Transitioning from cerebrospinal fluid to blood tests to facilitate diagnosis and disease monitoring in Alzheimer’s disease

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Abstract. Alzheimer’s disease (AD) is increasingly prevalent worldwide, and disease-modifying treatments may soon be at hand; hence, now, more than ever, there is a need to develop techniques that allow earlier and more secure diagnosis. Current biomarker-based guidelines for AD diagnosis, which have replaced the historical symptom-based guidelines, rely heavily on neuroimaging and cerebrospinal fluid (CSF) sampling. While these have greatly improved the diagnostic accuracy of AD pathophysiology, they are less practical for application in primary care, population-based and epidemiological settings, or where resources are limited. In contrast, blood is a more accessible and cost-effective source of biomarkers in AD. In this review paper, using the recently proposed amyloid, tau and neurodegeneration [AT(N)] criteria as a framework towards a biological definition of AD, we discuss recent advances in biofluid-based biomarkers, with a particular emphasis on those with potential to be translated into blood-based biomarkers. We provide an overview of the research conducted both in CSF and in blood to draw conclusions on biomarkers that show promise. Given the evidence collated in this review, plasma neurofilament light chain (N) and phosphorylated tau (p-tau; T) show particular potential for translation into clinical practice. However, p-tau requires more comparisons to be conducted between its various epitopes before conclusions can be made as to which one most robustly differentiates AD from non-AD dementias. Plasma amyloid beta (A) would prove invaluable as an early screening modality, but it requires very precise tests and robust pre-analytical protocols.

Keywords: Alzheimer’s disease, Blood, Cerebrospinal fluid, Diagnosis, Disease monitoring, Fluid biomarkers.

Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid beta; APP, amyloid precursor protein; AT(N), amyloid, tau (neurodegeneration); BBB, blood–brain barrier; CJD, Creutzfeldt–Jakob disease; CSF, cerebrospinal fluid; CU, cognitively unimpaired; DS, Down syndrome; ECL, electrochemiluminescence; ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer’s disease; FDG-PET, fluorodeoxyglucose positron emission tomography; MCI, mild cognitive impairment; MRI, magnetic resonance imaging; MS, mass spectrometry; NfL, neurofilament light chain; NFT, neurofibrillary tangle; NIA-AA, National Institute of Aging and Alzheimer’s Association; PET, positron emission tomography; P-tau, phosphorylated tau; SCD, subjective cognitive decline; Simoa, single molecule array; TBI, traumatic brain injury; T-tau, total tau.
Introduction

AD, biomarkers and the AT(N) criteria

Alzheimer’s disease (AD) is the most common form of dementia worldwide. It is characterized by (1) the presence of amyloid beta (Aβ) plaques in the brain parenchyma, which is often accompanied by Aβ in cerebral blood vessels (amyloid angiopathy); (2) intraneuronal neurofibrillary tangles (NFTs), composed of hyperphosphorylated tau; and (3) neurodegeneration [1-3]. According to the amyloid cascade hypothesis, accumulation of misfolded Aβ years before clinical symptom onset is the initial trigger of AD pathogenesis [4]. This accumulation of Aβ, as well as the production of toxic oligomeric species, results in aberrant tau phosphorylation and misfolding, ultimately inducing neuronal loss and plaque-induced synaptic dysfunction [5]. This pathophysiological process is summarized in Fig. 1. Histopathological analysis of the brain at autopsy remains the gold standard for definitively diagnosing AD. However, molecular biomarkers have been developed to increase the accuracy of diagnosing AD clinically [6].

A biomarker is a naturally occurring, detectable indicator that can be measured to assess a physiological or pathological state [7,8]. The importance of biomarkers is highlighted in the recent update of the National Institute of Aging and Alzheimer’s Association (NIA-AA) research framework in 2018, in which a clinical diagnosis of AD is supported by biomarker evidence of a disease-specific pathophysiological signature, rather than by clinical symptoms alone [9]. A key reason for this is the inaccuracy of a diagnosis based solely on symptoms, with one multi-centre study observing the sensitivity and specificity of clinically probable AD to detect Braak stages V/VI to be 76.6% and 59.5%, respectively [10,11]. There are marked phenotypic differences within AD, especially in younger patients, and the symptoms overlap with other neurodegenerative disorders, including vascular dementia, and mood disturbances such as depression [12]. A secure diagnosis is important to ensure patients receive the correct management (of AD, or of alternative conditions), and to provide prognostic information, advice and support. Furthermore, it is now clear that histopathological changes predate symptom onset by several years in both familial and sporadic forms of AD [13-16]. While not currently clinically indicated, in the future it may become important to make a diagnosis of AD before symptom onset – if a

Fig. 1  AD pathophysiology and AT(N) criteria fluid biomarkers.
disease-modifying treatment is shown to be effective at this early stage.

Detection of AD pathology pre-symptomatically is already important for research and for clinical trials that seek to show disease modification at this stage. Clinical trials aiming to halt, or significantly slow, AD progression have thus far proven ineffective. This is possibly due to the inclusion of symptomatic patients who have progressed too far along the disease process, and in whom significant irreversible neuronal loss has already occurred [17]. Conversely, it may be due to some participants having a false AD diagnosis. This is particularly true of the solanezumab trial, where some recruited participants were later found to be amyloid PET-negative, hence were unlikely to have AD [18]. Furthermore, the lack of success in recent clinical trials may be due to too short trial duration and is further complicated by some participants displaying AD mixed with other disease pathologies, rather than being pure AD cases. Identifying individuals with AD pathology years prior to symptom onset will enable recruitment into clinical trials at a much earlier, and potentially more tractable, disease stage, and hence may prove more effective at identifying treatments to slow, or perhaps even halt, the disease process. Moreover, as participants in such trials would not be displaying cognitive symptoms, conventional cognitive/symptomatic endpoints are unlikely to be effective for identifying response to treatment, and so dynamic biomarkers which are sensitive to progression in pre-symptomatic disease will be important. Table 1 summarizes the use of available CSF and neuroimaging biomarkers in clinical trials, along with upcoming blood-based biomarkers.

There are two main types of biomarkers for molecular AD brain changes – neuroimaging biomarkers (primarily positron emission tomography [PET] imaging) and fluid biomarkers (primarily cerebrospinal fluid [CSF]) [19]. The AT(N) criteria for AD diagnosis, which divide seven AD biomarkers into three groups based on the pathophysiological characteristic of AD they measure, include both of these classes of biomarkers [20] and are summarized in Table 2, where we also list a number of upcoming blood biomarkers. 'A' refers to Aβ pathology, as depicted by increased amyloid PET uptake, decreased CSF Aβ1-42 (Aβ1,42) or decreased Aβ1,42/Aβ1-40 ratio (Aβ1,42/1-40). 'T' refers to tau pathology, as depicted by positive tau PET tracer uptake or increased CSF phosphorylated tau (p-tau). Finally, '(N)' refers to neurodegeneration or neuronal injury, as depicted by decreased signal on [18F]-fluorodeoxyglucose (FDG)-PET, grey matter atrophy on structural magnetic resonance imaging (MRI), increased CSF total tau (t-tau) or increased CSF neurofilament light-chain (NfL) [20]. '(N)' is denoted in brackets to highlight that the biomarkers of neuronal injury are not specific to AD [9]. The fluid biomarkers in the AT(N) criteria can be seen alongside the pathophysiological process they reflect in Fig. 1.

While the AT(N) criteria highlight that both neuroimaging and fluid biomarkers can reliably confirm pathophysiological evidence of AD, fluid biomarkers offer the advantage of being able to detect the presence of multiple molecular pathologies in one bio-sample, as well as being of lower cost. However, a drawback of fluid biomarkers is the lack of anatomical information on the location and extent of pathologies, which can be gained from neuroimaging. Indeed, fluid biomarkers reflect a pathological process in the tissue, while neuroimaging, with a few exceptions, quantifies this pathology [21]. In this review, using the AT(N) criteria as a framework, we will address the

| Intended use in trial | CSF biomarkers          | Neuroimaging biomarkers | Blood biomarkers                |
|-----------------------|-------------------------|-------------------------|---------------------------------|
| Pre-screening         | T-tau, p-tau, Aβ1,42    | Amyloid PET, Tau PET    | NfL, p-tau, Aβ1,42              |
| Supporting diagnosis  |                         |                         |                                 |
| Drug effect monitoring| Dependent on the mechanism of action of the drug | Dependent on the mechanism of action of the drug | Dependent on the mechanism of action of the drug |
| Safety markers        | Markers of inflammation and BBB integrity | MRI                  | NfL, markers of inflammation    |
evidence behind current CSF-based biomarkers for AD, with a particular focus on those that have potential for translation into blood-based biomarkers.

**CSF and blood biomarkers for AD-related pathologies**

Before delving into potential blood-based biomarkers for AD, it is important to consider some advantages and potential drawbacks common to all. Although CSF has the advantage of being in direct contact with the cerebral extracellular space, blood is less invasive to collect. Consequently, it is more suitable for obtaining repeated measurements from patients and is more easily accessible in low-resource and non-specialist settings worldwide [22-24]. While blood-based biomarkers have the potential to function as an initial diagnostic screening tool in a primary care setting, prior to more in-depth investigations in specialist centres [22,25], measuring biomarkers of brain diseases in the blood is not without its challenges, namely (1) analyte concentrations are 10- to 100-fold lower in the blood compared with CSF as a direct consequence of the blood-brain barrier (BBB) [26]; (2) some AD biomarkers are expressed by extra-cerebral tissues; (3) proteases in the blood may break down analytes of interest prior to their measurement [27]. This puts extra demand on the pre-analytical and analytical processes of relevance to blood biomarker measurements for CNS diseases.

**Amyloid beta**

$\text{A}\beta_{1-40}, \text{A}\beta_{1-42}$ and $\text{A}\beta_{1-42/1-40}$ as amyloid biomarkers in CSF

$\text{A}\beta$ in CSF is already well established as a biomarker for AD. $\text{A}\beta$ is produced when amyloid precursor protein (APP) is processed along its plaque-forming (amyloidogenic) pathway. In this pathway, APP undergoes cleavage, first by $\beta$-secretase followed by $\gamma$-secretase, to produce an $\text{A}\beta$ peptide [28]. The length of the $\text{A}\beta$ peptide is dependent on the site (or extent) of $\gamma$-secretase cleavage [29]. While $\text{A}\beta$ peptides of varying amino acid lengths can be produced, the most abundant isoforms in CSF are $\text{A}\beta_{1-38}, \text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ [30], with $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ being the most widely studied isoforms. All $\text{A}\beta$ peptides differ in amino acid sequence mainly at the C terminus [31].

Initial studies looking at total CSF $\text{A}\beta$ in AD compared with controls had mixed results. While some showed a slight decrease in AD [32-35], others found no change in total CSF $\text{A}\beta$ concentration in AD compared with controls [36-38]. A major shift occurred following the discovery of $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ and the development of assays that are specific to these peptides. Investigations into the key differences between them revealed that $\text{A}\beta_{1-42}$ is more hydrophobic and hence is more prone to aggregation than $\text{A}\beta_{1-40}$ [31]. Furthermore, CSF concentrations of $\text{A}\beta_{1-40}$ remain unchanged in AD, whereas CSF concentrations of $\text{A}\beta_{1-42}$ decrease [39-41], suggesting that of the two, $\text{A}\beta_{1-42}$ provides a better biomarker for AD.

While CSF $\text{A}\beta_{1-42}$ concentrations have proven invaluable in diagnosing patients with probable AD dementia, $\text{A}\beta_{1-42}$ concentrations are to some extent dependent on the total $\text{A}\beta$ concentrations of each patient [42]. Although it is necessary to have a threshold concentration of CSF $\text{A}\beta_{1-42}$ concentrations, below which an AD diagnosis is likely, inter-individual differences make these thresholds somewhat arbitrary. Looking at CSF $\text{A}\beta_{1-42}$ concentrations alone may result in some patients being misdiagnosed as ‘normal’ when in fact concentrations may be abnormally low, if the CSF results had been related to their overall $\text{A}\beta$ production and vice versa [43]. Harnessing the fact that CSF $\text{A}\beta_{1-40}$ concentration is not altered in AD, but instead may provide a useful index of an individual’s rate of $\text{A}\beta$ production more generally,
using CSF Aβ1-42/1-40, may improve the reliability of results compared to using CSF Aβ1-42 alone. Lewczuk et al. [40] found measuring CSF Aβ1-42/1-40 alongside Aβ1-42 to improve diagnostic accuracy when comparing patients with AD to either controls or those with non-AD dementias. Although the differences in diagnostic accuracy between Aβ1-42/1-40 and Aβ1-42 were not statistically significant, likely due to low patient numbers. Additionally, Slaets et al. [41] reported that the addition of CSF Aβ1-42/1-40 to a biomarker panel for AD diagnosis consisting of Aβ1-42, Aβ1-40 and tau phosphorylated at threonine 181 (p-tau181) significantly improved diagnostic accuracy compared with the same panel without Aβ1-40 and Aβ1-42/1-40. However, it is worth noting that they observed no statistically significant difference in the area under the receiver operating characteristic curves between Aβ1-42 and Aβ1-42/1-40. Furthermore, Struyfs et al. [30] and Bousiges et al. [44] both found that the addition of Aβ1-42/1-40 improved the ability to differentiate AD from non-AD dementias, particularly frontotemporal lobe dementia and dementia with Lewy bodies. In non-shunted normal pressure hydrocephalus, all Aβ peptides are reduced in CSF and measuring CSF Aβ1-42 alone would result in a false positive, while the Aβ1-42/1-40 corrects for this [45]. Finally, the concordance of CSF Aβ1-42/1-40 with amyloid PET is higher than for CSF Aβ1-42 alone [46], and the use of Aβ1-42/1-40 mitigates against adsorption effects that could lead to falsely low Aβ1-42 [47,48]. These studies clearly highlight the important role CSF Aβ1-42/1-40 plays in detecting Aβ pathology in AD.

Aβ1-40, Aβ1-42 and Aβ1-42/1-40 as amyloid biomarkers in blood

Building on the success of CSF Aβ1-42 and Aβ1-42/1-40 in diagnosing AD, Aβ is an attractive blood-based biomarker of AD because it easily crosses the BBB [49]. However, early investigations into the use of plasma Aβ1-42 and Aβ1-42/1-40 as predictors of future AD development showed inconsistent results, with some reporting that high plasma Aβ1-42 concentrations or a high Aβ1-42/1-40 are risk factors for AD development, while others reported the opposite, and still others reported no significant differences in plasma Aβ1-40 and Aβ1-42 between AD cases and controls [50-54]. The potential reasons for this include the following: the limited analytical sensitivity of the enzyme-linked immunosorbent assay (ELISA)-based techniques in use at the time; sub-optimal or variable sample handling protocols; and, in many cases, the use of clinical criteria for diagnosis rather than evidence for Aβ pathology.

Recent advances in immunoassay technology to detect and quantify single protein measurements have increased their analytical sensitivity and have made it possible to quantify protein biomarkers at subfemtomolar concentration levels. There have been three main developments that have allowed for this. One has been to replace the enzyme label of the detection antibody with a molecule that emits light upon an electrochemical reaction, so-called electrochemiluminescence (ECL) [55]. The second is a refinement of the basic ELISA technology, so-called single molecule array (Simoa), compartmentalizing the detection reaction within femtolitre-sized wells using magnetic beads onto which the immunocomplexes are captured, and digitalizing protein detection [56-58]. The final advancement has been the development of sensitive mass spectrometry (MS)-based assays to quantify plasma Aβ peptides [59]. These technological advances have led to breakthroughs in efforts to detect and quantify Aβ present in peripheral blood.

A study by Janelidze et al. [25], which used ultrasensitive Simoa immunoassay technology to measure plasma Aβ1-40 and Aβ1-42 concentrations, found slight but significant correlations between plasma and CSF measurements of these analytes, but not of Aβ1-42/1-40. Furthermore, plasma Aβ1-40, Aβ1-42 and Aβ1-42/1-40 were all significantly decreased in AD patients compared with controls and patients with either mild cognitive impairment (MCI) or subjective cognitive decline (SCD). This was also observed in CSF, but the differences in CSF were much more pronounced. Additionally, plasma Aβ1-42/1-40 was lower in patients with MCI compared with both SCD and controls. The results from this study are in line with those seen in Rembach et al. [60], Jessen et al. [61] and Pesaresi et al. [62] and have been replicated by Vergallo et al. [63]. In addition to observing similar results to those above, Palmqvist et al. [64] showed that plasma Aβ1-40, Aβ1-42 and Aβ1-42/1-40 can accurately predict cerebral Aβ deposition. Of particular importance is a cross-sectional study conducted by Palmqvist et al. [65], which highlights that plasma Aβ1-40, Aβ1-42 and Aβ1-42/1-40 reflect the changes seen in CSF, albeit not as dynamically, and that CSF and plasma Aβ alterations precede positive amyloid PET findings. While Chatterjee et al. [66] did not observe a significant difference in plasma
Aβ1-40 and Aβ1-42 concentrations between the Aβ-positive (Aβ+) and Aβ-negative (Aβ−) groups, perhaps due to their small sample size, they did observe a significantly lower plasma Aβ1-42/1-40 in the Aβ+ group compared to the Aβ−. Finally, in a study which observed the utility of blood biomarkers without classification of CSF and PET, Simrén et al. [67] demonstrated significantly lower Aβ1-42/1-40 in AD patients compared with MCI and controls, however no change between MCI and controls. Interestingly, Aβ1-42/1-40 was associated with longitudinal change in grey matter volume, which is more strongly seen in cognitively unimpaired (CU) individuals than impaired patients.

Similar success in blood Aβ measurements has been observed using MS, which, due to detecting analyte ions (or gas-phase-produced fragments thereof) at their specific mass-to-charge ratio with high accuracy, has a greater analytical specificity and selectivity compared with immunoassays. An important difference compared with immunoassays is that while MS methods for plasma Aβ rely on antibodies for enrichment of the low abundance of Aβ peptides, quantification in MS is antibody-independent, as the stable isotope-labelled synthetic Aβ peptide analogues, that are used as internal standards, are co-enriched with the endogenous peptides [68]. Furthermore, because samples analysed by MS are typically handled under denaturing conditions, in aqueous-organic solvents, results are less influenced by matrix effects [69-71]. Of note, Ovod et al. [72] highlighted that the half-life of Aβ in plasma is one third that of CSF Aβ. Additionally, they observed lower absolute concentrations of plasma Aβ1-42 and Aβ1-42/1-40 in the blood of Aβ+ individuals, suggesting that plasma Aβ concentrations correlate positively with CSF Aβ. Furthermore, Nakamura et al. [59] showed that plasma Aβ1-42 and Aβ1-42/1-40 accurately predicted amyloid PET positivity and negativity in two separate data sets, highlighting that plasma Aβ is inversely proportional to brain Aβ burden. Schindler et al. [73] observed similar results; however, they only saw a 10–15% change in plasma Aβ1-42/1-40 between amyloid PET-positive and PET-negative individuals, whereas in CSF, this change is 50%. Interestingly, direct (same-sample) comparison of Simoa and MS-based quantification of Aβ1-40 and Aβ1-42 in a preclinical cohort suggests that the correlation with brain amyloid pathology is higher with MS than with Simoa, at least at this stage of disease [74].

Despite this array of positive results, the contradictory results observed by other studies investigating plasma Aβ cannot be ignored. Consistent with early investigations into plasma Aβ, Giedraitis et al. [75] and Tamaoka et al. [76] reported no association between plasma Aβ1-40 or Aβ1-42 concentrations and AD pathology. Both Hansson et al. [77] and Lövheim et al. [78] agree with this finding, with Hansson et al. [77] also finding no correlation between plasma and CSF Aβ. One possible explanation for the contradictory results is the inter-study variation in pre-analytical practices [66], which has been addressed by the development of a standardized guideline for pre-analytical practices in AD blood-based biomarker research [67,79]. Importantly, discrepancies between blood and CSF biomarkers may reflect sampling issues in both. A systematic review conducted by Hansson et al. [80] in 2018, looking at the variation in pre-analytical methods for handling CSF samples prior to AD biomarker measurement, revealed a broad range of protocols was used in the 49 studies investigated. Out of the 15 variables assessed, the only two variables that remained consistent were the storage conditions (–80°C) and the lumbar puncture sampling location (L3-5). In some cases, these variations have a significant effect on the biomarkers of interest and hence on results obtained from the study. For example, CSF Aβ1-42 is significantly affected by storage tube type [81-83], and some studies have found that centrifuging CSF samples prior to analysis may cause significant reductions in CSF Aβ1-42, likely due to the high propensity of Aβ1-42 to aggregate [84,85]. While Hansson et al. [80] focussed their review towards CSF samples and have recently published an updated standardized pre-analytical protocol for measuring AD biomarkers in CSF [86], the results obtained in their 2018 review highlight the need for universal pre-analytical protocols, not only for CSF, but also for blood sample handling.

Alternatively, these contradictory results may be due to the variation in patient cohort characteristics between studies. Aβ concentrations vary depending on the patient’s stage of disease, which reflects the increasing plaque burden as the disease progresses. This, combined with the fact that Aβ is ubiquitously expressed in extra-cerebral tissues, may explain the variations in results obtained when investigating plasma Aβ concentrations. Indeed, a large proportion of plasma Aβ is not brain-derived, resulting in a much lower (10-15%) reduction in plasma Aβ1-42/1-40 compared with CSF...
A\(\beta_{1-42}\) is more neurotoxic than A\(\beta_{1-42}\) and is observed prior to AD 

section being observed prior to AD 

progression, with deposition of both peptides being observed prior to AD diagnosis. Additionally, in mouse models of familial AD (FAD), Saito et al. [92] showed that not only A\(\beta_{1-43}\) has a greater propensity to aggregate and is more neurotoxic than A\(\beta_{1-42}\), but it also accumulates in AD brains more frequently than A\(\beta_{1-40}\), observations which are supported by the findings of Welander et al. [93] and Keller et al. [94]. Furthermore, Jäkel et al. [95] observed a positive correlation between A\(\beta\) peptide length and plaque load (A\(\beta_{1-43} > A\beta_{1-42} > A\beta_{1-40}\)). These results deviate somewhat from the observations of Iizuka et al. [89], who found A\(\beta_{1-42}\) to be the major component of plaques, with A\(\beta_{1-43}\) being a minor component, and A\(\beta_{1-40}\) only being present in cerebrovascular amyloid. These differences in results are possibly due to the very small cohort size used by Iizuka and colleagues. Similarly, Perrone et al. [29] found CSF A\(\beta_{1-43}\) to have a positive correlation with A\(\beta_{1-42}\) concentrations, with CSF A\(\beta_{1-43}\) concentrations being significantly reduced in FAD mutation carriers. These studies highlight that A\(\beta_{1-43}\) plays a role in AD, albeit less well investigated.

Finally, some of the improvements in diagnostic performance recorded for plasma A\(\beta\) tests during recent years may be due to improved diagnostic work-up of the study participants so that most of them have been classified as A\(\beta^\text{+}\) or A\(\beta^\text{-}\) based on CSF or PET biomarkers. This has made it less likely that the control group contains individuals with preclinical amyloid pathology and that the AD group contains individuals with cognitive deterioration, having already ruled out non-AD neurodegenerative diseases. Studies in memory clinic or population-based cohorts without prior stratification may be due to improved diagnostic accuracy to CSF A\(\beta_{1-42}\) in AD. One reason for this is that A\(\beta_{1-43}\) has a very similar diagnostic accuracy to CSF A\(\beta_{1-42}\); hence, it is unlikely to provide additional diagnostic value over existing biomarkers [96,97]. However, A\(\beta_{1-43}\) may prove useful in differentiating between different groups of AD patients. One study observed a significantly greater reduction in CSF A\(\beta_{1-43}\), but not A\(\beta_{1-42}\), in early-onset AD compared with late-onset AD [97], while another study showed that A\(\beta_{1-43}\), but not A\(\beta_{1-42}\), could identify amnestic MCI patients who progressed to AD [98]. In addition, Lauridsen et al. [98] observed a significant decrease in CSF A\(\beta_{1-43}\) over the 2-year follow-up period, with no significant difference seen in CSF A\(\beta_{1-42}\) concentrations. It is clear that A\(\beta_{1-43}\) plays a role in AD; hence, there is a need to investigate this peptide further, particularly in blood.

**Phosphorylated tau**

Tau is a microtubule-associated protein that is a natural component of healthy, mature neurones [99]. A very small percentage of tau may be phosphorylated in healthy individuals. However, in AD, tau is 3–4 times more phosphorylated and aggregates intraneuronally into NFTs composed predominantly of p-tau [99-101]. Tau was first identified as a CSF biomarker for AD in 1993 using ELISA [102]. Since 1993, ELISA methods for measuring t-tau that detect all tau isoforms, irrespective of their phosphorylation, have been developed. Along with the 6 different isoforms of tau in the CNS, produced by alternate splicing, there are up to 85 possible tau phosphorylation sites [103]. Studies have revealed that the concentration of
p-tau in CSF accurately depicts the extent of p-tau deposition within the AD brain [104], and in contrast to t-tau, there is essentially no change in concentrations of certain p-tau species in other neurological conditions like acute stroke [105] or Creutzfeldt–Jakob disease (CJD) [106], nor in other tauopathies and neurodegenerative diseases [107-111]. This suggests that several p-tau species are specific to AD when measured in biofluids, and can be used to distinguish AD from other neurodegenerative disorders. It is thought that both p-tau and t-tau increase in CSF as a direct response to Aβ pathology, as opposed to being markers of neuronal loss, as previously assumed [88,112]. Rather, it may be the resultant tau pathology caused by Aβ-induced tau secretion that causes neurodegeneration in AD, since neurodegeneration and cognitive loss do not occur in the absence of tau [113]. This is consistent with earlier studies in mouse models, which show increases in CSF endogenous murine tau concentration without evidence of neuronal loss in APP transgenic mice [114]. In addition to phosphorylation, increasing evidence indicates that both N-glycosylation and O-glycosylation are implicated in AD, emphasized by the fact that tau carries potential N-glycosylation and O-glycosylation sites [115]. However, no established biomarkers to study the pathophysiological relevance of this in humans exist yet. In this section, we will discuss tau phosphorylated at three sites – threonine 181 (p-tau181), threonine 217 (p-tau217) and threonine 231 (p-tau231).

P-tau181, 217 and 231 as tau biomarkers in CSF

Early studies looking at CSF p-tau concentrations in AD using ELISA revealed that irrespective of which p-tau epitope was measured, p-tau is significantly elevated in AD compared with age-matched CU controls, as well as patients with non-AD dementias [109,110,116,117,118]. Further investigations into the efficacy of combining p-tau measurements with CSF Aβ42 and/or Aβ42/1-40, and CSF t-tau have led to CSF p-tau, particularly p-tau181, being included in the AT(N) criteria for AD diagnosis and the NIA-AA research framework for defining AD [9,20]. However, more recently, there has been question as to whether certain p-tau epitopes function better than others as AD biomarkers.

Of all the p-tau epitopes, immunoassays detecting CSF p-tau181 are by far the most widely studied. Unless otherwise specified, ‘p-tau’ is almost always assumed to refer to mid-region p-tau181 [119,120]. However, CSF is known to predominantly contain a mixture of both N-terminal and mid-region tau fragments, with C-terminal fragments being relatively scarce [121-123]. CSF p-tau181 has proven useful in differentiating AD from controls and other tauopathies and neurodegenerative diseases, while also predicting cognitive decline in preclinical cases of AD [124-126]. However, in 2020, two separate studies – one using ELISA [127] and the other using MS [128] – observed that CSF p-tau217 displayed a larger-fold change with AD pathology than p-tau181. A third study concluded that CSF p-tau217 serves as a better marker of cognitive decline than CSF p-tau181 [129], and a fourth study, using a novel ultrasensitive immunoassay on the Simoa platform, observed much less overlap between diagnostic groups (AD vs controls and amyloid PET-positive vs amyloid PET-negative) with p-tau217 than with p-tau181 [130]. In summary, these studies argue that p-tau217 is the superior tau pathology biomarker; therefore, it should be used more widely in clinical practice. Both Janelidze et al. [127] and Barthelemy et al. [128] observed that while CSF p-tau181 clearly distinguished AD from the non-AD groups studied, CSF p-tau217 more markedly distinguished between the groups, and it showed a stronger correlation with tau PET and amyloid PET in AD patients.

To investigate these results further, Karikari et al. [131] conducted a head-to-head comparison of novel CSF p-tau217 and p-tau181 biomarkers, containing the N-terminal amino acid 6-18 epitope (N-p-tau217 and N-p-tau181, respectively), with the performance of already established p-tau181 biomarkers, which target the mid-region epitopes (mid-p-tau181), in AD and MCI patients in three cohorts. In their two validation cohorts, N-p-tau217 and N-p-tau181 increased in MCI-AD patients, whereas mid-p-tau181 remained within normal range. Additionally, N-p-tau217 and N-p-tau181 both equally identified increased Aβ pathology and differentiated MCI-AD from non-AD MCI and Aβ CU individuals significantly better than mid-p-tau181. The performance of N-p-tau217 and N-p-tau181 was virtually indistinguishable from one another, suggesting that CSF p-tau217 may not be a more accurate biomarker for AD pathology, but rather it functions better than the p-tau181 biomarkers to which it was compared to – mid-p-tau181. Furthermore, N-p-tau217 and N-p-tau181 both increase in synchrony with Aβ pathology changes, whereas mid-p-tau181 increases at a later
disease stage [120,131,132]. Interestingly, Emeršič et al. [133] found CSF p-tau217 to also be elevated in both AD and CJD, suggesting that p-tau181 is more specific to AD, and may serve to better confirm AD diagnosis.

Studies looking at CSF p-tau231 have shown huge promise, with early investigations finding CSF p-tau231 to identify AD with 85% sensitivity and 97% specificity [118], and more recent studies observing a more prominent increase in CSF mid-p-tau231 in AD compared with a gold standard mid-p-tau181 immunoassay [120]. Of particular importance is a study conducted by Ashton et al. [134], which observed that compared with CSF p-tau181 and p-tau217, CSF p-tau231 was more sensitive to the earliest changes in parenchymal Aβ pathology before amyloid PET positivity had occurred.

P-tau181, 217 and 231 as tau biomarkers in blood
The challenges of measuring biomarkers of brain diseases in the blood have already been mentioned above. Previously, the low concentrations of tau in blood made it difficult to measure. However, the development of ultrasensitive immunoassay technologies has mitigated these difficulties [17]. Nonetheless, there remains one specific challenge which appears to be particularly problematic for tau. Tau is extremely stable in CSF, whereas in blood, it has a very short half-life (~10h) [88]. This could be due to proteases causing an increased rate of tau degradation [27,88]. Indeed, several studies investigating plasma tau clearance following hypoxic brain injury have highlighted the efficient clearance mechanisms of tau in blood [135,136]. However, it is possible to minimize tau degradation by adopting fast and efficient pre-analytical sample processing measures.

In one of the first studies of its kind, Shekhar et al. [137] attempted to quantify serum p-tau181 in a small pilot study, consisting of AD dementia, MCI and control groups. They observed an elevated concentration of p-tau181 in both the AD and MCI groups compared to controls, as well as in AD compared to MCI. Shortly after, in another pilot study, Tatebe et al. [138] attempted to quantify plasma p-tau181 in AD dementia, Down syndrome (DS) and control groups, using a novel p-tau181 Simoa assay which detects N-p-tau181. They observed a significantly higher concentration of p-tau181 in both the AD and DS groups compared to their respective age-matched controls, as well as a strong correlation between plasma and CSF p-tau181 concentrations. These findings have been further corroborated by other studies in CU individuals and those with AD dementia, MCI and non-AD dementias [139-143]. In a much larger-scale study, Mielke et al. [139] found that plasma p-tau181 was more strongly associated with Aβ and tau PET imaging than plasma t-tau, and more sensitively and specifically predicted increased brain Aβ concentrations. This was further corroborated in a recent multi-centre study conducted by Karikari et al. [143], which showed that not only can p-tau181 identify AD with high diagnostic accuracy, but it also increases minimally in individuals diagnosed with AD but who are amyloid PET-negative, and increases more prominently in individuals with decreased CSF Aβ prior to amyloid PET positivity. Moreover, Janelidze et al. [140] showed that plasma p-tau181 can accurately predict future progression to AD dementia in individuals who were initially CU. In a longitudinal study, Lantero-Rodriguez et al. [144] observed that p-tau181 accurately predicts AD pathology and discriminates between AD and non-AD pathology, at least 8 years prior to death and subsequent neuropathological diagnosis. Similarly, O’Connor et al. [145] observed, in their longitudinal study of FAD, that plasma p-tau181 concentrations were higher in mutation carriers than non-carriers from 16 years prior to estimated symptom onset. Furthermore, Moscoso et al. [146] have recently shown that longitudinal changes in plasma p-tau181 are associated with longitudinal neurodegeneration in AD-specific brain regions, as measured by FDG-PET and grey matter volume. Together, this evidence suggests plasma p-tau181 poses a promising blood-based biomarker for both AD diagnosis and for patient recruitment into clinical trials. Furthermore, it may provide longitudinal information relating to AD-specific neurodegeneration that could be employed as a treatment response measure in therapeutic clinical trials.

Studies into the utility of plasma p-tau217 in AD diagnosis began relatively recently but have had promising results. An investigation into core CSF and blood AD biomarkers in relation to amyloid PET revealed that plasma and CSF p-tau217 concentrations change simultaneously [65]. Following on from this, one cohort study found plasma p-tau217 to be increased in CU individuals with abnormal (i.e. positive) amyloid PET but normal tau PET, suggesting changes in plasma p-tau217 precede the detectability of insoluble tau aggregates by tau PET [147]. Before conclusions can be
made as to whether plasma p-tau217 will function as a useful biomarker for early AD pathology, investigations must first be conducted to compare plasma p-tau217 in AD with other neurodegenerative diseases, particularly CJD, since CSF p-tau217 was found to be increased in this condition [133].

A recent study also demonstrates the high diagnostic performance of p-tau231 in blood [148]. While at the cognitive impairment stage p-tau181 and p-tau231 are seemingly similar in diagnostic accuracy, the p-tau231 epitope begins to increase early in the preclinical stage of the disease, similar to the findings in CSF [148]. The early increase is suggested to be a response to accumulating amyloid pathology under a threshold of amyloid PET positivity.

**Neurodegeneration**

*T-tau as a neurodegeneration biomarker in CSF*

CSF t-tau in AD has been proposed to reflect the severity of Aβ-induced neurodegeneration and neuronal or axonal injury [49,140]. As with p-tau, high concentrations of t-tau have been observed consistently in AD patients [119]. Changes in CSF t-tau are not specific to AD, as t-tau is also increased in other cases of neuronal injury, including stroke, traumatic brain injury (TBI) and CJD [49]. However, recent studies have suggested that the t-tau being measured in AD biofluids is secreted alongside p-tau, and reflects Aβ-induced tau secretion from living neurones [112]. While these neurones will eventually degenerate and die, the t-tau being measured in AD is not thought to be a direct marker of this [149]. In contrast, the high CSF t-tau with normal CSF p-tau, measured in conditions like stroke, TBI and CJD, is a direct result of massive neuronal death, and in these cases, t-tau is a marker of neuronal injury [149]. Therefore, in combination with raised p-tau, increased CSF t-tau does reflect AD pathology, rather than simply being a non-specific effect of neuronal damage.

*T-tau as a neurodegeneration biomarker in blood*

One of the earliest studies investigating plasma t-tau in AD yielded discouraging results, reporting no significant increase in plasma t-tau being seen in AD compared to non-AD dementias [150]. However, this study was most likely limited by the low sensitivity of the ELISA technology used. Since the development of more sensitive ELISA technology, particularly through the use of Simoa, numerous studies have reported increased plasma t-tau concentrations in AD [17,136,151,152], with some observing a strong correlation between plasma and CSF t-tau [151], and others observing a weak [152] or absent correlation [136]. Furthermore, one study reported reduced plasma t-tau concentrations in AD [153]. While the general consensus is that plasma t-tau concentrations increase in AD, Zetterberg et al. [136], Dage et al. [17] and Mattsson et al. [152] all observed significant overlap in plasma t-tau ranges between their AD and non-AD groups, including age-matched CU controls. An additional study found an association between elevated plasma t-tau concentrations and cognitive decline; however, this was independent of elevated brain Aβ [154]. It is possible that the inconsistent results thus far in measuring plasma t-tau may be due to the currently available assays measuring a form of tau that is particularly susceptible to protease degradation [140]. Interestingly, Pase et al. [155] showed in a multi-centre study that plasma t-tau can act as a risk-stratifier for progression to AD dementia. One strength of this study was post-mortem correlation with tau pathology observed in a subset of the cohorts investigated. Nonetheless, the current evidence suggests plasma t-tau may not be a useful diagnostic blood biomarker for AD, but high concentrations may provide prognostic evidence of incident neurodegeneration, similar to the performance of a t-tau assay using N-terminal anti-tau antibodies which were recently described [156,157].

*NfL as a neurodegeneration biomarker in CSF*

Neurofilaments are an important structural component of the neuronal cytoskeleton [158], and one specific subunit of neurofilaments, NfL, is primarily expressed in large-calibre myelinated axons [159]. Increased CSF NfL concentrations have been associated with white matter lesions and subcortical brain damage in AD [160], as well as other neurodegenerative and non-neurodegenerative diseases [161]. Hence, NfL is not specific to AD, but it functions as an excellent biomarker for neuronal death and axonal loss. Furthermore, CSF NfL concentrations are significantly increased in AD compared to CU controls, serving as an accurate marker of progression from MCI to AD and reflecting neurodegeneration independent of Aβ pathology [119,161,162,163,164].
**NfL as a neurodegeneration biomarker in blood**

Interest in NfL as a blood biomarker came about in relation to longitudinal studies, due to blood being easier to sample serially than CSF. Following the development and validation of the first assay to reliably measure serum NfL concentrations in 2013 using ECL [165], more sensitive assays have been developed using Simoa technology [166]. Indeed, in a comparison between three analytical platforms – ECL, standard ELISA and Simoa – Simoa was found to be the most sensitive at quantifying serum NfL concentrations [167]. Using this ultrasensitive Simoa assay, Mattsson et al. [166] showed for the first time that plasma NfL correlates with CSF NfL, but also with other hallmarks of AD. Furthermore, blood NfL has high diagnostic accuracy for AD, and it is increased prior to symptom onset, making it a promising biomarker for neuronal injury in this disease. These results have since been corroborated by the vast majority of studies across both sporadic and familial disease [168-173], with Schultz et al. [172] observing that similar to CSF NfL, plasma NfL concentrations correlate with white matter damage in the brain, and Ashton et al. [173] demonstrating that plasma NfL correlates strongly with the severity of NFT pathology in AD seen in post-mortem analysis. Due to the lack of specificity of NfL for AD, its value is unlikely to be in differentiating AD from other neurodegenerative diseases, but rather to distinguish neurodegeneration (including AD) from non-degenerative causes of cognitive impairment (e.g. primary psychiatric causes) [174,175]. Additionally, it can be used as a non-invasive screening tool to identify patients at risk of cognitive decline, as well as a dynamic biomarker to monitor treatment efficacy and to track disease progression.

**T-tau vs. NfL as neurodegeneration biomarkers in AD**

Both t-tau and NfL are useful markers of neurodegeneration in AD. CSF t-tau has the added advantage of correlating with Aβ pathology changes [88,112], which is not the case for CSF NfL [176]. However, the evidence presented suggests that NfL translates better into a blood biomarker for AD neurodegeneration than t-tau. Indeed, plasma NfL is robust to even a 48-h delay in centrifugation of whole blood, in contrast to the known issues with plasma tau being susceptible to degradation by proteases [177]. Therefore, it is possible that plasma NfL may replace t-tau in an initial blood-based diagnostic work-up for AD to confirm the presence of neurodegeneration, followed by CSF t-tau being used in tertiary centres to aid the confirmation of Aβ-induced neurodegeneration.

**An integrated hypothesis for AD pathogenesis**

AD is an extremely complex disease. To date, research has shown that microglia are the primary mediators of neuroinflammation in AD brains. However, the role of neuroinflammation in AD pathogenesis remains highly debated. Some papers argue that neuroinflammation is neuroprotective, designed to clear Aβ plaques, while others argue that it is neurotoxic by promoting AD progression through cytokine release, phagocytosis of synapses and consequent neurodegeneration [178-182]. Furthermore, one review argues that microglia play both a neuroprotective and a neurodegenerative role, depending on the stage of AD [183].

In their recent review, Edwards [113] proposed a unifying hypothesis for AD pathogenesis, whereby they suggest the primary driver for AD progression following amyloid plaque deposition and Aβ-induced synaptic damage is an inadequate microglial response. The authors introduce the idea that the magnitude with which microglia respond increases with disease progression, proposing that microglia are responsible for removing damaged synapses and hence play a neuroprotective role in AD. Consequently, this protective role of microglia prevents damage from propagating down the axon, thus breaking the cycle of Aβ-induced synaptic dystrophy. This provides an alternative explanation for why some elderly individuals without dementia are found to have a similar burden of plaques and tangles to that seen in patients with clinically advanced AD at post-mortem [184]. In essence, the plaque load an individual can tolerate prior to neurodegeneration occurring may be dependent on the genetic characteristics of their microglia, which determines the rate at which damaged synapses are phagocytosed [113].
proteins, e.g. C3). Tests for some of these proteins have shown promising results in CSF studies [185-188], but translating them into blood tests will be difficult. Investigations have revealed that neurogranin [189] and soluble TREM-2 [190] do not function well as blood biomarkers for AD. Additionally, YKL-40 was found to be significantly increased in the AD and MCI groups compared to controls [191]. However, there was a significant overlap between the groups, and it did not correlate with CSF Aβ1-42 or CSF p-tau181. The proteins discussed by Edwards [113] are highly expressed in extra-cerebral tissues. Consequently, any brain-derived signal in blood is likely to be overwhelmed by release of proteins from other tissues.

Conclusion

In conclusion, we have considered biomarkers which have the potential to be translated into blood biomarkers for AD. In particular, plasma p-tau181 and NfL show huge promise, with both having significant evidence highlighting that assays for these markers work in both research laboratories and in specialist settings. Plasma NfL could potentially screen for a range of pathologies, not just AD, and act as a therapy response marker. As plasma p-tau181 reflects both amyloid and tau pathology, it would be applicable in differential diagnoses compared to other dementias, as well as potentially functioning as a therapy response marker, given the changes seen in longitudinal studies. However, prior to clinical implementation, plasma p-tau181 requires further analysis comparing assays targeting N-terminal and mid-region p-tau181.

Plasma Aβ would have value in early, or even pre-symptomatic, screening and recruitment to clinical trials. However, it would need cautious interpretation due to the prevalence of amyloid positivity increasing with age in individuals who will not develop AD in their lifetime. Nonetheless, the inter-laboratory variation in pre-analytical protocols has led to inconsistent plasma Aβ results. Therefore, a new standardized guideline for pre-analytical variables in AD blood-based biomarker research must be established for worldwide use, with implications for protocols which deviate from the proposed guideline.

Finally, plasma p-tau217 and p-tau231 studies look promising. However, more head-to-head comparisons of assays measuring different phospho-forms of tau, using identical methods, are needed to reach a conclusion on which of these biomarkers most robustly separate AD from non-AD neurodegenerative dementias.

Given the rapidly changing field, it is unclear which of these biomarkers will ultimately prove most useful to answer different clinical and research questions. As is often the case with technical advances, there are associated ethical issues, including the fact that the ease of testing with blood-based measures may lead to inappropriate use, such as direct-to-consumer predictive testing without counselling or support being available. However, what is clear is that blood-based biomarkers are set to transform both clinical and research practice – and will have wide, even global, applicability.

Conflict of interest

DOTA has no conflicts of interest. JMS has received research funding and PET tracer from AVID Radiopharmaceuticals (a wholly owned subsidiary of Eli Lilly); has served as a consultant at advisory boards, or at data monitoring committees consulted for Roche, Eli Lilly, Biogen, Merck, GE and Axon Neuroscience SE; and is Chief Medical Officer for Alzheimer’s Research UK. KB has served as a consultant at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu, Julius Clinical, Lilly, MagQu, Novartis, 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. NCF has served as a consultant at advisory boards, or at data monitoring committees for Roche, Biogen and Ionis. HZ has served at scientific advisory boards for Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Piteon Therapeutics, Nervgen, AZTherapies and CogRx; has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program.

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