with the sequence for Aβ1–42 being DAEFR-HDSGY-EVHHQ-KLVFF-AEDVG-SNKGA-IIGLM-VGGVV-IA, and with IA missing from the Aβ1–40 peptide. Other peptides such as Aβ38 have not been investigated as much in AD blood but have shown some promising results in CSF in their ability to predict tau positive (T+) and neurodegeneration positive (N+) related pathology [15]. For Aβ42 and even more so for the Aβ42/40 ratio, a lower blood level has been shown repeatedly to correlate with development or existence of AD, increased brain amyloid pathology as indicated by amyloid burden and tau-related pathology as that confirmed by either imaging and/or CSF biomarkers at population/cohort level [16–21] and even at an individual level [22]. Interestingly, such change has been found to have both a cross-sectional and potential longitudinal character (3.9 ± 1.4 years with a range of 1.9–9.0 years from baseline) in diagnosing AD and brain amyloidosis in one study [20], while only a cross-sectional in another [21] as no future amyloid pathology-related changes were observed over 4–6 years in both preclinical and symptomatic stages. Of note, is that the Aβ42/40 ratio has been found to negatively correlate with amyloid deposition even in cognitively healthy individuals [23] and to predict brain amyloid changes decades prior these changes take place and can be detected [24]; however, it is not as strong in discriminating between AD and other neurodegenerative disorders [25]. As such an A+ status does not necessarily indicate a T+ status nor AD (this is currently being re-considered in the ongoing criteria revision, where some suggest that A+ is enough to indicate AD by itself, also at a pre-clinical stage).

One caveat to be aware of when examining the Aβ42/40 ratio in blood, is the very small differences seen between amyloid-positive and amyloid-negative individuals which are ~10% compared to the ~50% we see in CSF [20]. The most sensitive immunoprecipitation mass spectrometry (IP-MS) methods appear to be more robust than ELISA-based methods [26,27], but the most recently developed immunoassays now show identical diagnostic performance [28]. Several companies have started producing tests which can be used in a clinical setting, in the US, offering the service of detection of the ratio, or even straight-to-consumer /patient tests. Examples include the PrecivityAD™ test offered by C2N [29] where in addition to the Aβ42/40 ratio, the *APOE* status and patient age are used in a mass spectrometry (MSp) platform with an AUC of up to 0.9 in predicting a positive amyloid status by PET [18]. A different test incorporating p-Tau217 and giving a binary outcome when it comes to amyloid status by introducing cutoff levels in the amyloid probability score has also been

Table 1. Summary of AT(N) Biomarkers.

Criteria	Pathology	Neuroimaging biomarkers	CSF Biomarkers	Blood biomarkers
A	Aβ	Amyloid PET	Aβ1–42 or Aβ1–42/1–40	Aβ1–42/1–40
T	Tau	Tau PET	P-tau	P-tau
(N)	Neurodegeneration	MRI or FDG PET	T-tau or NfL	NFL

developed, namely PrecivityAD2™. Tests from other companies such as the Quest AD-detect™, Sysmex's HISCL kits, Fujirebio's Lumipulse-based assays, and Roche's Elecsys assays have also shown promise [30–33].

2.2. Tau

Tau is a highly abundant protein in the CNS, however it is also expressed in peripheral tissues such as the submandibular gland, sigmoid colon, liver, scalp, and abdominal skin, both in its phosphorylated forms but as a whole too [34]. Increased total tau (tTau) in CSF is a key characteristic of AD [35], however, its distribution and levels in tissues but also in peripheral blood change under certain conditions, including AD [34], TBI [36], and other tauopathies [37]. As a consequence, although studies on blood t-tau have shown differences between AD pathology and controls as summarized in [38] and [39] other studies have not [40]. There is also both poor correlation between blood t-tau and (the more accurate in predicting AD) CSF-tau, as well as a certain overlap in t-tau levels with symptoms and features with other conditions (including normal aging) for t-tau to be a good diagnostic marker for AD [41]. However, recently a brain-specific form of tau has been characterized, and measured in blood, which is able to surpass the issues with detecting tau that is not caused by CNS-related issues [40]. BD-tau was correlated with CSF-tau, other AT(N) biomarkers, and could distinguish AD-related neurodegeneration from other causes [40]; although investigations on the analytical validity of the assay also show promising results [42], more validation studies are needed for the confirmation of this biomarker as a potential diagnostic blood biomarker (BBM) for AD. Notably, both t-tau and potentially BD-tau could be considered N biomarkers in the AT(N) framework, as they indicate neuronal injury and neurodegeneration [40,43], rather than (in the case of t-tau) or in addition to (in the case of BD-tau due to its correlation with CSF-tau) T biomarkers, which would indicate tau pathology (neurofibrillary tangles). Nevertheless, across non-AD neurodegenerative disorders t-tau is typically unchanged, suggesting that, if it indeed is an N marker, it represents a somewhat AD-specific form of neurodegeneration. Additionally, the increase that has been seen in AD for plasma t-tau is very mild [44], which contrasts with what is seen in acute severe brain injury, *e.g.*, hypoxic brain injury due to cardiac arrest [45,46] in conclusion it would seem that whilst raised CSF tTau is a reliable biomarker for AD, tTau in blood is lot more variable and probably reflecting processes that are not only due to AD pathology.

2.3. Phosphorylated tau

In contrast with t-tau, blood p-tau is considered both a true T biomarker (at least a biomarker of Aβ-related tau pathophysiology that eventually may translate into tau tangle pathology [47]), and has been found to be consistently raised and discriminatory between AD and non-AD individuals (whether these are cognitively unimpaired controls, or non-AD dementia patients [48]). More specifically, the phosphorylated forms p-tau181, p-tau217, and p-tau231 are the most interesting as potential diagnostic BBM for AD due to their robustness and reproducibility of results [49]. P-tau181 levels in blood and CSF are highly correlated, and both were associated with subsequent development of AD dementia; blood (plasma) p-tau181 predicted positive tau-PET scans, detected AD neuropathology postmortem, and was able to differentiate between AD and non-AD-related neurodegeneration [50]. Elsewhere, plasma p-tau181 was able to differentially detect an A-T-N- profile from an A+ or T+/N+ CSF profile, outperforming the blood Aβ42/Aβ40 ratio measurement [51]. Interestingly, when comparing findings for p-tau181 measured by different methods, namely Simoa and MesoScale Discovery (MSD) assays, subtle differences were found in their ability to predict tau-PET pathology with MSD outperforming in accuracy the Simoa assay [52]. Differences in performance of p-tau between both methodologies and p-tau forms were also indicated by another study, where a mass spectrometry-based assay measuring p-tau217 outperformed nine other assays in its ability to detect Aβ+ status and progression to AD dementia [53]; for the same outcomes, in the same study, p-tau181 and p-tau217 were relatively consistent and highly accurate. Plasma p-tau217 has been confirmed in other studies as able to discriminate AD from other neurodegenerative diseases outperforming p-tau181 [25], and in the case of autosomal dominant AD as an early predictor of development of MCI; in the same study, it was also shown that it outperformed p-tau181 in the accuracy of prediction of abnormal tau pathology (as indicated by tau-PET scans and correlation with tau tangles in participants with amyloid plaques). The marker has also demonstrated associations between its longitudinal increase and cognitive decline, amyloid-dependent changes over 4–6 years, and clinical deterioration and brain atrophy [21]. Lastly, p-tau231 has been shown to perform equally well as p-tau181 in its ability to identify AD neuropathology and clinical stages; however, its predictive ability is better as it starts to increase before the Aβ-PET positivity has been achieved, and earlier than p-tau181 and p-tau217, making it an earlier and just as accurate indicator of AD compared to the other two p-tau forms [54] however, unlike p-tau217 its levels plateau in more advanced levels of Aβ pathology [21]. In a different study [55], and an effort to move from comparative studies to diagnosis based on a cutoff point, potential thresholds for p-tau181 and p-tau217 were suggested, which in that study improved sensitivity and negative predictive value, however it recognizes the fact that such thresholds depend not only on p-tau form for the same condition but also for different detection methods [55,56]. Recently, plasma p-tau217 demonstrated equivalence with a CSF reference test for diagnosing AD [57]

Accordingly, as all p-tau forms are relatively accurate in diagnosing AD, with an ongoing positive validation for their clinical efficiency, they can be proven valuable in different aspects. As such, tests such as PrecivityAD2 [58] include p-tau217 in addition to measuring the Aβ42/40 ratio are currently being validated and used in a clinical study [58].

2.4. Neurofilament light chain protein (NfL)

NfL has been shown to be marker of axonal injury and has been found elevated in the blood in a variety of neurodegenerative

and neuroinflammatory conditions, including MS, FTD, ALS, and AD [59]. Although increases in NfL are not disease-specific, as a marker it has been suggested to be able to discriminate between similar conditions in their clinical appearance (e.g. atypical parkinsonian disorders from PD) and was able to discriminate between AD and cognitively unimpaired controls [59]. Similar findings have been demonstrated in other studies, where plasma NfL was increased in MCI and AD-dementia patients vs. healthy controls, and it also correlated with poor cognition and more severe atrophy; it was also higher in MCI and AD patients with higher Aβ pathology [60]. Conversely, in other studies, no cross-sectional relationships between plasma NfL and cognitive decline were found [61,62], however in [62] longitudinal associations with cognitive decline were demonstrated. In other studies, higher plasma NfL was also shown to be associated with developing dementia (both AD and non-AD), and a faster increase in NfL was found in individuals who developed AD-dementia compared with those who did not develop any form of dementia; moreover, when combined with a low level of plasma Aβ42, a high level of NfL is also associated with an increased risk of AD-dementia [63]. In a study including postmortem neuropathology, an association between NfL and higher neurofibrillary tangle pathology, as stratified based on Braak staging was found, in addition to NfL being higher in AD plasma and correlating with cognitive decline [64]. Within AD, and more specifically in familial AD, as well as in AD due to Down syndrome, increase in NfL has been shown to predict future onset of symptoms decades before expected year of onset (22 years for individuals with the presenilin 1 E280A autosomal dominant AD mutation [65], around 20 years for individuals with Down syndrome [66], and 16 years in the Dominantly Inherited Alzheimer Network cohort [67]). Although on its own, it would not be useful in detecting AD, detecting neurodegeneration by using NfL in conditions that have been shown to be a risk for developing AD may be beneficial as a monitoring and predictive tool. Plasma NfL also has proven clinical utility to detect neurodegenerative causes of cognitive symptoms in people who are AD biomarker-negative [68]. From around 3 years ago, plasma NfL has been available as a clinical test in several laboratories around the world, and the development of fully automated tests on random access clinical chemistry analyzers will increase the accessibility of the biomarker further.

2.5. Glial fibrillary acidic protein (GFAP)

GFAP is not as extensively studied as amyloid, p-tau, or NfL as a potential diagnostic AD biomarker, however it has been shown to have consistent results among studies. GFAP is a marker of neuroinflammation, and more specifically reactive astrogliosis. As a finding, reactive astrogliosis is found in early AD but also in normal aging postmortem brains, underscoring that the biomarker is likely not specific to AD [69]. A study focusing on both the differences between Aβ+ and Aβ- individuals also examined the stability of GFAP in CSF and blood, found that Aβ+ individuals had higher levels of GFAP, and that the protein was not affected by freeze-thaw cycles in blood; however, it was affected in CSF [70]. Moreover, it has been shown that GFAP was positively correlated with Aβ-PET signal overall, but the relationship diverged in older adults depending on their cognitive status [71]. In another study comparing AD and cognitively healthy controls for GFAP blood levels, it was shown that GFAP levels were higher in pre-clinical individuals but also patients within the AD continuum, and that GFAP can discriminate between Aβ+ and Aβ- individuals, and within the Aβ+ individuals, its levels are positively associated with tau pathology [72]. Interestingly, the last finding may also suggest that reactive astrogliosis may be the difference between Aβ+ individuals who are cognitively unimpaired and those who go on to develop AD-related cognitive issues. The latter has been replicated in MCI subjects, as GFAP levels in individuals who go on to develop dementia were found higher than in those who do not [73]. Elsewhere, it was shown that Aβ pathology was associated with increased tau phosphorylation only in individuals who presented with reactive astrogliosis as indicated by their GFAP levels [74]. Additionally, it has been shown that plasma amyloid (Aβ42/Aβ40) and GFAP are associated with amyloid status determined by PET and that together they can identify positive amyloid status, whereas NfL and GFAP together predicted cognitive performance, while they were also separately negatively correlated with medial temporal lobe atrophy [75]. The Aβ42/Aβ40 ratio has been shown to also have a negative correlation with GFAP and that a low Aβ42/Aβ40 ratio and high GFAP is found in Aβ+ individuals, and the addition of GFAP may contribute to an increased diagnostic accuracy [76].

As previously mentioned, GFAP is not specific to AD (although the fold-change in response to Aβ positivity is impressive), but it reflects astrocytic activation/astrogliosis in a range of neurodegenerative diseases, as well as in neuroinflammatory and traumatic brain conditions. Tests to measure GFAP in plasma/serum in clinical laboratory practice have been developed, as the marker is useful in the diagnosis of TBI, such as the i-STAT TBI Plasma test [77] and the Banyan TBI test [78], so repurposing them for AD would not be difficult, if more studies show robust evidence of its usefulness in either being a diagnostic, predictive or monitoring marker for AD.

The results of efforts in validating such biomarkers, including assays available to measure them, clinical validation results for AD, known covariates, specificity in AD and examples of clinically and/or commercially available blood tests can be found in Table 2.

2.6. Other biomarkers

2.6.1. Other protein biomarkers

Although the four aforementioned biomarkers are the most often used in studies and considered good candidates due to their overall performance and advances in their detection, other proteins have also shown promise in their clinical relevance for AD. These can be roughly categorized in inflammation-related biomarkers (including complement system markers [79,80], cytokines [81–83]), coagulation markers [84,85], CNS-related markers (either indicating neuronal damage (e.g. Visinin-like protein 1 (VILIP-1) [86] and S100B [87]), or glial activation (e.g. YKL-40/Chitinase 3-like 1 (Ch3L1) [88–90] and soluble Triggering receptor expressed on myeloid cells 2 (sTREM2) [91]) or neuronal function (e.g. Brain-derived neurotrophic factor (BDNF) [92])). These however are not specific to AD [93], but they are also not as studied in clinical cohort studies or pinpointed in discovery studies.

Table 2. The most promising BBMs for the detection of AD, and a summary of their characteristics and applications. *1Specific to neurodegenerative disorders, including AD; *2Specific to diseases where astroglia activation is observed.

Biomarker	Aβ	Tau	NfL	GFAP
Assay(s) available	ELISA, SiMoA, MSD for Aβ 38, 40, 42, Aggregated/oligomers, Mass Spec for Aβ 40, and 42	ELISA, SiMoA, MSD, Mass Spec for t-Tau, p-Tau181, p-Tau217, p-Tau231, BD-tau	ELISA, SiMoA, MSD	ELISA, SiMoA, MSD
Clinical validation	+++++	+++++	++++	++
Known covariates that affect blood biomarker values	Age, race, sex	Age, sex	Age, sex, comorbidities with other neuroinflammatory conditions	Age, comorbidities with other neuroinflammatory conditions, sex in some studies
AD specificity	++++	++++	+*1	+*2
Commercial and clinical availability of approved and authorized blood tests	Yes: • C2N PrecivityAD and AD2 blood test (+APOE) (U.S.A.) • Sysmex's HISCL β-Amyloid 1–42 Assay Kit and HISCL β-Amyloid 1–40 Assay Kit (Japan) • Quest Diagnostic's AD-Detect • Provided as a service by companies (e.g. ABtestService by Araclon)	• Provided as a service by companies (e.g. Quanterix, ALZpath, Roche) • PrecivityAD2 is under testing	Several companies provide it as a service (e.g. Simens, Mayo Clinic, Labcorp, Quanterix (Simoa LDT NfL))	Yes, but not AD specific: • i-STAT TBI Plasma test • Banyan BTI

2.6.2. Non-protein biomarkers

Although most studies focus on protein or protein-related biomarkers, other studies have focused on non-protein blood analytes. Lipids, including cholesterol and some of its forms and derivatives, triglycerides, low-density lipoprotein, sphingolipids, and phospholipids have all been found to be associated with AD, as they have been found increased in AD individuals, as summarized and discussed by [94]. Interestingly, proteins involved with lipid regulation, metabolism or transport have been shown to be involved in AD, including apoE [95], amyloid precursor protein (AP) [96], Aβ and presenilin [97]. Cell-free DNA (cf-DNA) and RNA, including both mitochondrial and genomic, and focusing either on the amount of cf-DNA or cf-RNA or the methylation profile has also been successfully used to distinguish between AD and controls [98–101], and predict cognitive outcomes and dementia risk [102]. Of special interest are certain types of regulatory RNAs such as long non-coding RNAs (lncRNAs) and micro-RNAs (miRNAs) as not only have been found to be dysregulated in AD [103,104] but also have been shown to be differentially expressed in AD vs controls [105,106].

Although both lipids and cf-DNA do show potential, these are just emerging as biomarkers and currently not many of these assays exhibit a robust predictive value nor has been an effort to include them in the guidelines for validation, so they are not within the scope of this review.

The above biomarkers whether they are well established or not as well studied and often not specific to AD, may all have something to offer in the diagnosis of AD. Single biomarkers often do not offer enough information, as they may be involved in more than one processes, and often come from different sources. As no single biomarker has been found to accurately predict AD, it would be more beneficial should a heterogeneous panel comprising a combination of biomarkers be developed, after evaluation for their independent contribution in predicting future development or existence of AD, when models are adjusted for known demographic, clinical, and radiological factors.

3. Validating biomarkers in blood

To properly validate a fluid for biomarker use, there needs to be standardization in collection, processing, and storage. For CSF, this has been the focus of a large body of work to ensure that variability due to different practices in the pre-analytical timeframe is reduced to a minimum [107]. Additionally, the assays used to measure the biomarkers must be validated in terms of analytical sensitivity, precision, and robustness at minimum [108]. The actual adoption of CSF biomarkers for AD into clinical practice has been somewhat controversial and to this day is mainly used in research settings and some specialist centers, the issues and challenges discussed in more detail here [109], we can expect those same issues with adoption of AD diagnostic tests in blood but with a much larger impetus to solve them, with the new era of therapeutics becoming available.

3.1. Pre-analytical standardization of blood based biomarkers for AD

The quality and availability of the sample often plays a role in how feasible it is to measure certain analytes. First, pre-analytical conditions and sample handling can have an impact on the values detected in different studies and by using different protocols which has been shown for both CSF and blood biomarkers, including in measurements of AD samples [110–113]. These factors can be separated into pre- and post-storage factors. Examples of the former include sample type (serum vs. plasma when it comes to BBM), handling to avoid hemolysis and clotting, timeframes for plasma separation and until storage, anticoagulants (citrate vs. EDTA vs. heparin), and their concentrations and handling temperatures (RT vs. ice) following thawing. For the latter, storage temperature and time, but also freeze-thaw cycles, have been shown to affect different proteins in different degrees and ways. For the proteins of interest, in AD as

suggested by the AT(N) framework, the Standardization of Alzheimer's Blood Biomarkers (SABB) working group published guidelines in [111]; however, more recent studies have found additionally that differences may occur depending on whether the samples belong in a patient group or a control group [114], so the initial amount of protein in the sample may play a role in whether that will be affected by extrinsic factors.

Although cohort- and, in a clinical setting, person-specific variations should be considered when measuring sample values, validation, and standardization of assays in order to measure analytes often removes that flexibility, therefore a robust assay is better in clinical settings than a flexible one. To correct for that, and especially if an assay is given quantitative results related to a cutoff value or reference limit rather than absence/presence of an analyte, these values should be based on measuring values accurately, and relating these values to clinical features consistently. An excellent guide for the above, addressing the majority of the challenges mentioned, but also offering practical guidance in the form of SOPs is included in [115].

Altogether, a large body of work has shown that BBM for AD are generally quite insensitive to pre-analytical variation; they are stable for at least three freeze-thaw cycles and are measurable in both serum and plasma, including in different forms of plasma. The exception is plasma Aβ, the concentrations of which drop if the samples are not spun within 2 hours. Additionally, Aβ biomarkers in blood should be measured in plasma rather than serum [116,117]. Regarding P-tau, such markers are measurable in both serum and plasma with excellent correlation, but the absolute concentrations differ; hence, sample type-specific reference limits and cut-points are needed [118]. There are also some studies suggesting that it may be better to measure the biomarkers in fasting morning samples, as some influence of food intake and sampling time has been reported [119].

3.2. Assay choice

As proteins and other BBMs are diverse in nature and can range from peptides to protein complexes, to different forms (e.g. phosphorylated on different residues) of proteins the quality of the standards and calibrators of BBM immunoassays should fulfill certain criteria. According to EU guidelines (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf), 'the validation of bioanalytical methods and the analysis of study samples for clinical trials in humans should be performed following the principles of Good Clinical Practice.' These guidelines also recognize specific issues and considerations in ligand-binding assays – such as immunoassays – including sample-related issues, such as matrix-related issues, dilution issues (including minimum required dilution and linearity), parallelism-related potential-, and reagents/assay-related issues, and considerations including issues with reference standards, calibration curves, precision and accuracy, specificity, and selectivity.

Different manufacturers often follow different SOPs in the production of the assay standards, which may result in differences in the resulting product. The material used as a reference should be well documented and characterized, and even QC tests between batches should be used in order to ensure the characteristics of the standard remain the same. Moreover, for a biomarker to be fit-for-purpose, its stability in assay conditions and timeframes for both standards and samples should also be tested and considered.

Similarly with the antibodies used, as differences in cross-reactivity may contribute to big differences in results, but also different epitopes might be recognized in only certain forms of a protein, but not others, and certain antibodies only work well for some assays, while not in others (e.g. works only for western blot (WB), but not enzyme-linked immunosorbent assay (ELISA)). The different assays most often used for BBM research are presented in Figure 1 and are discussed within the text below.

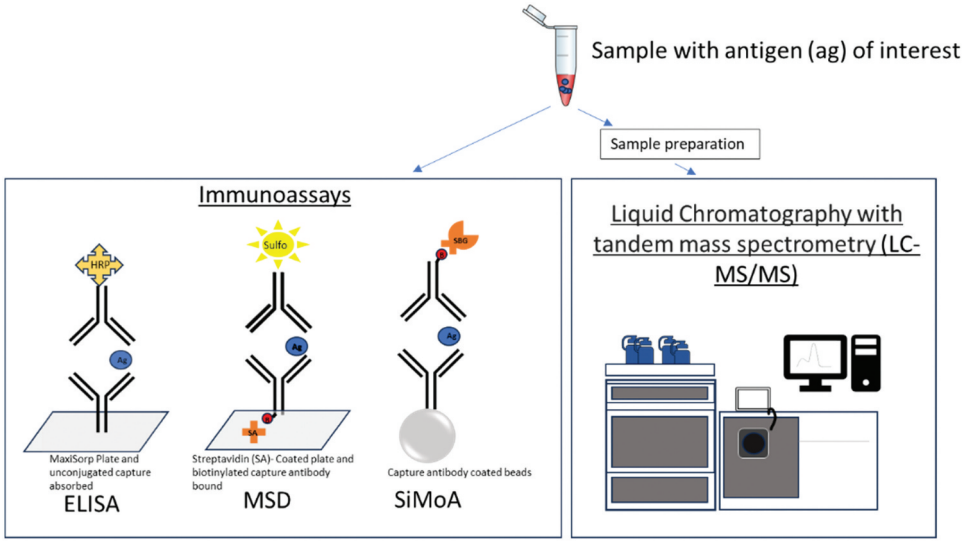

Figure 1. Description of the typical methods (immunoassays and mass spectrometry) used for the detection and quantification of blood-based biomarkers. HRP=Horseradish peroxidase, Ag=antigen, SA=streptavidin, B=biotin, SGB=Streptavidin beta galactosidase.

An antibody used for an assay must be not only specific for the analyte in question but also selectively bind only that analyte in the required form and not proteins with a similar structure, or be influenced by matrix-related factors. Therefore, testing the antibodies and standards near high and low detection limits, and under different matrix conditions (e.g. haemolysed or lipemic samples, as these are included in real-world cohorts) is recommended. The above should also be considered not only during the development of an assay, but when using theoretically-validated commercially available assays.

As an example, the aforementioned issues are put into test in [120] for a single protein, namely the complement factor H (CfH). A variety of studies have examined CfH in the context of AD, and although some studies have found differences between AD and controls (e.g. [121], and others finding that CfH is able to differentiate between AD stages (e.g. [122]), the results are not consistent. In the context of addressing the discrepancies between studies and even different levels of the protein found in studies in healthy donor plasma using different assays [120], first examined 7 commercially available assays using the same set of samples, which were collected, stored, and handled in exactly the same manner, where striking differences in the detected values between the commercially available assays were found, though there was a correlation between the findings for most of the assays. They also measured purified protein dissolved in PBS, in order to examine the selectivity and specificity of the antibodies, and two of the assays were not able to detect the protein at all. The other assays did detect it, but the accuracy was not high. Additionally, they measured depleted samples, where although most assays did not detect anything, as expected, one of the assays detected a relatively high amount of protein. Lastly, they exchanged the calibrators and while some of the assays had no issues detecting the calibrators of other assays, there were calibrators that were not recognized in some assays, whereas for one assay it was only able to detect its own calibrators. It is to be noted that although for assays specifically investigating the complement system a committee exists that aims to standardize and regulate the available commercial assays, this has clearly not been achieved. Similarly, a study was conducted focusing on tau variants in CSF and plasma, differences between the quantities detected by assays were apparent; however, there were consistent findings with regard to differences between control and AD samples [56]. In another study focusing on the performance of 10 p-tau assays in prodromal AD [53], it was shown that both the form of p-tau and the assay used play a role in the performance of the assay, with p-tau217, a mass spectrometry-based assay outperformed all other assays in its ability to identify MCI Aβ+ patients, or those who will develop AD-dementia; moreover assays detecting p-tau181 and p-tau217 were more consistent and accurate across both measurements. In a similar setting for Aβ42/40 assays comparing mass spectrometry-based detection assays to immunoassays, differences in performance among assays were also observed, with mass spectrometry-based assays outperforming most of the immunoassays [26]. Encouragingly, however, a study focusing on measuring different tau variants using the same method, namely, Simoa, found high analytical performance, and high clinical performance and correlations in the findings between the majority of the assays examined [123]. In the AAIC meeting in Amsterdam 2023, the results of a large p-tau Round Robin study suggested equivalent and excellent diagnostic performance of several new immunoassays with MSp-based p-tau assays (Zetterberg, personal communication).

After the above considerations have been taken into account, different assay-specific ones come into question. One of those are the amount of calibration standards needed which are ideal for the measurement of a wide range of values, internal calibrator samples, and repeats. It is usually recommended that a minimum of 6 calibration standards are needed in order to produce a reliable standard curve, however certain assays use as few as a 3-point calibration 'curve.' High and low value calibrator samples should also be considered, as well as a potentially additional calibrator sample(s) which could be a pooled sample to reflect an average expected value. These should be used in every measurement and treated in the same way as the other samples on the assay and could be also used to calculate the intra and inter-assay CV values. For the latter, different values are deemed acceptable depending on the lab and assay, but usually a CV of <20% is accepted as accurate in most cases. It is worthwhile to mention here, that although a CV of 20% is acceptable in laboratory/research settings, this may cause issues when there is a small difference between groups for a biomarker of interest, and basing a diagnosis on that biomarker should take into consideration a 'gray area' of values.

Oftentimes the range of an analyte within a sample cohort may be outside the one which can be calculated using a calibration curve. Therefore, it is useful to demonstrate that samples that exceed the upper limit of the calibration curve can be measured after being diluted, without affecting the accuracy of the measurement. The sample dilution buffer should be appropriate so it will not introduce artifacts during the measurement, so if certain samples are known to have higher concentrations, a parallelism experiment could be carried out to ensure that there are no matrix or dilution-related effects; if the sample is not linearly diluted, then this should be noted and accounted for.

3.3. Covariates

In addition to the accuracy issues and between and within assay variation, which we have already discussed, other factors should be controlled for when a biomarker is used in clinical settings. Such factors have been shown to differentially affect the development of AD, it is expected that age, sex, race, lifestyle, and potential co-morbidities may alter the levels of AD-related BBMs.

As an example, plasma tau has been shown to increase in normal aging and to be associated with sex and *APOE* genotype [124]. In a study focusing on both tau and GFAP, these were shown to be associated with sex in some studies (37), but this finding is not consistently shown in other studies (38, 39). Similarly, NfL is increased with age, but factors such as BMI and renal function also affect its levels in different age sub-groups, with increased BMI predicting decreased NfL in younger adults, and impaired renal function predicting increased NfL in individuals over 60 [125]; NfL is also increased in other

neurodegenerative and neuroinflammatory conditions, such as MS [126] and TBI where higher levels have been shown to persist even years after the brain injury incident [127]. Other conditions such as protein metabolism and clearance issues and co-morbidities (e.g. kidney and liver issues) may also influence the levels of these proteins in blood and lead to a mistaken diagnosis [128]. Finally, a study focusing on plasma p-tau181, Aβ40, and NfL found racial differences in the levels of all three biomarkers in blood [129] with the levels of the biomarkers in self-reported African American individuals being lower than white individuals. In addition, the timepoint of sample collection should be considered when the proteins of interest have been shown to be influenced by factors such as circadian rhythms [119,130] and pre-sampling activity, such as fasting [119].

Clinical studies and trials for the establishment of AD biomarkers often exclude participants with factors that may influence the biomarker of interest, which although clinically validates a biomarker for a subgroup of the general population, this does not translate in the general population. As an example, in a clinical trial focusing on plasma Aβ as a predictive biomarker (identifier #NCT01315639), exclusion criteria included the patients suffering from major depression, issues interfering with the cognitive assessment, and certain levels of education; for that trial differences in age groups between MCI and AD also existed, with AD patients being recruited for over 45 years of age, whereas MCI individuals were recruited for an age of over 70 years; no reference to race, BMI, or any of the factors mentioned above is made.

By excluding factors that have been correlated with AD development or are considered symptoms of AD or have been shown to affect BBMs (such as depression), groups with conditions with similar clinical characteristics (e.g. other types of dementias), by not including neuropathological confirmation of AD cases and thus having non-AD patients classed as AD, and ignoring or manipulating other factors that have been shown to affect the outcome (e.g. differences in race, age between groups which are not accounted for) the results of the such trials are often underwhelming, and while the biomarkers being investigated may be useful, that usefulness is potentially masked. An extensive list of potential controllable or uncontrollable characteristics for AD trial can be found in [131], where also a great effort was made to provide a set of guidelines for pre-analytical processing of specimens for AD clinical trials. In addition to these guidelines, in [132] the authors explain the roles that the academia and industry can play in the development of a clinically valuable, useful, and useable blood biomarker for AD, employing the strengths of both and giving examples of how by collaboration of the two such an idea might become reality faster. Examples of the steps where a blood biomarker could be useful and replace or complement present practices are also discussed in [132], with blood biomarkers being good candidates as a screening tool, but also predictive and to show response to medication.

4. Conclusion

Accurate diagnosis of neurodegenerative disease in life has always been a difficult endeavor, and to some extent, while no disease-modifying treatments have been available, there has been little urgency to improve this. Now that we are entering a new era of disease-modifying treatments, with the possibility to diagnose and treat patients with AD, it is essential that we prioritize this area. In this review, we have discussed briefly the AT(N) classification of biomarkers used for a biological definition of AD (Table 1), and the biomarkers currently most likely to be adopted for this purpose. We have highlighted studies looking to validate these biomarkers in various cohorts and the issues which must be investigated with respect to bringing a test into a clinical setting and can conclude that a number of the biomarkers studied are already ready to be validated and adopted for clinical use, most notably the Aβ42/40 ratio and phospho tau. Real-world considerations, such as co-morbidities and ethnicity are being taken into account in studies [128,133,134], to assess their impact. This is very much an intense area of research at present with many studies being set up to answer the questions that will allow a robust diagnosis that is available to all communities in an equitable way in order that as many people as possible have access to the new wave of therapeutics as possible. As both novel imaging techniques emerge (for example the use of far-red fluorescence probes for monitoring and potentially diagnosing AD [135]) and new biomarkers and assays to detect changes that happen during or even before the occurrence of AD, a more accurate, early, and less invasive detection and diagnosis of AD may be closer than ever.

5. Expert opinion

Work on blood biomarkers for AD has accelerated rapidly in the last 5 years with access to new technology and improved assays. The field is fueled by the prospect of new drugs being approved for clinical use, and the challenge is to be ready for this. BBMs are already highly indicated for use in pre-screening for clinical trial cohorts, potentially amyloid positivity can be established with a high performing BBM such as p-tau, reducing costs of patient selection substantially, especially when you have biomarkers that can detect amyloid pathology prior to PET positivity – essential when targeting drugs at the MCI stage of disease. Clinical implementation though will require further steps to be taken.

The best performing biomarkers need to be incorporated into robust assays fit for use in a clinical laboratory, efforts are ongoing at a very rapid pace to develop assays such as those for Aβ40, Aβ42, p-tau181, −217, and −231 for use on clinical chemistry analyzers which are used in routine hospital laboratories. Reference materials need to be formulated and normal values determined. This requires 'real world' cohorts, and research efforts are currently aimed at meeting this challenge.

Most patients will present to their primary care provider with cognitive worries or symptoms, few will have access to specialist memory clinics, so in order that diagnosis and possible access to treatments are available as widely as possible we need to make tests available to primary care providers but crucially this must come with education – how and when should the tests be used, how to interpret the results, does this individual need to go for further tests or is it a clear rule in/out AD. If a patient presents with cognitive problems, but is

amyloid-negative and NfL-positive, another neurodegenerative disorder is indicated, so there needs to be a protocol in place to deal with that, one extremely good reason we should have patient and public involvement (PPI) groups advising at all steps of implementation.

It is feasible with the rate of research at present that within the next few years we will have the materials and knowledge we need to be able to effectively screen populations for AD pathology, and there will be more cost-effective and equitable access to therapeutics in trials, which will increase in number. An ideal scenario would make the screening process as simple and cost-effective as possible, a blood spot for the measurement of BBMS, likely a p-tau isoform and NfL, a digital cognitive test and perhaps a saliva sample for genetic screening. Once therapeutics are approved, if it is appropriate, they could be prescribed but that may instead be a 'lifestyle' prescription and BBM monitoring at appropriate intervals, with therapeutic intervention only when necessary. Of course, once a course of drugs has been started BBMs, will be important to monitor disease-modifying effects, as well as safety (NfL and GFAP could be useful in the latter context as well, as general markers of neuronal injury and astrocytic activation).

To finish, whilst blood biomarkers are an incredibly exciting and important development in the AD research field, a biomarker relies on appropriate use and clinical expertise, all these must be in place for successful implementation, also researchers now need to consider biomarkers for other non-AD neurodegenerative diseases and the pathology that causes them. Efforts are needed to produce robust markers for alpha-synuclein (αsyn) pathology and TAR DNA-binding protein-43 (TDP-43) as well as other markers that may emerge as playing a role in the development of other neurodegenerative diseases, so that the progress we are celebrating for AD therapeutics can be translated across diseases.

Abbreviations

αsyn	alpha synuclein
APOE	apolipoprotein E
AAIC	Alzheimer's Association International Conference
AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid precursor protein
AT(N)	amyloid, tau (neurodegeneration)
AUC	Area Under the Curve
Aβ	amyloid beta
BBM	Blood Biomarker
BDNF	Brain derived neurotrophic factor
BD-tau	Brain Derived tau
BMI	Body mass index
cfDNA	cell-free DNA
CfH	complement factor H
Ch3L1	chitinase 3-like 1
CNS	Central nervous system
CSF	cerebrospinal fluid
CV	Coefficient of Variation
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FDG	Fludeoxyglucose
FTD	Frontotemporal dementia
GFAP	Glial fibrillary acidic protein
IP-MS	immunoprecipitation mass spectrometry
lncRNA	long non coding RNA
MCI	Mild Cognitive Impairment
miRNA	micro RNA
MS	Multiple sclerosis
MSD	MesoScale Discovery
MSp	Mass spectrometry
NfL	neurofilament light
NIA-AA	National Institute of Aging and Alzheimer's Association
PBS	Phosphate-buffered saline
PET	positron emission tomography
p-tau	phosphorylated tau
QC	Quality control
RT	Room temperature
SABB	Standardization of Alzheimer's Blood Biomarkers
SiMoA	single molecule array
SOP	Standard operating procedures
sTREM2	soluble Triggered receptor expressed on myeloid cells 2
TBI	Traumatic Brain Injury
TDP-43	TAR DNA-binding protein-43
t-tau	total tau
VILIP-1	Visinin-like protein 1
WB	western blot

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