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Blood-based biomarkers of Alzheimer's disease: challenging but feasible

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Abstract

Blood-based biomarkers present a considerable challenge: technically, as blood is a complex tissue and conceptually, as blood lacks direct contact with brain. Nonetheless, increasing evidence suggests that there is a blood protein signature, and possibly a transcript signature, that might act to increase confidence in diagnosis, be used to predict progression in either disease or prodromal states, and that may also be used to monitor disease progression. Evidence for this optimism comes partly from candidate protein studies, including those suggesting that amyloid- β measures might have value in prediction and those studies of inflammatory markers that consistently show change in Alzheimer's disease, and partly from true proteomics studies that are beginning to identify markers in blood that replicate across studies and populations.

Keywords

Alzheimer's disease; biomarkers; blood; dementia; plasma; transcriptomics

These are interesting times in Alzheimer's disease (AD) research; for interesting read challenging. When the amyloid that forms the core of the chief pathological lesion of AD was discovered in the mid-1980s and the amyloid cascade hypothesis articulated by John Hardy and others soon after [1], the translational path to disease modification became somewhat predictable – the revealed pathological process would lead to targets, targets would lead to compounds, and compounds would be further developed and tested in Phase I, II and then III trials. Currently, ten compounds are in Phase III trials for AD with more than 50 in Phase II and many of these compounds are directed, one way or another, towards amyloid, with others being putative disease modifiers directed at other parts of the cascade [2]. However, the first few disease-modification trials have failed and our confidence has taken a knock. The most

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optimistic view of these failures is that the compounds were simply not effective but the next set of compounds will be and a disease-modification therapy will reach the clinic. Another perspective argues that the targets are wrong; a view most often espoused by those advocating a new target. But another view gaining traction is that there is a bigger problem with trial design of disease-modification trials in AD. This view notes that pathology precedes clinical symptoms by years, perhaps decades, and those clinical measures, including those used as primary outcomes in clinical trials, show huge variation. As an example we reported that a significant number of people with AD demonstrate greater annual improvement in mini mental state examination (MMSE) scores than are achieved with the symptomatic treatments for AD even though their disease would have continued to progress [3]. These twin problems – very early pathology preceding symptoms and clinical outcome measures that poorly reflect pathological processes – may turn out to be the biggest obstacles in developing innovative medicines for AD. It may be that the drugs will work best for a group of people with prodromal disease that we cannot yet identify and may halt or slow the progression of disease in ways that we cannot yet measure. This is arguably the strongest driver for biomarkers in AD; not so much markers for clinical diagnosis, but markers to identify pathological processes before the onset of clinical symptoms and markers to measure pathological processes or disease progression. If a marker for pathological processes could be found then it could be used for screening to identify trial participants for early intervention, for patient stratification in trials and to measure the effect of intervention on pathological processes. The ultimate, but very rarely achieved, marker for disease process is the surrogate, a marker that can substitute for clinical measures [4]. In addition to these disease-related markers, there is an important potential role for compound-related markers in early-stage trials as a pharmacokinetic/pharmacodynamic marker and to establish dosing regimens.

As reviewed elsewhere in this issue, cerebro-spinal fluid (CSF) and imaging markers have made the most progress and are the closest to utility. However, there are promising signs that blood-based biomarkers are feasible and if they can achieve success, would have a particular place, especially in the context of clinical trials. The potential advantage of blood-based biomarkers is obvious – obtaining blood is easier than almost any other body fluid, and blood-based tests lend themselves to high-throughput and cheap measurements. A blood-based marker could be obtained routinely in the community in primary care or in the patient's home. Blood-based markers lend themselves to repeated measurement even in frail, elderly people. The technology surrounding biomarker analysis in blood is developing rapidly, and microfluidics, multiplexing and miniaturization raise the prospect of complex lab-on-chip type tests. However, the challenge of blood-based biomarkers in brain diseases is equally obvious – peripheral blood has no direct connection with the brain. Moreover, blood as a fluid poses some additional challenges, for proteins the challenge is of dynamic range, for RNA the challenge is of cell type and for metabolomics the challenge is of the potential dominance of the effects of the environment (internal and external). In this article, we discuss some of these challenges in the context of progress that has been made and conclude that despite these challenges, the prospect of a blood-based marker, perhaps in combination with other markers and for specific indications, is good.

Multiple approaches to blood-based markers

Blood, as a source for biomarkers, contains a number of different tissues. Proteins, lipids and other metabolic products can be examined in plasma, serum or cellular compartments. The latter can be red cells, platelets or white cells examined either crude (buffy coat) or separated by flow cytometry into separate cell types. Cell-based protein studies can be static or cells can be cultured for short periods and the response of proteins to a challenge measured. RNA can be obtained from cells, obviously, but it is also present in exosomes in plasma, an intriguingly interesting potential source of biomarkers [5,6]. DNA in blood is present in nucleated cells.

Each of these compartments in the blood offers its own advantages and disadvantages. As well as having multiple different compartments, the range of types of biomarkers in blood is, at least potentially, substantial and includes: protein concentrations; protein isoforms and post-translational modifications; RNA, the complexity of which is only beginning to be revealed; DNA, including single nucleotide polymorphisms, copy number variants and other 'static' variation and epigenetic changes, which may be more responsive to the internal and external environment; lipids and other metabolic products, which represent huge variation and the constituents of the blood itself, such as the relative proportion of cell types or simple protein and glucose levels. This list serves simply to emphasize that, in comparison to other fluids, such as CSF, blood is a highly complex tissue.

Comparing the different potential uses of blood as a tissue for biomarkers in AD, there are some advantages and disadvantages of each approach. This article concentrates on the most advanced and most studied approach – the use of plasma as a source for proteins – but will briefly discuss some other potential uses of blood in the biomarker field. Most obviously, blood is the most common source of material for genetic studies. Whether genetic variation is a biomarker or not is something of a moot point. Most genetic variation is a trait rather than a state marker and, therefore, not a biomarker of either disease or biological processes, in the strictest sense of the term. Nonetheless, in relation to AD, APOE variation is currently the most used marker for stratification in clinical trials and the best marker, thus far, for predicting progression from prodromal states to full dementia [7]. It is possible that as genome-wide studies report more genes associated with disease then the predictive power of genomic variation will become greater. Moreover, if a genotype is related to response to a therapy then pharmacogenomics is likely to drive more frequent use of genetics in AD. The acceleration of genomic technologies continues apace, with individual genome sequencing probably not far away, and so it is likely that genetic variation, including epigenetic variation, will increase in importance as a biomarker in AD, perhaps in combination with other markers, including imaging, CSF and other blood-based markers.

As well as the relatively static genome, with respect to gene variation, the hugely variable expression genome is a potentially rich source of biomarkers. There is relatively little evidence published of a transcript signature in blood in AD that might act as a biomarker, although there is commercial activity in this area with at least two companies claiming biomarkers based on transcripts derived from blood cells. Using a candidate-gene approach, expression changes in AD blood have been demonstrated in genes previously shown to be altered in the brain in AD and to have mutations in other neurodegenerative diseases [8,9]; at the very least suggesting the feasibility of using blood-based mRNA as a source for biomarkers. It is also worth noting in this context that miRNAs, which act as regulators of gene expression, have been the focus of recent studies in AD. While specific miRNA expression profiles in the AD brain have been linked to BACE1 expression, their potential role as peripheral biomarkers has also been studied. This is an exciting and new area of research that appears to hold considerable promise [10–12]. The transcriptome has many tantalizing advantages as a possible source of biomarkers. It is readily obtained and is tractable by truly genome-wide technologies, in marked contrast to the proteome. Obtaining RNA from blood is not without its challenges, although there are widely used proprietary solutions that stabilize blood-based RNA after collection such as the PAXgene™, LeukoLOCK™ and similar proprietary systems. However, it is important to realize that although offering all the advantages of stability and uniformity, these approaches may collect a somewhat different set of RNAs and it remains to be seen which system will have the most utility in AD. Both the total expression of a gene as well as splice variants can be examined and there is a growing body of evidence of transcript changes in the brain in AD allowing interesting bioinformatic approaches seeking a common signature across brain and blood. In other brain disorders, such as schizophrenia and Huntington's disease, blood-based transcript signatures do seem to reflect disease, although some of these studies have not been replicated

[13–17]. This begs the question, why? How is it that a blood-based transcript signature can act as a marker of a brain disease? This has not been satisfactorily answered but it might be that a disorder involves a systematic insult or a systematic process that results in a systematic change in gene expression measurable in blood and possibly the brain but only results in organ dysfunction in the brain [18]. Alternatively, brain disease might itself cause a systematic insult either directly or indirectly – changes in diet or behavior resulting from the brain disease being one example of this.

Despite the near-universal use of APOE as a marker and the possibility of transcript-based markers, it is proteins that have the most potential as truly dynamic biomarkers of state and for use as markers in diagnosis, prediction and in monitoring progression. The immediate problem facing any search for protein markers is the huge dynamic range – perhaps as much as 15-fold – in proteins in plasma [19]. In cells, an important potential source of biomarkers, the range is less but still presents a challenge. With such a huge dynamic range, from the most to the least abundant proteins, there are unlikely to be technologies able to accurately measure all proteins in plasma, at least for the foreseeable future. An additional challenge for proteomics is the complexity of proteins relative to other molecular categories. Proteins come in many different isoforms, are metabolized, have different biophysical states, are complexed with other proteins, have altered activities and have a very large and variable number of post-translational modifications. In sharp contrast to genomics, where measuring a million or so single nucleotide polymorphisms captures a significant part of the genome, the full extent of the proteome in blood is more complex and largely unknown. These twin challenges – dynamic range and complexity – mean that any proteomic technology currently in use is only analyzing a small component of potential biomarker-relevant changes.

Despite these challenges, developments in both bioinformatics and mass spectrometry have led to very significant advances in proteomic analysis of complex tissue, including blood. The Plasma Proteome Project, part of the Human Proteome Organization [201], aims to map all proteins in plasma and in its first phase identified over 9000 proteins with some confidence and over 900 with very high confidence [20,21]. A range of proteomic approaches are used in biomarker discovery, all relying on mass spectrometry for protein identification. The major differences are in the separation of the hugely complex protein tissue either by gel-based techniques, typically 2D gels with either silver or fluorescent-tag staining, or nongel-based separation, using either liquid chromatography or utilizing matrices to differentially bind and release proteins. These various approaches have been extensively reviewed elsewhere [22–26]. Our view is that it is premature at this point to dismiss any particular approach and that a combination of techniques is almost certainly necessary to discover biomarkers for disease. Comparing the different 2D-gel approaches, for example depletion of the most abundant proteins, allows better resolution of the least abundant proteins and yet no depletion technique is entirely specific and, indeed, many of the proteins depleted using the most specific approaches turn out to be potential biomarkers. With regards to the staining of gels, there are those who advocate the use of differential gel electrophoresis, which undoubtedly offers considerable advantages in experimental design in allowing within-gel case and control comparison, and yet there is evidence that silver staining of gels is more sensitive and hence, has some advantages for some proteins. Similarly, in mass spectrometry-based proteomics a case can be made for the use of multiple reaction monitoring, gel-free tandem mass spectrometry, isobaric tagging, MALDI, SELDI and many other approaches. No one method is suitable for all approaches. We have reviewed methodological aspects of various proteomic techniques currently employed for biomarker discovery in AD in an earlier article [27].

An alternative approach to the discovery of protein-based biomarkers is to assay candidate proteins. Typically this will be by immune capture but increasingly other approaches are being explored, including aptamer-based capture. Using antibodies to capture a protein depends on

having a suitably specific and sensitive antibody available. This remains a major limitation although multiplexing of immune-capture assays including, but not limited to, the widely used Luminex xMAP[®] system, has resulted in a major step forward in the speed with which multiple proteins can be assayed. Theoretically, this approach can multiplex up to 100 proteins, although in practice a decline in assay performance means that multiplex assays rarely complex more than 30 proteins at a time. Nonetheless, this approach ensures that where assays are available, dozens to hundreds of proteins can be assayed relatively rapidly and with excellent reliability. The use of these candidate arrays is increasing with some promising data in the field of AD. However, it is important to remember that such approaches remain candidate-protein approaches and experience from genetic fields was that candidate gene studies were rarely replicated. There may be lessons to be learnt here for the proteomic field.

Having discovered a potential protein or panel of proteins acting as a biomarker it is necessary to validate as well as replicate the finding. The biomarker terminology is confusing and the terms validation, replication and qualification are sometimes used interchangeably and can mean different things. We use the terms in this context to mean three separate stages leading from discovery through to clinical or research utility. Validation is the step after discovery, necessary to demonstrate that the finding is correct, for example that a particular protein is altered in plasma in disease. This step necessitates an alternative method, complementary to that used in discovery phase. If the primary discovery was using an antibody capture approach then validation might use an alternative antibody or for example a mass spectrometry-based approach, such as multiple reaction monitoring. If the primary discovery was made using mass spectrometry then validation by an immune-capture approach (ELISA or xMAP) would be one way to validate. Replication in this context refers to repeating the observation, now validated using a complementary assay, in another and ideally much larger study population. In the third phase, qualification refers to whether the biomarker qualifies for use in a given context. Does it meet the standards required to be used in clinical trials as an indicator of drug response? Does it have sufficient positive and negative predictive power to act as a diagnostic tool? Might it qualify for that hardest to achieve status as a surrogate marker? These are issues that involve regulators and typically a huge volume of data. There are no markers yet qualified in AD and more widely, very few fully qualified biomarkers in medicine. These are hard targets to achieve.

Amyloid- β peptides as markers of disease status (cross-sectional studies)

Several cross-sectional studies have measured plasma amyloid- β ($A\beta$) levels in patients with AD, healthy control subjects and those with mild cognitive impairment (MCI) to test its utility as a diagnostic biomarker that can discriminate between disease and control states. These studies include assays of $A\beta_{40}$, $A\beta_{42}$ and the ratio of $A\beta_{42}:A\beta_{40}$. However, the majority of these reports have not detected statistically significant differences between diagnostic groups [28–32]. The broad overlap between mean plasma $A\beta$ levels in AD and control groups in most of these studies suggests that, at least in cross-sectional analyses, assays of $A\beta$ peptides have little clinical utility as diagnostic biomarkers. A recent multicenter study reported lower plasma $A\beta_{42}$ and $A\beta_{42}:A\beta_{40}$ ratios in AD and amnesic MCI subjects relative to non-AD dementias [33].

Cross-sectional studies are rather limited in their ability to assess the association of $A\beta$ with disease progression. Recent studies have addressed this issue by measuring these peptides at multiple time points in cohorts of subjects that were longitudinally followed, or by relating baseline measurements to clinical progression assessed at serial time points in order to assess their role as risk/progression biomarkers.

A β peptides as markers of disease progression or risk (longitudinal studies)

In a cohort of 1125 nondemented older individuals (>75 years at baseline) followed over a 4.5-year interval, Schupf and colleagues found that greater levels of A β_{42} at baseline (but not baseline A β_{40} concentration or baseline A β_{42} :A β_{40} ratio) and an interval decline in A β_{42} concentration over the duration of follow-up were associated with a significantly higher risk of incident AD [34]. A similar association was found between decreasing ratio of A β_{42} :A β_{40} and risk of incident AD. This is one of the few studies to relate both baseline as well as interval changes in A β to the risk of AD.

In the Rotterdam study, 1756 nondemented older individuals were followed over 8 years and assays of A β_{40} and A β_{42} in plasma were performed at baseline [35]. Higher concentration of A β_{40} at baseline was associated with a greater risk of incident AD. This association was especially strong in individuals who also had a concomitantly lower A β_{42} concentration. A higher ratio of A β_{1-42} :A β_{1-40} at baseline was associated with a reduced risk of subsequent dementia. An important difference in the design of these longitudinal studies that may explain the discrepancy in the results may be that the Rotterdam study only analyzed samples at baseline whereas Schupf and colleagues assessed both baseline and interval change in A β .

Younkin and colleagues studied a cohort of 563 cognitively normal older individuals over a median follow-up interval of 3 years and reported that a lower ratio of A β_{1-42} :A β_{1-40} at baseline was associated with a higher risk of conversion to AD or MCI [36].

Adding further complexity to the interpretation of plasma A β concentration and risk of incident AD, the Uppsala Longitudinal Study of Adult Men (ULSAM) followed two cohorts of elderly males, with mean baseline ages of 71.0 and 71.6 years over a median interval of 11 and 5 years, respectively [37]. In the older cohort of subjects, low baseline concentrations of A β_{40} were significantly associated with a risk of incident dementia at follow-up. A similar trend was observed for a low baseline concentration of A β_{42} that did not reach statistical significance. No significant associations were observed between plasma A β levels at baseline and incident AD in the younger cohort of subjects. There was no association between the lowest tertiles of A β_{42} :A β_{40} ratio and incident AD. In the same study, 630 individuals had plasma samples drawn at two time points, specifically at 70 and 77 years of age. In these subjects, there was no association between interval change in plasma concentrations of A β and subsequent AD.

The Three-City study prospectively followed 8414 nondemented older individuals over a 4-year interval and measured baseline concentrations of A β_{1-40} , A β_{1-42} , A β_{n-40} and A β_{n-42} [38]. Subjects with higher high baseline A β_{1-42} :A β_{1-40} or A β_{n-42} :A β_{n-40} ratios were found to have a significantly lower risk of developing AD. This study is among the first to use the multiplex xMAP technique for assays of A β peptides.

In patients with established AD, low plasma levels of A β_{1-40} and A β_{1-42} were associated with a more rapid cognitive, as well as functional, decline in a longitudinal study of 122 patients followed over a 4-year interval [39].

Taken together, results of the aforementioned large longitudinal studies suggest that assays of plasma A β_{1-40} , A β_{1-42} and the ratio of A β_{1-42} :A β_{1-40} may be potentially useful biomarkers of incident AD in nondemented older individuals, perhaps in combination with *APOE* genotype [40]. The emerging finding common to most of these studies indicates that a decrease in plasma A β_{1-42} is a proximate event to disease onset and may reflect sequestration of this A β species within the brain. However, the data are inconsistent and may reflect variability due to technical reasons, such as the assay methods employed (ELISA or xMAP), differential affinities of the antibodies used for different A β species (truncated vs full-length, oligomeric vs monomeric), variable sensitivity of detection for free or protein bound fractions of A β , as well as timing of

the sample collection in relation to the disease prodrome or onset. Nevertheless, the results to date are promising and indicate that future studies in prospectively followed cohorts of subjects using standardized assays of A β peptides, allowing for comparison of results across centers and subject cohorts may yield conclusive data on the clinical utility of these peptides as markers of disease risk/progression.

A β peptides as markers of treatment response

There are little conclusive data that relate changes in peripheral concentration of A β to treatment response either in the setting of clinical trials of disease-modifying treatments or in AD patients treated with cholinesterase inhibitors. In a recent Phase II clinical trial examining the safety of the γ -secretase inhibitor LY450139, there was a dose-dependent reduction in plasma A β _{1–42} after oral administration of the drug [41]. In another recent study, Roher and colleagues reported that there was no association between plasma A β levels and duration of treatment with donepezil in AD patients [42]. However, relating changes in peripheral concentration of A β in response to treatments targeting amyloid precursor protein (APP) processing or cholinesterase inhibitors is challenging. Owing to a lack of evidence correlating peripheral plasma A β levels and those in the CNS as well as the lack of data relating plasma A β concentration to severity of cognitive impairment, the utility of these assays appears limited at present [32,43–46].

Candidate plasma biomarkers: single proteins

A large number of proteins, peptides and aminoacids, other than A β , have been examined in plasma based on their putative role in AD pathology (see recent reviews [30,47]). Perhaps the two most consistent findings are the increase in homocysteine and C-reactive protein (CRP) seen in AD. First reported by Clarke *et al.* [48], an increase in plasma total homocysteine (tHcy) has been widely replicated and a recent systematic review of large and prospective studies found a relative risk of elevated tHcy for AD of 2.5 [49]. Increased tHcy and CRP are also both associated with a risk of cardiovascular disease but it is worth noting that neither contribute to risk estimation, even in cardiovascular disease, enough to warrant recommendation for use in clinical practice [50]. Given that the association with AD may well be weaker than with cardiovascular disease, this emphasizes the gap between finding an association with disease and proof of utility as a biomarker. Nonetheless, CRP and tHcy may have independent effects on the risk of developing AD [51] and an elevation in CRP has been associated with AD, rate of progression of AD, MCI and Down's syndrome [39,52–57]. However, other large longitudinal studies found that other general markers of inflammation, such as TNF- α but not CRP, were associated with risk of dementia or with brain volume [58,59] and others found an association between CRP and vascular dementia [60]. Differences in inflammatory pathways are, in fact, a very consistent finding in plasma AD and are being increasingly examined not by single or small numbers of proteins, but in large-scale arrays. We discuss some of these studies later but other pathways associated with disease have also been probed for biomarkers. Members of the wnt signaling and associated pathways have been claimed as markers of AD, including, for example, the proteins dickkopf homolog-3 and glycogen synthase kinase-3 [61,62], although other studies have not fully replicated these findings [63]. Some of these and other candidate protein studies are summarized in Table 1.

Candidate plasma biomarkers: multiplex approaches

Perhaps the most significant recent finding in the field of plasma AD biomarkers was reported by Ray *et al.*, who used protein array technology to assay the relative concentrations of 120 signaling proteins in plasma [64]. The authors observed that the concentrations of 18 plasma proteins were able to discriminate AD samples from control subjects with approximately 90% accuracy. More importantly, baseline plasma concentrations of these 18 proteins were able to

identify subjects with MCI who subsequently converted to AD from those who remained stable or converted to other (non-AD) dementias. Understandably, these findings have received wide attention both because of the application of array technology to biomarker discovery in AD for the first time as well as the demonstration of a robust peripheral signature from cell-signaling proteins, which might be relevant to pathological processes in AD. Attempts to replicate these striking findings by other groups in independent subject cohorts have not been fully successful [65,66].

Soares, Breteler and colleagues used an alternative array approach (Luminex xMAP) in their attempt to replicate the original finding of cytokines as a marker for AD [66]. This technology is a flow cytometric-based platform and uses microspheres loaded with a specific ratio of two different fluorescent dyes. The capture antibodies are covalently coupled to the microspheres, and immunoassays run under standard sandwich immunoassay formats. The assay read-outs are generated by lasers exciting the internal dyes identifying each microsphere particle, and also any reporter dye captured during the assay. Using an expanded 151-analyte xMAP panel that contained eight of the original 18 cell-signaling proteins reported by Ray *et al.*, Soares, Breteler and colleagues observed a diagnostic accuracy for discriminating AD from control samples of only 61% compared with 83% with these eight proteins in the original Ray *et al.* data set [66]. However, this approach holds promise as a composite xMAP panel of other analytes appears to have a superior diagnostic accuracy. Although an exhaustive analysis of the reasons for these inconsistent results is beyond the scope of this article, some points that may be worth considering in the interpretation of the index study include the considerable difference in the mean age of the MCI converters, who were 10 years older than nonconverters. It is unclear whether any adjustment was required or made for this variable in the prediction analysis. Moreover, the original training set did not contain samples from MCI subjects and the application of the composite panel to the test set may have overfit the data. This is suggested by the subsequent finding that as few as five analytes from the original 18-protein panel could provide a greater than 90% diagnostic accuracy for samples from AD patients [67]. Despite these concerns, the application of multiplex immune-based assays is a significant technological advance in the field of biomarker discovery for AD and may pave the way for larger studies using standardized methodology, thereby allowing the comparison and replication of findings from independent groups.

Use of proteomics to discover novel plasma biomarkers

An alternative approach to the candidate proteins discussed previously is to employ so-called 'unbiased' methods to interrogate the plasma proteome to identify AD-related protein biomarkers. In general, the proteomic methods use either gel-based or gel-free separation techniques allied to mass spectrometry-based identification and/or quantification of protein biomarkers [27]. Although the proportion of the plasma proteome analyzed in either of the proteomic approaches is small, the high-dimensional data generated from unbiased proteomic approaches as well as multiplex immunoassays present particular challenges that must be considered in the design of biomarker discovery studies.

A biomarker discovery program consists of an initial discovery-phase study where the primary objective is to derive protein signatures that are differentially expressed in cases relative to controls followed by a validation phase using a higher-throughput methodology. In a typical experiment using 2D gel electrophoresis (2DGE) of human plasma samples, optical densities of 200–700 spots, each representing a small number of proteins, can be compared across the case and control groups to identify differentially expressed proteins in AD patients. We have previously used this approach in our proteomic analyses of plasma and using p-values adjusted to the false discovery rate to allow-multiple comparisons, we identified 15 protein spots, whose mean integrated optical densities on silver-stained 2DGE gels were significantly different in

AD plasma samples (n = 50) relative to age-matched controls (n = 50) [68]. Liquid chromatography tandem mass spectrometry (LC/MS/MS) of the excised protein spots was then applied to identify these proteins. As most experiments in the discovery-phase proteomic studies use nonspecific methods, such as silver staining, to visualize protein expression and quantify such expression merely by integrating the optical density of a given spot over the mean optical densities of all spots on a gel, it is imperative that these results be confirmed in independent experiments using specific and sensitive methods directed against the protein targets of interest. In these confirmatory/validation studies, careful attention must be paid to define the groups of interest and, including plasma samples from non-AD dementias or other neurodegenerative conditions besides healthy controls, is likely to provide valuable information on the specificity of the putative biomarkers for diagnosis of AD. Using the example of our prior proteomic study, our validation experiments used quantitative immunoassays (ELISA) against complement factor H (CFH) and α 2-macroglobulin (A2M), the two proteins showing the greatest difference in expression between AD and control plasma samples. These experiments were performed in an independent cohort of subjects that, besides AD and healthy control included plasma samples from patients with vascular dementia, Huntington's disease, motor neurone disease, multiple systems atrophy and progressive supranuclear palsy. By confirming that plasma concentrations of CFH and A2M were significantly higher in AD relative to controls and not different in other neurodegenerative diseases, our validation studies confirmed these proteins as AD-specific biomarkers. Subsequently, using ^1H -magnetic resonance spectroscopy, we have also demonstrated that CFH and A2M are associated with hippocampal metabolite abnormalities in patients with AD [69].

Biomarker discovery paradigm: beyond case-control

The overwhelming majority of biomarker discovery studies, including ours, have used the aforementioned strategy, comprising of binary distinction between disease and control samples. However, this approach may not be suitable for the identification of biomarkers aiming to reflect disease state, especially in a disease such as AD with a long preclinical prodrome. Many controls in these studies will have AD pathology even in the absence of clinical symptoms. However, biomarkers reflecting disease state, independent of clinical symptoms, and ideally before the onset of any symptoms, are critically important if preclinical identification and treatment is to be achieved. Furthermore, current approaches to biomarker discovery in AD do not address the considerable heterogeneity in disease progression in patients with established AD [70,71]. The predominant biomarker discovery study design, comparing cases to controls, is, therefore, unlikely to identify biomarkers that reflect early disease pathology or disease progression, and yet it is markers such as these that would be most useful not only in clinical practice but would be invaluable in clinical trials for the enrichment of at-risk patient populations and for patient stratification. A further consideration in biomarker studies using proteomics or multiplex immune-based assays is the possibility of the generation of high-dimensional data containing numerous analytes that may provide excellent binary distinction between AD and control samples, but lack any relevance to the core pathological features of AD and, therefore, are of doubtful clinical utility. In this context, plasma proteins that are associated with specific and well-established endophenotypes of disease pathology, such as brain amyloid burden or hippocampal atrophy, might be biologically relevant biomarkers of AD and, thereby, also sensitive to disease progression.

In order to overcome the above limitations, we have recently employed a novel approach to derive proteome-based plasma biomarkers of AD based not upon their ability to discriminate AD from healthy controls, but by their association with brain atrophy as well as rate of clinical progression in patients with established AD [72]. In two independent studies, we sought markers correlating with other measures or endophenotypes of disease, including imaging. We

identified clusterin (also known as apoJ) as the only protein in these discovery studies and then we confirmed that plasma clusterin concentration is associated with brain atrophy, disease severity and rate of progression. We also undertook further studies seeking to relate peripheral clusterin concentration with yet another distinct endophenotype of AD pathology, specifically *in vivo* brain amyloid burden, in both man using ¹¹C Pittsburgh Compound B PET imaging, and in a transgenic mouse model of AD overexpressing APP and presenilin (PS)1 mutations. To the best of our knowledge, this is the first biomarker discovery study that has employed unbiased proteomic analyses to identify plasma proteins associated with specific pathological and clinical endophenotypes of AD. We believe that such a strategy is likely to yield biomarkers that accurately reflect AD pathology and are sensitive to disease severity and progression.

Proteomic identification of AD biomarkers in blood

As noted previously, a well-recognized limitation of gel-based proteomic studies of human plasma is the inaccessibility of low-molecular-weight proteins (<30 kDa) to this method. This fraction of the plasma proteome contains peptides and protein fragments, bound to highly abundant carrier proteins such as albumin. Therefore, peripheral signatures arising from these fragments are masked by highly abundant proteins. Moreover, a standard approach to sample preparation, prior to 2DGE, followed by several groups consists of depleting the sample of proteins, such as albumin, thereby further precluding any meaningful analysis of the plasma 'fragmentome', which is likely to contain analytes with important and novel biological functions. Two independent proteomic studies have used MS-based methods to analyze carrier protein-bound signatures derived from blood in AD. Lopez *et al.* isolated albumin-bound peptides from serum samples by affinity chromatography [73]. MALDI-TOF MS was used to analyze the peptide mass spectra and the m/z profiles were used to build predictive models capable of identifying AD with a sensitivity and specificity of 90 and 72%, respectively. Using a similar approach, German and colleagues observed four spectral peaks that were useful discriminators of AD samples from control subjects [74]. Equally important, three of the four peaks were common to the study by Lopez *et al.*, suggesting that mass spectrometric analysis of carrier protein-bound signatures may be a robust method for the discovery of novel AD biomarkers in blood.

Zhang and colleagues employed multidimensional LC in combination with 1D electrophoresis and 2D electrophoresis to detect serum-based biomarkers in AD [75]. Using MALDI-qTOF and ion-trap MS, they identified several proteins, including inflammatory response mediators, such as CFH, complement components C3 and C4 and A2M, which were significantly increased in AD serum compared with controls. It is worth noting that our own proteomic studies described earlier identified CFH and A2M as AD-specific biomarkers.

Cutler and colleagues used gel-based proteomic analysis coupled with LC/MS/MS in plasma samples from AD and control subjects in an experimental design that included a discovery phase study of 47 AD patients and 47 control subjects [76]. Immune-based detection of proteins differentially expressed in these experiments was then used both in the original discovery cohort as well as in a larger independent cohort of 100 AD cases and 100 control subjects. However, while subjects in the discovery-phase studies were all females, the validation cohort included both males and females, and subjects older than those in the discovery studies. These differences must be taken into account while comparing results between the discovery and validation experiments. The authors also carried out proteomic analyses in plasma from TASTPM transgenic mice overexpressing APP and PS1 mutations. This allowed for the identification of proteins common to both AD patients as well as those that might be related to known pathogenic mutations associated with familial AD. These results demonstrated eight proteins that were confirmed by immunoassays to be differentially expressed in AD relative to controls in the original cohort of subjects, including four (clusterin, complement component

C1r, α 1-antitrypsin and EGF receptor) that were also differentially expressed in transgenic TASTPM mice. Only α 1-antitrypsin and complement C1 inhibitor were confirmed to be differentially expressed in the larger validation cohort. Nevertheless, it is striking that our own proteomic experiments have independently identified clusterin as a plasma biomarker of AD associated with both clinical progression and brain atrophy. In addition, we have also observed in independent proteomic experiments that plasma concentrations of complement-related proteins are associated with brain atrophy in AD, suggesting that the overlap in our results with those of Cutler *et al.* indeed represent peripheral signatures associated with pathological processes relevant to AD [72].

Proteomic studies of plasma have also established a two- to sixfold increase in oxidized forms of fibrinogen and α 1-antitrypsin in AD patients relative to controls, thereby extending a large body of evidence for the role of oxidative damage in the pathogenesis of AD [77–79].

Few studies have applied unbiased proteomic analyses of blood to relate peripheral protein concentrations to treatment response following drug administration in patients with AD. Akuffo and colleagues employed 2DGE and LC/MS/MS to identify a panel of proteins associated with a dose response to the peroxisome proliferator-activated receptor- γ agonist rosiglitazone in a clinical trial in AD patients [80]. They observed a significant association between plasma concentrations of CFH, A2M, APOE and complement C1 inhibitor and ADAS-Cog scores in AD patients treated with the highest doses of rosiglitazone (4 or 8 mg). In a similar study design, proteomic analysis of peripheral leucocytes was performed in AD patients before and after treatment with divalproex sodium [81]. Several proteins were differentially expressed in plasma after treatment and may be relevant to both disease processes in AD as well as to the mechanism(s) of action of divalproate sodium. In interpreting data from biomarker studies using unbiased approaches, such as those described previously, due care must be paid to sample sizes used and definitive conclusions regarding clinical utility/biological relevance reserved until independent replication of the results are reported.

Conclusion: plasma biomarkers are possible & plausible but we are not there yet

The work reviewed here allows one very firm conclusion – the null hypothesis ‘there is no biomarker signature in blood in AD’ can be robustly rejected. Despite the blood–brain barrier and the immense challenge of finding a biomarker in a very late onset, slowly progressive disease in people who often have many other pathologies and accompanying medications, it is clear that proteins, transcripts and probably other constituents of blood, are different in people with AD. Therefore, a biomarker in blood is possible. It is also plausible in the sense that those biomarkers that show the most promise in AD – A β and inflammation-related proteins – are not brain specific but are normally present in the periphery and could plausibly change in response to AD even if the site of pathology is central. However, at the present time, although significant progress is being made, there are no proteins, transcripts or metabolites in blood that have been sufficiently replicated to be established as AD biomarkers.

The prospect of a blood-based biomarker, however, is an important one owing to the ease and potential low cost of obtaining samples and analysis. If the progress that has been made to date is to be sustained and even accelerated then attention could profitably be paid to the design of studies, the collection and accessibility of cohorts and samples, and the use of technologies. With respect to design, the predominant approach of comparing AD cases to normal elderly controls is only one route to the discovery of biomarkers. As distinguishing cases from controls is not the most difficult or important task, then studies in this area might also consider the discovery of biomarkers predicated on comparing AD to other non-AD neurodegenerative disease, comparing progressors to nonprogressors (either MCI conversion or speed of

progression in AD) and establishing biomarkers against endophenotypes, such as imaging or other biomarkers. With respect to cohorts and samples, the public–private partnerships in both the USA through the Alzheimer’s Disease Neuroimaging Initiative (ADNI) study [82] and in Europe through the AddNeuroMed study [83] have both established large longitudinal cohorts with extensive sample collection, neuroimaging and clinical data. These two studies are now joined by others in Europe and ADNI-like studies in Japan, Australia and China. This is an immensely important step forward as it allows ever larger and more powerful studies and cross-validation between studies. However, if biomarkers are to achieve utility in clinical trials then the key will be to include plasma markers in large international multicenter trials, to share the data obtained in these trials and to make the samples available to the scientific community. It is only when markers are validated against trials outcomes that they will become accepted as outcome measures. Finally, technologies are advancing and MS and array-based proteomics in particular have made major steps forward in the last 5–10 years. These, and newer technologies in development, are to be welcomed and will significantly increase our ability to detect markers in blood. However, older technologies, such as 2DGE, remain of real value and it is intrinsically unlikely that they will be completely surpassed in the near future. Researchers should remain open-minded and use a variety of approaches to gain the most value in their search for markers.

Future perspective

The prospects for blood-based biomarkers are excellent. The increasing availability of large sample sets, the range of technologies available and the evidence that there is a signature of changes in blood in AD that might contribute to diagnosis, prediction and monitoring progression, all suggest that blood-based biomarkers, perhaps accompanying other markers, will find utility in AD. If this trend increases, if sample collection and precompetitive collaborative studies continue to flourish between academia and pharma, and if the technologies to examine the plasma proteome continue to develop then we think it is very likely that plasma markers will become part of the armamentarium for the investigation of AD.

However, it seems unlikely to us, from this review of the literature to date, that there is a single plasma marker or that a plasma marker will be in itself a sufficient marker. Looking forward, it seems most likely that markers will be combinatorial – multiple proteins or multiple proteins combined with other blood-based or nonblood-based markers, such as imaging. It is unlikely that there will be one set of markers for all possible uses in AD, and more likely that there will be a marker to aid diagnosis, a different marker or set of markers to predict outcome in people with AD or conversion in MCI and yet another set to be used for monitoring of progression. Some of these markers may be sequential; it might be that plasma protein markers could be used to identify a set of patients or subjects who would then go on to have more complex and expensive investigations, such as PET for example.

In 10 years time we would be surprised if blood-based biomarkers were not used in the context of research and development, and were not an increasingly routine part of early-stage clinical trials. They might even be used in later-stage trials to either stratify subjects or to screen for early disease before subjects went on to have more invasive investigations. Whether plasma markers could be used to monitor progression or even act as a surrogate for clinical measures is more doubtful – there are very few examples of such surrogates and, so far, there is little in the literature to suggest blood-based markers will achieve such a goal. Equally uncertain is the prospect of a miniaturized lab-on-a-chip to detect early AD in the community – such ready access to biomarkers is already available in pharmacies and even in supermarkets, for cholesterol for example, and is being developed for other conditions. Today the prospect of such a test for AD not only seems far off and unlikely but also, in the absence of a disease-modifying treatment, unwanted. In the decade to come this might change.

Executive summary

Multiple approaches to blood-based markers

- Blood is a complex tissue with different fluid and cellular compartments; this complexity impacts on biomarker discovery. Attention should be paid to collection, extraction and curation of samples.
- Blood is a source of protein, metabolites, RNA and DNA, and all of these might contribute to a blood-based biomarker.
- Measuring proteins in blood is especially problematical owing to the very large dynamic range and the complexity of the proteome, which not only includes levels of proteins but also different isoforms, complexes, activities and post-translational modifications.
- Similar to genomic research, the proteomics field has tended to concentrate first on candidate studies and is increasingly moving towards larger-scale, multianalyte studies. However, in contrast to genomics, proteomics is only able to assay a relatively small proportion of the total proteome.

Amyloid- β peptides as biomarkers

- Cross-sectional studies suggest that amyloid- β (A β) peptides have little clinical utility as diagnostic biomarkers.
- Longitudinal studies suggest that plasma A β_{1-40} , A β_{1-42} or the A β_{1-42} :A β_{1-40} ratio may be potentially useful biomarkers of very early or even prodromal Alzheimer's disease (AD).
- Overall, the evidence suggests that a decrease in plasma A β_{1-42} is a proximate event to disease onset and may reflect sequestration of A β within the brain.
- As altering A β is a key target for therapy, measures of A β may be useful in the drug development process. However, evidence that plasma measures predict clinical utility of a therapy are currently lacking.

Candidate proteins as plasma markers

- The two most replicated findings in plasma of people with AD are total homocysteine and C-reactive protein.
- Many other potential biomarkers have been suggested but few have yet received independent replication.
- Plasma markers of inflammation are consistently identified as potential biomarkers in both single-candidate and candidate-array studies. However, the precise inflammatory markers differ from one study to another – although evidence for a change in inflammation is convincing, the evidence for any one protein is not.

Discovery with proteomics

- Few studies have used a proteomics approach to identify potential biomarkers.
- There is a consistency amongst proteomics studies with α 2-macroglobulin, complement factor H and other complement-related proteins being identified in multiple independent studies.
- Discovery-phase studies might usefully use endophenotypes such as imaging, other biomarkers and speed of progression rather than case versus control to

identify potential biomarkers. Using independent variables, such as endophenotypes, biases studies towards the discovery of biomarkers for clinical and research utility.

Conclusion

- No blood-based biomarker has been fully validated or qualified but an increasing number have been replicated.
- Progress in the field depends upon good access to large and well-characterized sample collections from longitudinal studies, from collaborative research and from the use of a variety of technologies and study designs.

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201. Human Proteome Organization. www.hupo.org

Table 1

Candidate plasma protein markers for Alzheimer's disease.

Pathway	Biomarker(s)	Association with AD	Ref.
Cholesterol metabolism	Total serum cholesterol	High midlife concentration of serum cholesterol is associated with increased risk of incident AD. This finding appears to be consistent across several longitudinal studies	[84,85]
	Oxysterols	Decreased ratio of plasma 24S-hydroxycholesterol:cholesterol and 27-hydroxycholesterol:cholesterol in AD versus controls. Not replicated in independent samples	[86,87]
	APOE	Inconsistent findings relating differences in plasma APOE concentration between AD and control subjects. Cited references show increase and decrease as well as no differences Previous studies in large population-based cohorts of subjects have established that APOE protein concentration in plasma is highest in ε2, intermediate in ε3 and lowest in ε4 carriers	[88–90] [91,92]
Oxidative stress	F ₂ -isoprostanes	Increased in AD plasma Not confirmed in other studies	[93] [94]
	Total hydroxyoctadecadienoic acid and oxidatively modified peroxiredoxin	Increased in AD plasma. Not independently replicated	[95]
Coagulation and fibrinolysis	Fibrinogen	n = 2835 subjects. A longitudinal study with 5.7-year follow-up. Increased fibrinogen was associated with an increased risk of incident dementia This has been independently replicated in other studies	[96] [97]
	D-dimer, TAT and t-PA activity	Cross-sectional, case–control study demonstrating increased concentrations associated with a higher prevalence of dementia D-dimer finding was replicated in a longitudinal study against incident dementia	[98] [99]
Inflammation	α1-antichymotrypsin, IL-6 and CRP	Increased concentration at baseline was associated with a risk of incident AD. Longitudinal study with 6713 subjects of whom a subcohort of 727 randomly selected individuals were studied along with 188 subjects with dementia at follow-up The CRP finding has been replicated against prevalent MCI and prevalent dementia Low levels of CRP in patients with established AD are associated with a more rapid cognitive and functional decline	[52] [53,56] [39]
	Homocysteine	Increased baseline plasma concentration is associated with higher risk of incident AD in longitudinal studies This was independently replicated but not consistently observed	[100,101] [102]
Signal transduction	GSK-3	Increased in WBCs from AD and MCI subjects relative to healthy controls. Not independently replicated Another study demonstrated a decrease in GSK-3 concentration in peripheral mononuclear cells in AD and MCI relative to controls	[61] [63]
	Dkk-3	Significantly increased Dkk-3 levels in plasma from AD patients relative to controls. Not independently replicated	[62]

AD: Alzheimer's disease; CRP: C-reactive protein; Dkk: Dickkopf homolog; GSK: Glycogen synthase kinase; MCI: Mild cognitive impairment; TAT: Trans-activator of transcription; t-PA: Tissue plasminogen activator; WBC: White blood cell.