alzheimer's research&therapy

REVIEW

Biomarkers for Alzheimer's disease in plasma, serum and blood – conceptual and practical problems

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Abstract

Substances produced throughout the body are detectable in the blood, which is the most common biological fluid used in clinical testing. Biomarkers for Alzheimer's disease (AD) have long been sought in the blood, but none has become an established or validated diagnostic test. Companion reviews in *Alzheimer's Research & Therapy* will review specific types of biomarkers or applications; in this overview, we cover key concepts related to AD blood biomarker studies in general. Reasons for the difficulty of detecting markers of a brain-specific disorder, such as AD, in the blood are outlined; these pose conceptual challenges for blood biomarker discovery and development. Applications of blood tests in AD go beyond screening and diagnostic testing; other potential uses are risk assessment, prognostication, and evaluation of treatment target engagement, toxicity, and outcome. Opportunities and questions that may surround these different uses are discussed. A systematic approach to biomarker discovery, detection, assay development and quality control, sample collection, handling and storage, and design and analysis of clinical studies needs to be implemented at every step of discovery and translation to identify an interpretable and useful biomarker.

Introduction

The road to developing a blood biomarker for Alzheimer's disease (AD) is paved with good intentions. Without question, developing validated biomarker tests by measuring analytes in the blood would greatly enhance many aspects of AD clinical practice and research. Despite several decades of investigation into potential peripheral biomarkers, among which blood tests have been the main focus, none has been established or accepted as an aid to diagnosis. A series of reviews in *Alzheimer's Research & Therapy* will examine the field and cover traditional and novel approaches. In this overview, we briefly survey concepts and methods that are critical to developing blood, plasma, or serum biomarkers for AD (which we will refer to generally as blood biomarkers).

The biological plausibility and rationale that underlie specific diagnostic blood biomarkers for AD need to be justified. A prominent reason for the failure of many attempts to identify biomarkers in the blood for AD is that AD is a brain disease with little evidence of peripheral manifestations. Pathological changes in the brain result in changes that are detectable with structural and biochemical brain imaging and that also are reflected in altered cerebrospinal fluid (CSF) levels of Aβ42, tau, and phospho-tau. By analogy, blood biomarkers would make obvious 'biological sense' if they reflected changes related to amyloid protein precursor (APP) processing or amyloid deposition in the brain, neurofibrillary tangle formation, or other pathological processes in AD. However, candidate biomarker approaches that measure proteins, lipids, or other substances in blood that are involved in AD neuropathology and whose levels are changed in the brain or CSF have not yielded supportive findings. Some of these approaches could benefit from greater attention to issues such as assay methodology and study design. Alternative approaches to biomarker discovery, including assumption-free (-omic) methods that measure large numbers of a particular type of biomarker (for example, multiplex protein analysis, proteomics, or mRNA expression), will also be reviewed in this series.

Uses for biomarkers for Alzheimer's disease

Biomarkers have many potential uses in blood. First, they could help to support the diagnosis of AD. One approach is to use a blood biomarker as a screening test and, if it is

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positive, follow up the evaluation with a more sensitive and specific CSF or imaging biomarker. However, in view of the serious implications of a diagnosis of AD and the cost of a more definitive workup, the value of the readout from a screening test that has only moderate sensitivity or specificity is unclear. For patients who have memory or other cognitive impairment, blood biomarkers that have reasonably high diagnostic accuracy in their own right would be the most helpful. The preclinical diagnosis of AD is an emerging research priority. For prevention studies, a simple and cheap screening method is highly desirable. A blood test with moderate sensitivity and specificity, in combination with factors such as age and genetic profiling, could be used to help to select people at risk for developing AD (presumably at a stage when they harbor presymptomatic AD pathological changes in the brain). Positive screens could trigger a more definitive biomarker testing.

A panel representing pharmaceutical companies and the US Food and Drug Administration reviewed the qualification of biomarkers for different uses and suggested that the weight of evidence for a biomarker depends on the value of a true result versus the value of a false result, which needs to be placed in the context of the use of a biomarker and determined by stakeholders such as those involved in the process of developing studies and regulatory agencies [1]. The extensive discussions of weight of evidence that may lead to the uses and interpretation of amyloid positron emission tomography imaging as a test for AD pathology in patients with cognitive problems are an example of this process [2]. The field would benefit by reaching a consensus about the minimal target specificity and sensitivity of blood-based biomarkers for AD for these to be clinically useful in different diagnostic settings.

Biomarkers may be used to stage AD or to predict progression or prognosis. Through integration of data on central biomarkers related to amyloid deposition and neurodegeneration, a plausible biomarker map of AD progression has been developed [3]. Changes in peripheral biomarkers may arise at different stages of AD, and it is possible – though challenging in view of the current lack of validated peripheral biomarkers – that a model based on a combination of biomarkers could be developed to help to stage AD. Predicting the progression of AD once symptoms are present has proven difficult. At present, factors such as age, comorbid illness, and apolipoprotein E (APOE) genotype may be used to crudely assess prognosis; the role of biomarkers (central or peripheral) in improving the accuracy of this prediction is unproven but worth investigating.

Measurements from plasma, serum, or blood cells could provide an index of risk of AD. Studies of risk typically involve longitudinal assessment and the clinical outcome measure of a diagnosis of AD at the stage of dementia. These can be conducted in population-based cohorts rather than be limited to clinic populations. Although some of these large-scale studies may suffer from the lack of confirmation of specific diagnoses, they provide data from which relative risks and effect sizes of biomarkers can be determined for typical clinical settings. In recent years, studies have examined whether plasma or serum biomarkers can 'predict' the risk of having an AD pathology biomarker (such as positive amyloid imaging). These are typically cross-sectional correlational studies, which are often agnostic to clinical diagnosis. They may provide more value in understanding the biology of the peripheral biomarker(s) in relation to brain pathology than in defining a clear readout of risk.

Given the importance of Aβ in the pathogenesis of plaques and as an initiating factor in AD, plasma Aβ has been studied extensively in relation to AD diagnosis and risk. Research into factors that influence Aβ in the periphery and increased attention to assay methodology have helped to clarify the potential and limitations of plasma Aβ levels as indices of AD risk [4]. Although many other peripheral biomarkers have been linked to AD risk, the mechanisms or pathways that mediate this risk are not always well understood. For example, some peripheral biomarkers may reflect genetic risk factors for AD, whereas others may identify processes, such as inflammation, that may predispose patients to AD risk. Research into candidate and -omic approaches to biomarkers in the periphery in relation to AD risk is also reviewed in this series.

Finally, blood biomarker tests may be used in clinical trials of treatment for AD. Potential uses and standards of evidence to support the validity of biomarkers in clinical trials have been outlined previously [1]. Biomarkers can be used to select patients or define subsets in clinical trials. If selection is aimed at increasing the likelihood that patients have AD pathology (enrichment), then biomarkers with high diagnostic accuracy or with strong correlations with the presence of amyloid or tau pathology typical of AD would be needed. Plasma measurements may help to characterize target engagement in the periphery, which includes both interaction with the target and aspects of a pharmacologic mechanistic response. In addition, off-target or adverse effects of treatment can be identified. A biomarker can be linked to clinical outcomes at different stages of drug development. An example is measuring plasma Aβ levels in pharmacodynamic studies of γ- or β-secretase inhibitors. Characterization of plasma effects in relation to doses of these secretase inhibitors can help to predict central nervous system (CNS) effects as clinical trials enter phase 2 or 3. Unfortunately, plasma biomarkers are not available for most non-Aβ mechanisms of action. For clinical trials,

biomarker validation is critical. Important considerations are (a) measurement accuracy and precision of the biomarker and (b) data implicating the biomarker across a range of preclinical and human studies.

Assays and study design for blood biomarkers

Factors that influence the plausibility that a peripheral biomarker change is present and detectable in the blood in relation to AD will influence the design of assays and studies. As mentioned above, seeking diagnostic markers in the blood in a disease with CNS-specific pathology, such as AD, raises basic questions about how the biomarker gets into the blood. Changes in proteins, lipids, DNA, or other substances in the brain are often reflected in CSF. However, CSF undergoes substantial dilution as it passages into the blood, and this raises challenges in trying to detect brain-specific biomarkers in plasma – their concentration is likely to be orders of magnitude lower than in the brain or CSF. Many analytes are produced in both the brain and the periphery. This complicates the analysis of blood levels because the fraction of the biomarker attributable to the brain may be masked by the amounts produced in the periphery. Processing and post-translational modifications of proteins may differ in the brain and the periphery, and careful biochemical characterization of candidate biomarkers may be able to tease these differences apart. The use of animal models has been undervalued in biomarker development. Studying peripheral and brain biomarkers in genetically engineered animals that express selected aspects of AD pathology may clarify how biomarker changes relate to mechanisms of pathology.

Another problem is that changes in the blood may reflect the systemic effects of having AD rather than specific brain changes. For example, weight loss accompanies AD even during its early stages and can affect the levels of many analytes measured in the blood. A nonspecific inflammatory response may accompany the presence of a chronic disease such as AD and again may lead to changes in inflammatory proteins measured in plasma or patterns of mRNA measured in lymphocytes or other peripheral cells. The first study that systematically measured levels of a host of secreted proteins in plasma with multiplex assays in AD [5] also studied a small number of plasma samples from patients with inflammatory arthritis as a control. Comparisons with disorders with known systemic effects (for example, arthritis, cancer, or diabetes) would provide useful information about the biology underlying the blood biomarker changes and also will help to identify the most specific members of a putative biomarker panel.

The APOE e4 allele has an increased frequency in people with AD relative to controls. Effects of e4 on lipids may lead to a series of changes in plasma that may be driven by genetic background rather than AD. Several recent studies that measured multiple proteins in plasma in patients with AD and controls identified plasma APOE concentration as one of a panel of diagnostic markers for AD [6-9]; however, the extent of additional predictive value beyond APOE genotyping [10] remains to be clearly established.

Similar questions surround biomarkers of risk. For example, plasma levels of Aβ have been widely studied as a predictor of incident AD. Aβ is produced in both the brain and the periphery and is rapidly cleared from plasma by the liver. Many studies have shown that plasma levels of Aβ do not correlate with CSF Aβ or with brain amyloid burden [11,12]. This is the case for both plasma Aβ40 and Aβ42. Plasma levels of Aβ are influenced by genetic factors and by aging and renal function. Therefore, interpreting changes in plasma Aβ as a predictor of AD is complicated. Although absolute levels of plasma Aβ have not proven to be informative, some studies support the potential utility of a ratio of Aβ42:40 [13-15]. Furthermore, given the spectrum of Aβ species deposited in the AD brain [16], future studies that examine plasma levels of specific Aβ species or modifications could be informative. However, the levels of these species may be even lower than those of Aβ42; therefore, it will be a considerable technical challenge to develop assays that are sensitive enough to allow detection in the blood.

Vascular risk factors and disease processes have systemic and CNS effects and increase in prevalence with age; they are also more likely to be present in patients with a clinical diagnosis of AD relative to controls – older people with dementia often have combined AD and vascular pathology at autopsy. This may drive many of the reported associations between biomarkers that are influenced by vascular factors and AD risk. Risk biomarkers also may be related to genetic risk factors for AD. An important question is whether measuring the protein in plasma provides a measure of risk stronger than simply characterizing the genetic variant itself. For example, levels of clusterin (or Apo-J) in plasma are slightly increased in people who later develop AD in some (but not all) studies [17]; whether this reflects variation in the clusterin gene [18], effects of inflammation, or vascular risk is not certain.

Procedural and technical details are important in biomarker research because many factors other than the disease of interest may influence measurements of potential biomarkers in the periphery. Standardization of procedures – ranging from acquisition, handling, and storage of biosamples, through assay procedures, together with rigorous documentation – is critical. These laboratory medicine, sample handling, and processing issues, which typically are not evaluated in initial AD candidate biomarker studies, can have a huge impact on the levels

of the analytes being studied. Indeed, studies have shown that changes in the candidate biomarker following blood collection can be larger than the expected changes based on the underlying biology. For example, storage can change levels of certain chemokines and cytokines by fivefold or more, time on ice before blood is spun can dramatically alter levels of protein analytes, and the anticoagulant that is used can also change analyte levels [19]. For proteomic studies using plasma or serum, attention to details of sample preparation and storage can also help to reduce variability [20,21]. Thus, one forward-looking recommendation is to require much more rigorous analyses of how sample handling and processing alters a candidate biomarker as well as much tighter control of sample processing before initial publication of human study results. These issues could present a formidable challenge for large multi-center studies, but given the known confounds related to sampling handling and processing and the lack of reproducibility across studies of most peripheral AD biomarkers to date, this challenge needs to be addressed. The effects of time of day (diurnal variation occurs for many analytes), fasting, renal function, and medications need to be carefully considered. In proteomic (and other -omic) studies, detailed examination of how technical variables (sample collection, processing, and storage) and biological variables influence the analytical readout should precede large-scale analysis of biosamples.

Assay methodology is important and includes determining sensitivity, cross-reactivity, and test-retest (short-term) reliability. Traditional platforms such as enzyme-linked immunosorbent assay for protein quantitation have been most widely studied. Multiplex methods, though popular and potentially efficient, have not always undergone rigorous quality control. Calibration of assays with standards (for example, recombinant proteins or reference standards prepared from large pools of patient samples) can help to improve consistency and reproducibility across assay runs. Methods of calibration for proteomic techniques such as mass spectrometry – in particular, the use of isotope-labeled internal standards – have enhanced the early phases of diagnostic biomarker discovery [22]. Plasma may contain heterophile antibodies or other sources of interference or cross-reactivity with assays, which need to be defined before large-scale studies are undertaken. For biomarkers that are intended for use in regulatory studies (for example, clinical trials), use of validated assays with documented analytical precision and clinical sensitivity is critical. As an example, extensive validation of a commercial assay for plasma Aβ, to serve as a readout for a clinical drug development program, has been reported [23]. For mature assays that are ready for widespread use, harmonization efforts can help to ensure assay and data quality and to facilitate comparisons of results of studies across different sites [24].

The design of clinical studies requires careful attention at every stage. During the discovery stage, samples from well-diagnosed cases and controls need to be used. Because older individuals may often have preclinical AD pathology, characterization of controls by using methods such as amyloid imaging or CSF biomarkers can add to stringency at this stage of the study. Controls should be matched with cases for demographic variables such as age and sex. To study how aging affects the biomarkers under consideration, controls representing a wider age range may be worth including. Statistical considerations include adequate sample size to be able to detect reasonable discrimination effects. Replication and validation cohorts in diagnostic studies are essential. These cohorts should include separate sets of patients with AD at whatever stages are being studied as well as cognitively normal healthy controls. Controls with other neurodegenerative disorders as well as systemic diseases may be helpful in interpreting mechanisms related to biomarker changes and are important in determining the disease specificity of putative biomarkers. Comparison with a subset of patients and controls who have been followed to autopsy provides the highest-quality gold standard. For studies of risk biomarkers, incident cases of AD are essential. In studies looking at multiple biomarkers or using proteomic, genomic, or other multi-analyte approaches, data analysis and study design are critical because of the potential for false-positive discovery in these studies; validation using multiple sample sets is essential. These and other issues that are important in reporting the accuracy of diagnostic tests are being summarized in the STARDdem initiative [25].

Conclusions

The concept of blood tests as biomarkers for AD is appealing, and these could be put to many uses, such as screening, diagnosis, and risk assessment, and as an aid to drug development in clinical trials. However, the plausibility that changes in the blood reflect mechanisms of neurodegeneration in the brain, and the dilution of proteins and other analytes as they traffic from the brain to the CSF and then to the bloodstream, results in a considerable analytical detection challenge. Awareness of the potential problems at each stage of discovery, development, and clinical validation of a blood biomarker is important in formulating a comprehensive plan that will yield clearly interpretable data. The survey of peripheral biomarkers to be covered by *Alzheimer's Research & Therapy* will include plasma Aβ, multi-parameter plasma, and serum biomarkers and a review of biomarkers of risk that have emerged from population-based and longitudinal studies. Novel approaches to identifying biomarkers

in plasma include measuring immune responses to changes that presumably originate in the brain in AD. As sensitive and novel technical approaches are developed and study design receives greater care, the potential of blood biomarkers for AD will be clearly tested.

This article is part of a series on *Peripheral Biomarkers*, edited by Douglas Galasko. Other articles in this series can be found at http://alzres.com/series/biomarkers

Abbreviations

Aβ, amyloid beta (protein); AD, Alzheimer's disease; APOE, apolipoprotein E; CNS, central nervous system; CSF, cerebrospinal fluid.

Competing interests

DDG has served as a paid consultant for Elan Pharmaceuticals, Inc. (Dublin, Ireland) and serves on Data and Safety Monitoring Boards for clinical trials for Janssen Pharmaceuticals, Inc. (Titusville, NJ, USA), Elan Pharmaceuticals, Inc., and Balance Pharmaceuticals, Inc. (Santa Monica, CA, USA). He has received research funding from the National Institutes of Health, the Michael J Fox Foundation, and the Alzheimer's Drug Discovery Foundation. TEG is a co-inventor on several patents relating to Alzheimer's therapeutics and has consulted or served on advisory boards for Elan Pharmaceuticals, Inc., Novartis (Basel, Switzerland), Genentech (South San Francisco, CA, USA), Roche (Basel, Switzerland), Bristol-Myers Squibb Company (Princeton, NJ, USA), and Pfizer Inc (New York, NY, USA).

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