Biofluid-based biomarkers for Alzheimer's disease-related pathologies - an update and synthesis of the literature

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ABSTRACT

The past few years have seen an explosion in sensitive and specific assays for cerebrospinal fluid (CSF) and blood biomarkers for Alzheimer's disease (AD) and related disorders, as well as some novel assays based on pathological seed-induced protein misfolding in patient samples. Here, I review this exciting field that promises to transform dementia diagnostics and disease monitoring. I discuss data on biomarkers for amyloid β (Aβ) and tau pathology, neurodegeneration, and glial activation, mention the most promising biomarkers for α-synuclein and TDP-43 pathology, and highlight the need for further research into common co-pathologies. Finally, I consider practical aspects of blood-based biomarker-supported AD diagnostics and emphasize the importance of biomarker interpretation in a full clinical context.

RESEARCH IN CONTEXT

Systematic review: In preparing my plenary lecture for the Alzheimer's Association International Conference 2021 and this accompanying perspective article, I used knowledge gained over many years in collaborative projects with colleagues all over the world. I also searched PubMed for fluid-based biomarkers for each of the following pathologies: amyloid β (Aβ), tau, and neurodegeneration, as well as glial activation, α-synuclein and TDP-43. This is a narrative rather than a systematic review.

Interpretation: The fluid biomarker field of neurodegenerative dementias has developed enormously during the past few years. Valid CSF tests for most of the Alzheimer's disease (AD)-related pathologies are in clinical use. Some of the most promising blood tests are now also entering clinical practice. The full set of CSF biomarkers inform on the clinical importance of AD and non-AD pathologies in cohort studies and clinical practice. Plasma phosphorylated tau and amyloid β peptides may be useful as early biomarkers to determine who needs further evaluation before start of disease-modifying treatments, as well as to monitor these treatments.

Future directions: More data on diverse and real-world clinical populations are needed. We also need improved biomarkers for non-AD pathologies, especially in blood. We should develop appropriate use criteria for the existing blood biomarkers and do all we can to implement them in clinical practice in a thoughtful manner to best serve the patients.

1. Introductory narrative

Alzheimer's disease (AD) is a progressive neurodegenerative dementia-causing disease in which disease-modifying treatments, likely to be the most effective in early disease stages (or even in primary prevention settings), are now being developed at a very rapid pace. AD-related pathologies appear in the brain decades before symptom onset. Making a clinical diagnosis in this early phase is difficult or (in the case of pre-clinical disease) impossible. Genetic risk is important (causative mutations for autosomal dominant disease have been identified, and personalized polygenic risk scores are available for sporadic disease), but we need biomarkers to determine the onset, profile, and intensity of AD-related brain changes in individual patients, independent of genetic background. In parallel with the development of novel treatments, intense research has given us fluid and imaging biomarkers that that may serve this purpose. In the current Perspective paper, which is a summary of the plenary talk that I gave during the Alzheimer's Association International Conference (AAIC) 2021, I give an updated account of fluid biomarkers for the following AD-related pathologies: amyloid β (Aβ), tau, and neurodegeneration (*i.e.*, the classical ATN concept[1]), as well as glial activation, α-synuclein and TDP-43. I discuss their strengths and weaknesses, and how they could be used in clinical trials and practice to help determine whether AD-related brain changes are present in a person and how they may change in response to novel disease-modifying drug candidates. Imaging biomarkers are not covered but mentioned when relevant to the fluid biomarker context. Biomarkers for AD-related synaptic degeneration and loss were recently summarized elsewhere.[2, 3]

2. Methods and results

2.1 Amyloid β

The first discernible pathology in AD is the accumulation of 42 amino acid-long amyloid β (Aβ) protein in extracellular plaques in the brain, occurring decades before clinical onset. Biomarker studies suggest that Aβ accumulation is closely accompanied by tau phosphorylation, potentially as a pruning or neuronal dedifferentiation mechanism, and microglial and astrocytic activation, *i.e.*, a general tissue reaction to Aβ, which eventually (many years later) translates into frank neuronal degeneration and loss.[4] Cerebrospinal fluid (CSF) concentrations of Aβ42 in AD patients are lower than in Aβ-negative controls and correlate with the amount of plaque pathology determined at autopsy,[5] or by amyloid positron emission tomography (PET).[6] The most likely mechanism is that Aβ42 is retained in the brain

tissue in people who accumulate Aβ, leaving less soluble Aβ42 in the CSF. However, it is also possible that microglia, activated by Aβ pathology, could take part in depleting the brain interstitial fluid from Aβ42 and thereby contribute to the CSF Aβ42 reduction.[7] The latter hypothesis could be tested in mouse models of AD by depleting them of microglia using established protocols[8] and determine if this prevents from CSF Aβ42 reduction when plaques appear.

In neuroinflammatory conditions (autoimmune or infectious), as well as in CSF dynamics disorders, *e.g.*, normal pressure hydrocephalus, CSF Aβ42 concentration is reduced.[9-11] However, in these conditions, Aβ42 is lowered together with Aβ40 and Aβ38, as well as soluble amyloid precursor protein fragments in the CSF, whilst plaque pathology in AD results in a selective Aβ42 reduction. The solution to the problem of non-specific Aβ reduction, not related to amyloid plaque pathology, is to create a ratio of 42 to 40 or 38 amino acid-long Aβ (Aβ42/40 or Aβ42/38).[12] CSF Aβ42/40 is the most specific biofluid-based Aβ plaque pathology biomarker that we have. It shows almost 100% concordance with amyloid PET and, from a diagnostic standpoint, CSF Aβ42/40 and amyloid PET can be used interchangeably to determine if a patient has Aβ pathology or not.[12]

CSF Aβ42/40 shows a distinct bimodal distribution in people older than 50 years of age (unpublished results from clinical laboratory practice at Sahlgrenska University Hospital over four years). Young people (below 40 years of age) all have a normal ratio (if they do not carry an autosomal dominant AD-causing mutation), but from the age of 40 and onwards some people drop in their ratio as a sign of onset of Aβ accumulation. This is related to *APOE* genotype, with each ε4 allele being associated with approximately 10 years earlier onset of the Aβ accumulation.[13] The drop occurs quite fast (over a year or two) and from a clinical standpoint, we often recommend resampling if the results are close to the cut-point for positivity; CSF re-analysis a year or two later may give a clearer result.

How about blood tests for Aβ pathology? Plasma Aβ concentrations have been possible to measure since the 1990s, but no consistent pattern in AD has been seen.[14] In 2017, Ovod *et al*. published an immunoprecipitation mass spectrometry (IP-MS) method in which all Aβ from plasma is extracted and subjected to MS-based quantification.[15] A clear group-level reduction in plasma Aβ42/40 in amyloid PET-positive people could be seen with an area under the curve of almost 0.9. These results have been replicated in independent studies using similar

methods,[16, 17] and immunochemical tests, easier to implement in clinical practice, with similar diagnostic performance have been developed.[18]

The major problem with plasma Aβ42/40 as an Aβ pathology test is robustness. This is a vague but at the same time quite practical concept that was adopted by clinical chemistry from statistics. A statistical test is defined as "robust" if the α risk (the probability of rejecting the null hypothesis – the hypothesis of no difference or effect when there is none) has little variation when the conditions for applying the test are not fully met. For a clinical chemistry test to be considered robust, the total pre-analytical and analytical error must be substantially lower than the percent fold change observed in the condition to be detected. The problem with plasma Aβ42/40 as an Aβ pathology test is the small fold change between Aβ-positive and -negative individuals (a 10-15% reduction compared with 50% in CSF).[19] The explanation is that the Aβ pathology-related reduction in plasma Aβ42/40 occurs on top of peripheral Aβ (Aβ produced in extracerebral tissues not affected by Aβ pathology). In short, plasma Aβ42/40 is a less robust Aβ pathology biomarker than CSF Aβ42/40 for biological reasons. It will therefore be a challenging test to standardize and maintain stable in clinical laboratory practice. Random variation in the measurements, and especially upward or downward drifts in the assay (*e.g.*, related to small changes in the calibrators, which are difficult to avoid), could result in significant numbers of people moving across the cut-point for positivity and bias across cohorts, as recently described.[20] Stringent pre-analytical and analytical protocols can help mitigate the problem, but given the biological reasons for lack of robustness, we should also look for other and more robust blood biomarkers for Aβ pathology.

2.2 Tau

The aggregation of hyperphosphorylated forms of the axonal protein tau in the cell soma and proximal dendrites, forming neurofibrillary tangles, is a key pathological feature of AD. Total tau (T-tau) and phosphorylated tau (P-tau) have been proposed, together with CSF Aβ42/Aβ40 ratio, as core biomarkers for biologically defining AD,[1] and are used in clinical laboratory practice around the world. Both CSF T-tau and P-tau concentrations reflect AD-related pathophysiology but do not detect non-AD tauopathy.[21] The most likely explanation for this is that the increased CSF levels of tau in AD are due to increased phosphorylation and secretion of tau from neurons as a neuronal response to Aβ exposure.[22-24] Tau can be phosphorylated at several amino acids, and assays that quantify different P-tau forms (*e.g.*, P-tau181, -217 and -231) in CSF produce results that correlate strongly with each other and

show similar diagnostic performance.[25, 26] P-tau increase has been seen in CSF from newborn with active synaptic pruning.[27] Further, tau phosphorylation increases upon sleep deprivation[28] and is an important mechanism in the downregulation of neuronal activity that occurs in hibernation.[29] Together, these data suggest that tau phosphorylation may be a physiological mechanism related to brain plasticity processes. Maybe Aβ plaques, directly or indirectly (via microglia and/or astrocytes), highjack this mechanism, giving a constant pruning signal to synapses in their vicinity, which results in tau phosphorylation and axonal destabilization and retraction.

We still lack quantitative assays for hyperphosphorylated tau (assays that require multiple phosphorylations on the same tau molecule to generate a signal), which could potentially be more related to pathological tau phosphorylation than the P-tau forms that we are measuring now using assays with one antibody specific against a particular phosphorylated tau epitope and another antibody against a non-phosphorylated epitope in the N-terminus or mid-domain of the molecule. Regarding positive biofluid-based biomarkers to detect non-AD tauopathies, *e.g.*, progressive supranuclear palsy and some forms of frontotemporal dementia, we still lack such; this is another field in need of further research.

Several research groups have developed very sensitive P-tau assays for use as blood biomarkers for AD. Mielke *et al*. originally demonstrated a correlation between P-tau181, and amyloid and tau PET, which indicates that plasma P-tau181 detects brain AD pathology.[30] These findings were replicated by Palmqvist *et al*., demonstrating that plasma P-tau181 correlates with amyloid PET positivity and CSF P-tau181.[31] Interestingly, in this study, the change in plasma P-tau181 occurred before amyloid PET, but after CSF and plasma Aβ42, *i.e.*, already at sub-PET threshold Aβ pathology.[31] Thus, plasma P-tau181 might be useful both diagnostically to detect early Aβ-related tau pathophysiology, as well as for disease staging (albeit without anatomical precision, which is a general limitation of all fluid-based biomarkers). Recent large validation studies show similar results,[32-36] corroborating plasma P-tau as a robust blood biomarker for AD pathology that should be relatively easy to standardize and implement in clinical laboratory practice. Additional assays specific for tau phosphorylated at amino acid 217 or 231 have been developed.[37-39] P-tau231 may be the earliest marker, whilst P-tau217 often ranks the highest regarding diagnostic performance. Nevertheless, most studies suggest that these different P-tau tests are more similar than different,[40] which bodes well for successful clinical implementation. (In clinical chemistry,

the importance of standardization is often emphasized. However, for individual patients, especially in cases with unexpected or unusual results, where assay interference may be suspected, it is great if other assays are available for confirmation; these assays do not necessarily have to be standardized to each other.)

An emerging use of plasma P-tau is to detect and monitor effects on tau pathophysiology by anti-Aβ antibodies in clinical trials. During AAIC 2021, Lilly reported reduced plasma P-tau217 concentration in response to donanemab treatment (unpublished results), and during the Clinical Trials on Alzheimer's Disease (CTAD) conference in November 2021, similar results (in this case P-tau181 reduction) were presented for aducanumab (unpublished results).

2.3 Neurodegeneration

For many years, CSF neurofilament light (NfL) has been used as a neuroaxonal injury marker in amyotrophic lateral sclerosis, frontotemporal dementia, and multiple sclerosis.[41, 42] Taking all published results into account, NfL now appears like the most promising neurodegeneration marker across the neurodegenerative dementias. The biomarker can be measured in both CSF and plasma (or serum), and virtually all CSF findings have been replicated in blood with sensitive assays.[43] The highest NfL levels are seen in frontotemporal, vascular and HIV-associated dementias.[44] However, the findings in familial AD are also very clear; mutation carriers show a sudden change in their blood NfL levels around a decade before expected clinical onset, which probably marks the onset of neurodegeneration, and the higher the increase, the more rapid clinical disease progression.[45-47] In sporadic AD, there is a clear association of increased plasma NfL concentration with Aβ and tau positivity, as well as with longitudinal neurodegeneration as determined by MRI, but with a larger overlap across groups than in familial AD,[48] most likely due to the multitude of neurodegenerative changes that may result in NfL increase in people older than 70 years of age. The dynamics of NfL changes are similar in CSF and blood; following acute brain injury, CSF and blood NfL concentrations reach their maxima after around 2 months and their apparent half-lives are 2-3 months.[49] In spinal muscular atrophy, the biomarker normalises within a few months following initiation of successful antisense oligonucleotide treatment.[50] Antisense-mediated silencing of huntingtin did not translate into any discernible clinical benefit and CSF NfL did not normalize (in fact, it increased in the high-dose group, potentially pinpointing a side-effect).[51] It has become clear that NfL might be a very slow biomarker in some conditions; it took 2-3 years for CSF NfL concentration to decline in children with enzyme replacement

therapy against neuronal ceroid lipofuscinosis.[52] In anti-Aβ antibody trials, attenuated increases of CSF NfL have been reported,[53, 54] which may indicate a positive effect on neurodegeneration by the treatment, although longer and larger studies are needed.

2.4 Glial activation

In CSF, numerous biomarker candidates related to astrocytic and/or microglial activation have been examined and there is a vast literature showing changes in biomarkers (*e.g.*, YKL-40, a glycoprotein expressed in both astrocytes and microglia, and the soluble form of TREM2, which is a microglia-specific protein) reflective of these processes soon after onset of Aβ buildup in the brain.[55] A recent study suggests that some microglial and astrocytic proteins increase already in pre-amyloid disease stages (according to CSF Aβ42/40 ratio or amyloid PET).[56] One interpretation of this finding is that microglial and astrocytic activation may play a role in the onset of Aβ deposition. An alternative interpretation is that microglial and astrocytic activation markers may be more sensitive to Aβ accumulation than the Aβ biomarkers themselves. For glial activation biomarkers, blood tests are difficult, due to high extra-cerebral expression of many of the proteins, making the blood tests less reflective of brain changes. However, one biomarker shows promise in this context: glial fibrillary acidic protein (GFAP). The strongest expression of this protein is seen in brain astrocytes and its blood level is strongly reflective of Aβ accumulation in the brain.[57, 58] In fact, the association with Aβ pathology is stronger for plasma GFAP than CSF GFAP.[57, 58] This is unusual for a fluid biomarker for AD and may reflect direct release of the protein into the bloodstream by astrocytic end-feet in the neurovascular unit and/or stability issues for GFAP in CSF.

2.5 α-Synuclein and TDP-43

Misfolding of α-synuclein plays a major role in the development of common neurodegenerative diseases, such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB).[59] α-Synuclein is the main constituent of Lewy bodies. TAR DNA-binding protein 43 (TDP-43) is another inclusion-forming protein that is frequently seen in some forms of frontotemporal dementia and in amyotrophic lateral sclerosis.[60] Further, both these pathologies are often seen together with classical AD pathology and seem to contribute to late-life cognitive decline.[61] There are currently no established imaging biomarkers for α-synuclein or TDP-43 inclusions, and it has been difficult to develop fluid biomarkers that are pathology-specific; both proteins can be measured in biofluids, but there is no correlation with pathology nor reproducible group differences,[62, 63] except for a slight decrease in CSF α-

synuclein concentration in PD.[64] Nevertheless, the fact that α-synuclein oligomers may spread in a prion-like manner has sparked the idea that seeding aggregation assays, such as real-time quaking-induced conversion (RT-QuIC) or protein-misfolding cyclic amplification (PMCA), could be used to qualitatively detect pathological forms of α-synuclein in CSF. Studies analyzing lumbar CSF with RT-QuIC or PMCA of α-synuclein have been able to distinguish synucleinopathies from non-synucleinopathies with excellent diagnostic accuracy and neuropathological confirmation.[65, 66] Recently, a proof-of-concept study using a similar approach to detect TDP-43 seeds in lumbar CSF with promising results in need of replication was published.[67]

2.6 The AlzBiomarker database

The AlzBiomarker database, developed in a collaboration between the ALZFORUM team and my research group at the University of Gothenburg, organizes data on fluid biomarkers for AD (https://www.alzforum.org/alzbiomarker). Biomarker measurements are curated from published studies and meta-analyzed. Versions 1.0 through 2.1 contained studies comparing measurements in AD patients with cognitively healthy individuals and studies comparing progressive mild cognitive impairment (MCI) with stable MCI.[14] Version 3.0, which was just released, includes cross-disease comparisons of biomarker levels in AD and non-AD neurological conditions and covers both biomarkers reviewed here and additional candidate biomarkers in development.

3. Conclusions

From this review, it is possible to conclude that we have well-replicated ATN and glial activation biomarkers in CSF and blood (Table 1). There are also promising and well-replicated results on synuclein pathology markers in CSF through assays based on seeded aggregation of recombinant α-synuclein (qualitative) in patient samples. Pilot data suggest that this approach may work to detect TDP-43 pathology as well, although more results are needed before a definitive conclusion on this can be drawn. A lot of work has been performed on standardization and implementation of CSF biomarkers for AD-related processes in clinical laboratory practice,[68, 69] and similar work is ongoing for the most promising blood tests (the CSF T-tau, P-tau, Aβ42/40 and NfL tests are up-and-running in clinical laboratory practice since years around the globe; plasma Aβ42/40, P-tau181, and NfL are available for clinical use in some laboratories in North America and Europe).[70] The whole set of CSF biomarkers should inform on the clinical importance of AD and non-AD pathologies in

clinical cohort studies (established AD biomarkers can be examined in parallel with new biomarkers for glial activation and α-synuclein and TDP-43 pathology). The blood biomarkers should be useful as additional diagnostic tools when patients undergo initial evaluation, *e.g.*, in primary care, prior to full evaluation at specialist clinics to determine who is likely to benefit from disease-modifying treatment. The blood biomarkers may also be useful to optimize drug selection and dose finding (*i.e.*, the right drug at the right dose to the right patient as evidenced by biomarker normalization, *e.g.*, a reduced plasma P-tau concentration 3-6 months after initiation of treatment with an anti-Aβ antibody), and to detect side effects (*e.g.*, plasma NfL increase in patients with a neurotoxic drug response). To facilitate clinical implementation, more studies on diverse and real-world clinical populations are needed. We should develop appropriate use criteria for blood biomarkers and de-dramatize their use; the new blood biomarkers for AD are simply additional tools in the diagnostic toolbox, which need to be interpreted in a full clinical context (this is a given when considering the use of clinical chemistry tests in other fields of medicine). It may also be worth mentioning that AD is not a condition that fulfils the Wilson and Jungner classic screening criteria,[71] at least not yet, and hence the use of the blood biomarkers for screening purposes outside clinical trials cannot be recommended. Finally, we need improved biomarkers for some of the non-AD pathologies, *e.g.*, non-AD tauopathy.

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Conflicts of interest

HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, Annexon, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Pinteon Therapeutics, Red Abbey Labs, Passage Bio, Roche, Samumed, Siemens Healthineers, Triplet

Therapeutics, and Wave, 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 (outside submitted work).

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Table 1. Biomarkers for Alzheimer's disease-related pathologies

Biomarker	Matrix	Methods	Process
Aβ42/40 ratio	CSF and plasma	Mass spectrometry assays Immunoassays	Cerebral Aβ pathology
P-tau	CSF and plasma/serum	Mass spectrometry assays Immunoassays	Neuronal tau phosphorylation and secretion
NfL	CSF and plasma/serum	Immunoassays	Neurodegeneration
GFAP	CSF and plasma/serum	Immunoassays	Astrocytic activation
α-Synuclein seeds	CSF	Assays based on seeded amplification	α-Synuclein pathology
TDP-43 seeds	CSF	Assays based on seeded amplification	TDP-43 pathology

Abbreviations: Aβ, amyloid beta; P-tau, phosphorylated tau; NfL, neurofilament light; GFAP, glial fibrillary acidic protein; TPD-43, TAR DNA-binding protein 43.