From cerebrospinal fluid to blood: the third wave of fluid biomarkers for Alzheimer's disease

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Abstract

The past five years have seen an enormous development in the field of fluid biomarkers for Alzheimer's disease (AD) and related disorders. The molecules that constitute the foundation for the CSF tests for the classical AD pathologies are now being explored as potential blood-based biomarkers, thanks to the recent implementation of ultrasensitive measurement technologies in academic and clinical laboratories worldwide. The current blood-derived data are still less clear than those obtained using CSF as the sample type but independent research suggests that there are biomarker signals in blood that relate to plaque and tangle pathologies in AD, which are relevant to explore further. Additionally, neurofilament light has emerged as the first robust blood-based biomarker for neurodegeneration in a broad range of central nervous system diseases. Here, we briefly recapitulate the first and second waves of fluid biomarker analysis in AD, *i.e.*, the development and validation of established and novel CSF biomarkers for the disorder, respectively, followed by a focused discussion on blood-based biomarkers for AD, which we consider the third wave of fluid biomarker analysis that hopefully will gain further momentum during the coming five years.

Introduction

The best established fluid biomarkers for Alzheimer's disease (AD) are cerebrospinal fluid (CSF) concentrations of total tau (T-tau), phospho-tau (P-tau) and the 42 amino acid form of amyloid β (Aβ42) [1]. The discovery and validation of these biomarkers and the development of robust tests for them may be described as the first wave of fluid biomarker analysis in AD research. During the past five years, it has been confirmed that CSF Aβ42 indeed is a reliable marker of amyloid (senile plaque) pathology in the brain (as determined at autopsy or through amyloid positron emission tomography [PET] studies), especially when measured in a ratio with CSF Aβ40 [2]. For CSF T-tau and P-tau, the interpretation is less clear; tau markers are robustly increased in AD CSF [1], but the exact mechanism remains unclear [3]. Some data suggest that neurons exposed to Alzheimer-associated factors such as Aβ may increase their secretion of both tau proteins [4]. Neurons who respond in this way may eventually accumulate tangle pathology and degenerate. In spite of these uncertainties, the diagnostic performance and clinical utility of CSF T-tau, P-tau and Aβ42 are undisputed: new diagnostic algorithms including CSF biomarkers have been formulated [5], automated routine clinical chemistry assays for the markers are now becoming available [6] and standardization efforts to harmonize assays are well underway; reference methods for Aβ42 have been formally certified by the Joint Committee for Traceability in Laboratory Medicine (JCTLM database accession numbers C11RMP9 and C12RMP1) [7, 8] and validated against amyloid PET [9], and a reference material for CSF Aβ42 is soon to be certified and released [10]. Similar work is ongoing for CSF tau biomarkers.

During the past five years, a number of additional CSF biomarkers for AD-related pathological processes have become available (the second wave). These include neurofilament light (NF-L) as a marker of neurodegeneration [11], neurogranin (Ng) as a marker of synapse dysfunction and/or loss [12], and sTREM2 and YKL-40 as markers of microglial and astrocytic activation [13, 14]. These biomarkers have been extensively reviewed elsewhere [1] and updated meta-analyses regarding their association with AD can be found in the AlzBiomarker database (http://www.alzforum.org/alzbiomarker).

Here, we will focus on what may be considered the third wave of fluid biomarker analysis in AD: the development of blood-based biomarkers for AD-related pathologies, which we believe will gain further momentum during the coming five years.

Methodological considerations of relevance to blood-based biomarker measurements in neurodegeneration

Blood as a biomarker matrix

Whereas CSF is a well-established sample type for the analysis of biomarkers for neurodegenerative diseases (it communicates freely with the brain interstitial fluid that bathes the neurons and has relatively low turnover and protease activity), blood has emerged more recently after decades of relatively disappointing results. Blood communicates with the brain across the blood-brain barrier, via lymph vessels [15] and through the glymphatic system [16]. This interchange, however, is less direct than for CSF and there are several challenges, both biological and technical, with the measurement of central nervous system (CNS)-related biomarkers in blood. First, a biomarker that has its origin in the CNS has to cross the blood-brain barrier in order to be detected in the periphery and, if the concentration is low in CSF, it will be even lower in the blood due to the blood:CSF volume ratio causing a substantial dilution of the analyte. Second, if the biomarker is not specific for the CNS but also expressed in peripheral tissues, the contribution from CNS will potentially drown in the high biological background caused by non-CNS sources (a good tool to assess the risk for this is the publicly available web-based Human Protein Atlas, http://www.proteinatlas.org/, which presents mRNA and protein expression in 44 different human tissues of close to 20,000 proteins) [17]. Third, the huge amount of other proteins in blood (*e.g.*, albumin, immunoglobulins, antitrypsin, transferrin, haptoglobin and fibrinogen) introduces analytical challenges due to possible interference [18]. Fourth, heterophilic antibodies may be present in blood, which may interfere in immunoassays [19]. Fifth, the analyte of interest may undergo proteolytic degradation in plasma and clearance in the liver or by the kidneys that may introduce variation [20]. Finally, there may be additional pre-analytical factors that may be more relevant for blood- than CSF-based biomarkers, including diurnal variation and influences of, *e.g.*, food intake and medication.

Ultrasensitive measurement techniques

Many, but not all, of the challenges reviewed above may be overcome with more sensitive assays with adequate blocking of heterophilic antibodies and improved pre-analytical standardization. Most biomarker assays of relevance to AD are immunochemical, *i.e.*, utilize

antibodies to quantify a substance in a sample. The most common assay format is the sandwich enzyme-linked immunosorbent assay (ELISA) in which the target analyte is captured between two antibodies in a complex and one of the antibodies carries a signal generator, *i.e.*, an enzyme that converts a substrate into a detectable form (coloured, fluorescent or luminescent), which, in combination with a calibrator curve (derived from artificial samples with known analyte concentrations), allows for quantification of the analyte of interest. ELISA is a theme with many variations, such as the choice of signal generator where the enzyme can be replaced by, *e.g.*, a fluorophore or a DNA-based detection system.

The technical issues are mainly a question of antibody sensitivity and specificity. In theory, if the time for the enzyme reaction is simply extended, this should increase the sensitivity of the assay. However, the substrates used are inherently unstable and therefore produce signal even in the absence of enzyme. This leads to a technical background signal that can mask the signal generated by the sandwich complex, making quantification difficult at low concentrations. In the end, the ability of the sandwich complex to correctly represent the concentration of the biomarker in a sample strongly depends on the quality of the antibodies used. If the antibodies cross-react with other substances, a signal can be measured even in the absence of the target analyte. Since the blood is much denser in protein content than is CSF, the risk for this is higher in the former, where even minor (*e.g.*, 0.1%) cross-reactivity against proteins present at one million times higher concentrations will have a large impact on the measured concentration.

Most of the ultrasensitive technologies rely on antibody-based detection of the target molecule, but in Single molecule array (Simoa), the detection reaction is compartmentalized into a small volume (50 femtolitres), so that the reporter molecule accumulates at a very high concentration [21], in Single molecule counting (SMC), the labelled detection antibodies, specifically captured by the target molecule/capture antibody complex, are released and counted one by one in a small detection cell, which allows for a single molecule read-out [22], and in proximity extension assay (PEA), partly overlapping complementary DNA strands are attached to the different antibodies allowing the strands to form a polymerase chain reaction-amplifiable template if immobilised close to each other on the same molecule [23]. These variations in signal generation/detection may result in assays that can be 10- to a 1000-fold as sensitive as the corresponding regular ELISA using the same antibody pair.

Mass spectrometry (MS)-based assays are increasingly important in clinical laboratory medicine, mostly to measure small molecules, such as drugs, amino acids, hormones and vitamins in an antibody-independent manner [24]. Mass spectrometers are also used in explorative proteomics studies to identify new biomarker candidates. However, explorative proteomics has so far failed to generate validated AD biomarkers and, in general, MS-based standardised quantification of peptides and proteins for routine diagnostic use remains rare [25]. However, this is changing and for example Aβ can be reliably quantified in plasma using immunoprecipitation and matrix-assisted-laser-desorption/ionization time-of-flight/time-of-flight mass spectrometry [26, 27].

Blood-based biomarkers for AD-associated pathophysiological processes

Blood-based biomarkers for amyloid pathology

It has been difficult to establish robust blood biomarkers for Aβ pathology in AD. Aβ proteins can be measured in plasma but historically the correlation with AD and/or cerebral β-amyloidosis has been absent or weak (statistically significant but clinically meaningless) [1]. Plasma Aβ concentrations have been interpreted as potentially influenced by production in platelets and other extra-cerebral tissues and the measurements have been confounded by matrix effects from plasma proteins [28]. However, this view is now starting to change. Recent mass spectrometric studies suggest that a ratio of a certain amyloid precursor protein (APP) fragment (APP669-711) to Aβ42 or Aβ42/Aβ40 identifies Aβ-positive individuals with high sensitivity and specificity [26, 29]. The latter result is in line with earlier data obtained using ultrasensitive Simoa technology by which the sample can be diluted to remove confounding matrix effects in the Aβ measurement [30]. Pilot data suggest associations of the concentrations of a number of plasma proteins (*e.g.*, pancreatic polypeptide Y, IgM, chemokine ligand 13, interleukin 17, vascular cell adhesion protein 1, α2-macroglobulin, apolipoprotein A1 and complement proteins) with amyloid burden in the brain [31-33]. However, these data should be interpreted with some caution, as they are derived from multi-marker panels and as a mechanistic understanding of the associations is currently lacking.

Blood-based biomarkers for tangle pathology

There are so far no validated blood biomarkers for neurofibrillary tangle pathology, although there is an emerging literature on P-tau concentrations in neuronally derived blood exosomes with varying results in regards to the association with AD [34, 35]. A recent study employed Simoa technology to measure P-tau phosphorylated at amino acid 181 in plasma (without exosomal enrichment) from AD patients (n=28), individuals with Down's syndrome (DS, n=20) and matched controls (n=15) [36]. The mean plasma P-tau concentration was about 3-4-fold higher in AD patients and DS individuals than in controls, but the variation and the numbers in each group were too small to determine the diagnostic accuracy of the test with certainty (pilot receiver operating characteristics curves suggested optimal sensitivity and specificity of 60% and 86%, respectively, for the AD-control comparison). Importantly, however, plasma P-tau correlated with CSF P-tau concentrations in a sub-cohort composed of 8 AD patients and 3 patients with other neurological diseases. In another recent paper, plasma P-tau (phosphorylated at amino acid 231) was measured in patients with traumatic brain injury (TBI) using a fiber optics technique in which antibody-based detection was combined with rolling circle amplification to increase the analytical sensitivity so that P-tau could be quantified in most samples [37]. Increased concentrations of plasma P-tau in TBI patients were reported but no data on AD were presented. Taken together, plasma P-tau is a hot topic in AD biomarker research and it will be interesting to follow how it develops during the coming five years.

Blood-based biomarkers for neurodegeneration

CSF assays for T-tau and NF-L were recently developed into ultrasensitive blood tests using Simoa technology [38]. Serum or plasma NF-L concentration (either sample matrix works well) correlates with CSF (correlation coefficients of 0.75 to 0.97) and most CSF findings (increased NF-L concentrations in AD, FTD, VaD and atypical parkinsonian disorders) have been replicated in blood [11]. Recent data show that serum NfL effectively identifies onset of neurodegeneration in familial AD [39] and Huntington's disease [40]. Plasma NF-L concentration is increased in patients with Charcot-Marie-Tooth disease (CMT) and correlates with disease severity, suggesting that peripheral nerves may also release NF-L [41]. This could potentially smudge the association of plasma NF-L with central axonal degeneration, but the robust association of plasma/serum NF-L with CSF NF-L suggests that most of the NF-L signal in blood is CNS-derived [42-44], at least in the absence of significant peripheral nerve disease.

For tau, the situation is promising but less clear. Firstly, for unknown reasons, tau concentrations are higher in plasma than in serum (unpublished observation). Secondly, the correlation with the corresponding CSF concentration is absent [45] or weak [46]. Plasma T-tau concentration in AD is increased but the effect size is smaller than in CSF and there is no detectable increase in the MCI stage of the disease [45, 46]. In a recent paper, Mielke and colleagues examined the relationship of plasma T-tau concentration, determined by Simoa, with cognitive decline in 458 participants from the Mayo Clinic Study on Aging [47]. Included subjects were cognitively normal at baseline and followed for up to 4 years. Plasma T-tau correlated with cognitive decline in the sense that higher plasma levels in both the cognitively normal and MCI groups predicted steeper drops in global cognition, memory, attention and visuospatial ability over three years. During follow-up, 67 of 335 cognitively normal people developed MCI. Those in the highest and middle tertiles of plasma t-tau were likelier to progress than those in the lowest. Over that same period, 28 of 123 people with MCI progressed to dementia, however, plasma T-tau did not predict who would. Altogether, the published studies on plasma T-tau as an AD biomarker so far point toward the feasibility of finding a predictive tau signal in blood. However, the lack of correlation of plasma with CSF T-tau suggests that researchers should look for additional tau biomarkers in plasma, *e.g.*, degradation end-products that may be more stable and potentially reflect CNS tau better. It will also be important to examine if there are forms of tau that could separate tau produced in the CNS from tau produced in peripheral tissues, *e.g.*, kidney and muscle (https://www.proteinatlas.org/ENSG00000186868-MAPT/tissue).

In regards to synaptic degeneration in AD, CSF neurogranin has emerged as the most promising fluid marker [48-53]. However, when examined in plasma, neurogranin is unchanged in AD and there is no correlation with CSF, most likely due to expression in peripheral tissues [54].

Blood-based biomarkers for microglial activation

Recent reports suggest that the CSF concentration of the secreted ectodomain of triggering receptor expressed on myeloid cells 2 (Trem2), a molecule that is selectively expressed on microglia in the CNS [55, 56] and genetically linked to AD [57, 58], is increased in AD in a disease-specific manner and correlates with CSF T-tau and P-tau [59-61]. These results are backed by an abundant literature showing increased CSF concentrations of several other microglia- and/or macrophage-derived proteins, including chitotriosidase [62, 63], CD14 [64]

and YKL-40 [65, 66]. Another microglial marker, the C-C chemokine receptor 2, is expressed on monocytes and one of its ligands, C-C chemokine ligand 2 (CCL2), that can be produced by microglia, is present at increased concentration in AD CSF [67-69]. Most studies suggest that these increases are modest with large overlaps between cases and controls, if compared to the more prominent changes seen in traditional neuroinflammatory conditions, such as multiple sclerosis [70] or HIV-associated neurocognitive dysfunction [71]. When measured in plasma or serum, the concentrations of most of the microglia-related proteins mentioned above are higher than in CSF and probably reflect release from monocytes and macrophages in peripheral blood rather than CNS-related changes. However, a few studies suggest a slightly increased plasma concentration of YKL-40 in blood from AD patients [1].

Blood-based biomarkers for AD-associated protein accumulations other than tau and Aβ

α-Synuclein is the major component of Lewy bodies that are characteristic inclusions of Parkinson's disease (PD) and DLB [72] but often also seen in AD [73]. In PD and other synucleinopathies, CSF α-synuclein concentrations are typically lower than in controls [74, 75], whilst in AD and CJD, the concentrations are increased and correlate with T-tau, suggesting that α-synuclein may also be an non-specific marker of neurodegeneration [75-79]. This has been reported not only in AD and CJD, but also in DLB, where there may be a competition between aggregation of α-synuclein into Lewy bodies and release of the protein from degenerating synapses, making the data complex to interpret [80]. Currently available assays for α-synuclein measure total amounts of the protein and not Lewy body-specific isoforms; sensitive and specific assays for the latter would resolve this issue. However, there are some preliminary reports on increased CSF concentrations of α-synuclein oligomers in CSF from PD patients [81, 82] and recently sensitive assays that detect and amplify the biochemical signal of what appears to be α-synuclein seeds in CSF have been published [83, 84]. α-Synuclein is highly expressed in red blood cells, a reason why blood contamination during CSF collection may limit the diagnostic value [85, 86]. For the very same reason, blood tests for α-synuclein pathology in the brain may prove hard to develop. Nevertheless, as peripheral Lewy body pathology, *e.g.*, in the salivary gland and gut, has been reported in PD [87], blood or salivary tests for α-synuclein seeds may be something to explore in the future.

Another pathology that commonly co-occurs with classical AD pathology is inclusions of hyperphosphorylated transactive response DNA-binding protein 43 (TDP-43) [88],

traditionally linked to frontotemporal dementia. TDP-43 can be measured in CSF but, unfortunately, most of the protein appears to be blood-derived and its CSF concentration does not reflect TDP-43 pathology and is unaltered in FTD [89]. Similarly, no reliable blood test for TDP-43 pathology in the CNS exists to date, but intense research efforts are ongoing.

Miscellaneous

There is vibrant research activity on other biomarker categories, such as exosomes and micro-RNA, lipid and metabolite profiles, using both CSF and blood as sample types in explorative studies to identify novel AD biomarkers. These fields are still in their infancy but may well represent an emerging fourth wave of AD biomarkers within the coming five years.

Concluding remarks

The past five years have seen an enormous development in analytical tools for ultrasensitive biomarker quantification in the context of neurodegenerative diseases. The development in the field has been much faster than we ever could have imagined. Assays that are 100-1000-fold as sensitive as standard ELISA or mass spectrometry-based techniques have opened up a new biomarker window in the CSF and made it possible to quantify the traditional CSF biomarkers in blood. NF-L is the only CSF biomarker for which the transition from CSF to blood has been relatively uncomplicated but for tau and Aβ biomarkers there is a signal also in blood, albeit with a smaller effect size than what can be obtained using the corresponding CSF measure. We believe that new ultrasensitive techniques will allow for the development of assays for the quantification of fragments or protein subforms that are more stable in blood and/or more specific to CNS pathologies. This will hopefully lead to more robust blood-based assays that eventually could be used as diagnostic and/or screening tools also in primary care. During the coming years, it will be important to continue to build biobanks from deeply phenotyped cohorts with access to both CSF and blood samples, as well as data on advanced neuroimaging, genetics and clinical follow-up. This will hopefully facilitate the development of even better tests. This will be particularly useful the day we have the first disease-modifying treatment. At present, we do not think blood-based analysis will substitute CSF analysis, but perhaps sequential testing, starting with blood analysis followed by referral for CSF analysis and additional examinations at expert centers in the case of results that point towards the need of refined diagnostic work-up, will be the future.

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