

Biomarkers for Alzheimer's disease – Clinical needs and application

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Abstract. Biomarkers for Alzheimer's disease have many diagnostic and therapeutic uses. They can be used to facilitate diagnosis, particularly early diagnosis and possibly presymptomatic diagnosis. In evaluating treatment aimed at modifying the course of AD, biomarkers can help to assess whether the drug is hitting a target or influencing disease mechanisms in the brain, and can help with dose-finding. In definitive clinical trials, biomarkers can be used as surrogate outcome measures. Progress has been made in investigating candidate biomarkers in cerebrospinal fluid and plasma. At present, cerebrospinal fluid levels of amyloid- β , tau and phospho-tau come closest to meeting criteria proposed for useful biomarkers for diagnosis. Other biomarkers can provide insight into processes such as inflammation and oxidative damage. Unbiased and broad-based searches for novel biomarkers using proteomics have much potential. As efforts to diagnose and treat AD move towards earlier intervention and even towards prevention, biomarkers will gain significance as tools to aid research and ultimately clinical practice.

Keywords: Alzheimer's disease, amyloid- β , biomarkers, tau

1. Introduction

Alzheimer's Disease (AD) is the most common form of dementia in the elderly. Much research progress has been made in understanding mechanisms and molecules implicated in the disease, but there are many unmet needs. In particular, with increased awareness among clinicians and the population at large that memory abilities can change with aging and that AD is common, there are increased demands for evaluation of memory complaints. Although it is often straightforward to make a clinical diagnosis of dementia and recognize a pattern as indicating AD, the diagnostic arena can pose challenges even for expert clinicians. First, very early diagnosis can be tricky, because deficits are mild and more likely to overlap with changes associated with aging, general medical disorders, or conditions such as depression. Second, the differential diagnosis of unusual presentations of dementia can be difficult. Finally, it may be difficult to decide whether AD is contributing to multifactorial causes of demen-

tia, with more than one underlying disease process, for example, Lewy Body disorders, dementia in the setting of depression, or AD plus vascular dementia, termed mixed dementia.

The treatment of AD is currently unsatisfactory. Symptomatic treatments are available, notably acetylcholinesterase inhibitors, and show mild clinical benefits without affecting the fundamental pathology underlying AD. No treatment has been shown to be disease-modifying – i.e., to slow the clinical progression of dementia or delay the onset of disease. Clinical trials examining disease-modifying effects typically use clinical rating scales that measure cognitive test performance and assess global daily performance or activities of daily living as outcome measures. These are subject to much variability, and it may be difficult to distinguish symptomatic from disease-modifying effects.

This brief background sets the stage to consider roles for biomarkers in AD. In principle, biomarkers could provide more precise measures of disease state or activity than clinical measures, with less variability, and

could enhance diagnosis and measurement of treatment effects. Research into biomarkers has taken many directions. Advances have been made in neuroimaging techniques that assess regional structure, function and biochemistry of the brain, and in identifying biochemical indices of brain dysfunction, measured in body fluids such as cerebrospinal fluid (CSF). This review will focus on progress and problems related to biomarkers in CSF and plasma, but many of the principles of biomarker development and uses are also relevant to imaging. Integrating information from biomarkers that provide information about the anatomy and biochemistry of the brain is likely to provide the most robust indices of processes and structural changes that are important in AD.

2. Principles of biomarker development

In 1998, a Working Group convened by the Alzheimer's Association and the National Institute on Aging proposed a set of guidelines for biomarkers for AD [1]. The key elements of their recommendations were that biomarkers should reflect a basic pathogenic or pathologic feature of Alzheimer's Disease, and needed to be able to be measured sensitively and reliably. If used for diagnosis, a biomarker should have sensitivity and specificity of at least 80%. Diagnostic sensitivity should be present even in very mild AD, and the diagnostic accuracy should be verified in autopsy-confirmed patients. The marker should be convenient and inexpensive. While the ideal has not been met, currently available markers satisfy many of these proposed criteria.

2.1. Assay development

Biomarkers in CSF and peripheral body fluids are being developed using two approaches. First, candidate biomarkers are identified and measured, based on their presence in lesions of AD or their roles in disease pathways implicated in AD. Second, broad-based approaches such as proteomics can be used to identify and measure a large number of molecules in an unbiased manner, and apply statistical methods to develop combinations or panels that distinguish disease state from control. Initial approaches to develop candidate biomarkers were derived from knowledge about pathologic markers of AD. Amyloid deposition in the brain is associated with aging, but is more prominent in AD, where deposits develop into plaques that mature and

are surrounded by degenerating processes of neurons (neurites) and inflammatory cells such as activated microglia and reactive astrocytes. Amyloid deposition reflects changes in the balance between production and clearance of amyloid- β protein ($A\beta$), resulting in its aggregation to form oligomers and fibrils that can be deposited. $A\beta$ is produced by proteolytic cleavage of the parent molecule amyloid- β protein precursor ($A\beta$ PP), which releases several species of $A\beta$ [reviewed in 2]. Most of the $A\beta$ ends at amino acid 40 in the molecular sequence, but longer forms of $A\beta$ ending at amino acid 42 comprise the vast majority of $A\beta$ that is deposited in the brain. $A\beta$ 42 is more prone to aggregate to form oligomers and fibrils, and has greater *in vitro* toxicity than $A\beta$ 40 [2,3]. Biomarker research has thus focused on measuring $A\beta$ 42 as a specific index related to amyloid. Because oligomers may have toxic effects in the brain, recent efforts have been made to develop assays specific for these forms of $A\beta$. Neurofibrillary tangles (NFT) are the other major structural lesion of AD [4]. They are composed of paired helical filaments (PHF), made of insoluble and highly phosphorylated forms of the microtubule-associated protein tau. Many research groups have used sensitive assays to measure different forms of tau in CSF as markers of neuronal pathology or tangle formation. Other processes implicated in AD include oxidative damage, inflammation, neuronal death, synaptic damage, and microglial and astrocytic activation [5]. A host of candidate molecules can be named in relation to these processes, and many have been sought in CSF.

Assay technology for measuring biomarkers is relevant to clinical needs. In recent years, most quantitative assays for proteins have used sandwich ELISAs, which depend on the availability on specific antibodies directed against different epitopes on the analyte of interest. As combinations of biomarkers are measured, multiplex assay technologies, that allow simultaneous measurement of several markers, will become increasingly important [6]. Other ways to measure biomarkers, for example mass spectrometry, or liquid chromatography, while very sensitive, are more difficult to standardize and apply uniformly at different laboratories. Assay convenience would be optimal if the biomarkers could be measured at the place where the patient is seen, i.e., the point of care. This has not been achieved for AD biomarkers, and currently available markers need to be measured at a research or custom laboratory. Standardization of assays is important to permit their use on a widespread basis. This can be a challenge, as illustrated for ELISAs for $A\beta$ 42 and tau, in which

published studies have yielded widely varying ranges of normal values and cutoff points by different research groups. Even though assay kits are commercially available, quality control and standardization need to be implemented to allow different laboratories to compare results. This problem can attain even greater magnitude for multiplex approaches, where groups of biomarkers are assayed, and in proteomic assays.

2.2. Study design

Many studies on CSF biomarkers for AD have included patients evaluated at tertiary referral clinics, often with clinically obvious AD, compared to small numbers of controls. While this may be appropriate for a pilot study of whether a novel biomarker or assay discriminates between these groups at all, it is not an appropriate dataset to derive estimates of sensitivity and specificity. A more definitive study should include a large number of patients, with causes of dementia that include AD and non-AD, including early stages of dementia, and controls who are matched for age. For the data to have the widest generalizability, the patients should be typical of those seen in community rather than referral settings. A 'gold standard' clinical diagnosis should be made by expert clinicians, against which the biomarkers are calibrated. This may require follow-up, for example in the case of subjects with Mild Cognitive Impairment (MCI), in whom clinical progression to dementia is the decisive outcome. Although the guidelines for biomarkers for AD emphasize validation against neuropathological examination of the brain, this is impractical because of the many years of follow-up required and the difficulty in obtaining this information on a large scale. The storage of CSF and plasma from subjects who take part in longterm studies that include autopsy permits the development of archival sets of samples to facilitate these comparisons. Postmortem CSF samples are problematic, because many constituents are significantly altered by postmortem autolysis and other biochemical changes, even after short postmortem times.

Handling of CSF and plasma is important. To minimize degradation of markers, samples of CSF and plasma should be aliquotted and frozen as soon as possible after collection. Centrifugation of samples of CSF before storage, and freezing at ultracold temperatures does not affect levels of markers such as A β and tau [7]. Polypropylenes tubes are best for storage, to minimize adhesion to the tubing by sticky molecules such as A β . Effects of circadian rhythms on biomarkers have not

been well studied; one way to avert this problem is to standardize the time of biomarker collection, e.g, to the early morning. CSF concentration gradients have been examined for many biomarkers. As one example, ventricular CSF levels of A β 42 are lower than those in lumbar CSF, but there is no appreciable concentration gradient for A β 42 or tau across the first 30–40 ml of CSF obtained from lumbar puncture.

Calculation of cutoff points, and determining sensitivity and specificity is quite straightforward. An important aspect of diagnostic tests that has not been well studied in AD is the additive value of the test to clinical practice. Most AD biomarker studies have used patients and controls evaluated at tertiary referral centers. Sensitivity and specificity is then calibrated against clinical diagnosis by experts as a gold standard. What is missing is a clinical diagnosis made before and after the application of a biomarker, to determine the incremental diagnostic value added by the biomarker. For studies of predictive biomarkers in asymptomatic or minimally symptomatic individuals, there is a different problem, namely following up a cohort of asymptomatic individuals for long enough to have a number of them develop AD so that the risk associated with different levels of the biomarker can be assessed.

3. Diagnostic biomarkers for AD

The clinical diagnosis of established AD is relatively straightforward. When made by expert clinicians, it carries a likelihood of 90% or higher of finding AD pathology at autopsy [8]. The clinical diagnosis of non-AD dementias, on the other hand, is less accurate. For clinicians who are not dementia specialists, the amount of time available to assess a patient in practice is also a limitation. Therefore the clinical diagnosis of dementia and AD in routine clinical practice could potentially benefit from incorporating biomarkers into the evaluation. In recent years, very early diagnosis of cognitive decline has been emphasized, and the syndrome of Mild Cognitive Impairment (MCI) was proposed, to describe patients who have symptoms of cognitive decline, usually involving memory, with mild but objective deficits measured on testing, but where performance does not meet sufficient impairment for a diagnosis of dementia [9]. MCI carries a high risk of progression to AD, ranging from 5 to 15% per year, particularly when it is defined to include memory decline as the cardinal feature (amnestic MCI) [9,10]. MCI usually needs to be evaluated and characterized with a panel of psychome-

tric test, and clinical follow-up is the “gold standard” for determining whether the patient is showing progressive deficits consistent with mild dementia. Biomarkers could help to increase the likelihood that AD or other brain lesions are present in patients with MCI, which could identify most clearly those patients most likely to benefit from specific treatment.

3.1. Candidate biomarkers: tau, P-tau and A β

A large number of studies have shown that CSF tau is increased and A β 42 is decreased in patients with AD relative to controls [reviewed in 11,12]. Sensitivity for distinguishing AD from controls exceeds 80% in most large studies. However, specificity has not been as high, and increased levels of tau can be seen in a percentage of patients with other dementing disorders, particularly those with rapid courses, such as Creutzfeld-Jakob disease [13]. A reason for this overlap is that assays for CSF tau measure total tau, i.e., all forms of this molecule in CSF. Tau can be released from damaged neurons or axons regardless of the cause, and therefore any disorder that damages or destroys enough neurons will release tau into CSF. Thus very high total tau levels are found in the CSF after acute stroke or trauma to the brain.

Low levels of A β 42 can be seen in patients with non-AD dementias, though less commonly than in AD, and may reflect age-associated amyloid deposition or coexisting AD, which may be a common finding in vascular dementia. Thus low CSF A β 42 lacks disease specificity for AD. To improve diagnostic discrimination, the combination of A β 42 and tau has been assessed, either as a ratio or by using more complex statistical approaches [14,15]. This improves specificity, sometimes at the expense of sensitivity. Another approach has been to measure forms of these proteins that may be more closely linked to AD. For example, ELISAs have been developed against several forms of phospho-tau (P-tau), notably P-tau181 or P-tau231, both of which are residues of tau that are phosphorylated in tangles. Phospho-tau appears to be a more specific biomarker than total tau. For example, phospho-tau levels are not elevated after acute stroke [16], and are much less frequently elevated than total tau in Lewy body dementia [17].

A number of studies have assessed A β , tau and P-tau levels in patients with MCI [reviewed in 18]. In general, the findings parallel those in AD studies, namely that increased tau and P-tau, and decreased A β 42 are present in patients with amnesic MCI compared to

controls, and appear to have high sensitivity to identify those MCI patients likely to progress to AD. To date, the size and scope of these studies has been relatively small, with most studies examining fewer than 50 MCI patients. Nevertheless, the findings that these markers are altered in many patients with MCI mirror those of AD. CSF A β 42 may decrease even before the development of memory complaints, as recently shown in a group of asymptomatic carriers of presenilin or A β PP mutations, whose A β 42 levels were lower than those of age-comparable controls [19], and in a study of asymptomatic 85-year old women, some of whom progressed to develop dementia [20].

A β aggregation and oligomer formation may be an important step in AD. For example, oligomers can produce synaptic damage and dysfunction *in vitro* and have been implicated in transgenic mouse models of AD. It has been difficult to develop sensitive detection methods specific for oligomers. Recently, oligomer-specific antibodies were used in an extremely sensitive nanoparticle-based assay. A preliminary study reported the detection of attomolar levels of A β oligomers in CSF, with increased levels found in AD compared to controls [21]. More widespread studies of oligomers are eagerly awaited.

3.2. Other candidate biomarkers

Processes such as inflammation or oxidative damage are not necessarily unique to AD, but may occur in other brain disorders. Biomarkers reflecting these processes are therefore likely to discriminate between AD and controls, but less well between AD and other dementias. F2-isoprostanes were identified as stable markers of oxidative damage, as discussed in detail by Montine in this issue. Assays have been developed against total isoprostanes or a specific form of an F2 isoprostane. Studies indicate that levels are elevated in CSF in AD, but to a lesser extent in other disorders [22]. Markers of inflammation such as cytokines and chemokines are altered in the brain in AD [reviewed in 23]. However, they have been less consistently shown to be altered in CSF in AD, and although some studies raise the possibility of increased levels of some cytokines early in AD [24], findings have not been widely confirmed and diagnostic specificity has not been established. S100B is a molecule secreted by reactive astrocytes. Although it has been found to be increased in AD in CSF [25], it shows overlap with values in controls and lacks disease specificity. Because AD pathology develops gradually, and most likely predates the clinical syndrome of AD,

it may not be possible to discover a unique molecule that is altered in AD but unchanged in controls or any other condition.

3.3. Peripheral biomarkers

Blood or urine would be a more convenient source of a biomarker than CSF. However, it is unclear how a protein or other substance that originates as a result of brain pathology would survive transit from the brain to the blood and dilution from the CSF space to the much larger blood volume, and still be measurable by conventional assays. Tau and phospho-tau have been undetectable when ELISA methods were applied to plasma samples. $A\beta$ species can be measured in plasma by ELISA, at concentrations that are 20 to 30 fold lower than those seen in CSF [reviewed in 26]. There is no correlation between plasma and CSF levels of $A\beta_{40}$ or $A\beta_{42}$, therefore it is unclear how much of plasma $A\beta$ is produced by the brain versus in the periphery [27]. The reduction of CSF $A\beta_{42}$ seen in patients with AD is not reflected by a similar reduction in plasma. Plasma $A\beta_{40}$ and 42 increase with age and also are increased in relation to renal function as indexed by serum creatinine. There were no significant differences between patients with AD, MCI or controls in one large cross-sectional study [28]. However, a prospective study has suggested that elderly individuals whose plasma $A\beta_{42}$ levels were in the highest quartile had an increased risk of developing AD in the next 2–3 years [29]. Different antibody sets were used in these studies, and the discrepant findings need to be resolved.

Several interesting findings regarding peripheral markers need further study to determine their utility and generalizability. One research laboratory measured levels of a specific isoprostane in plasma and reported increased levels in AD and Down's syndrome [30]. Two other groups have measured the ratio between different forms of $A\beta$ PP in platelet preparations, and found that in AD, there was a relative decrease in high molecular weight forms of $A\beta$ PP compared to lower molecular weight forms [31,32]. The underlying biology of these changes in $A\beta$ PP processing has not been explained, and further research is required.

At present, no plasma tests are supported by adequate data to serve as diagnostic aids to AD or other dementias. Because of the ease of procurement of blood, using biomarkers to predict the risk of AD among older individuals is an appealing application. Higher levels of markers such as cytokines, homocysteine and $A\beta_{42}$ measured in plasma have been found to be associated

with increased risk of AD in some studies [reviewed in 26], and this topic is discussed in more depth by Holtzman. Replication studies and accurate estimates of the extent of risk are needed to make a stronger case for the value of antecedent biomarkers.

4. Treatment and biomarkers

There are many potential applications of biomarkers in the treatment of AD. Disease-modifying treatment and anti-amyloid strategies are priorities in AD. The quest to develop and validate such treatments has been difficult, and many questions need to be answered to demonstrate drug safety and efficacy. An important role for biomarkers is to show that the drug hits a target or affects mechanisms or pathways of interest. Even though data from animal models may predict successful clinical trials, this has not yet been demonstrated in AD. Since animal and cellular models often involve overexpression of pathogenic genes, it is unclear whether interventions in the model systems will carry over to patients with late-life sporadic AD. Dose-finding cannot always be inferred in extrapolating from small animal models to adult humans. To guide drug development, CSF and plasma biomarkers are increasingly being used in Phase I studies of novel compounds directed against AD. Several issues are important in planning such studies. The degree of variability of the biomarker, both within and between subjects, needs to be known, because it is a determinant of sample size. For example, CSF levels of $A\beta_{42}$ and total tau show a persistent pattern of change in patients with AD, with a strong correlation of absolute levels on repeated lumbar punctures separated by periods as long as 6 to 12 months [33–35]. The half life or clearance of the marker from the CSF is important, particularly in planning short-term studies. Data from patients with acute stroke who underwent serial lumbar punctures suggests that tau has a half life in CSF of about 6–8 weeks [16]. Data from animal studies suggest that $A\beta_{42}$ in CSF and plasma has a half life of about 30 to 60 minutes [36]. The choice of patient population may be important in selecting biomarkers and analyzing drug effects. For example, early phase and dose-finding studies are often done in healthy individuals. For $A\beta_{42}$, this may be advantageous because CSF levels are not altered due to deposition in the brain and drug effects may be more clearly identified by measuring CSF $A\beta_{42}$ than if the study is done in patients with AD. On the other hand, CSF tau, P-tau and other markers related to damage or

degeneration are not altered in normals, therefore potential neuroprotective effects of treatment on biomarkers need to be shown in patients with AD. For example, CSF tau remains stably elevated in patients with AD. This suggests that tau reflects ongoing neuronal damage and continued release of this protein into the CSF. If a biomarker leads to a decrease in neuronal damage or neurodegeneration, then it may decrease levels of tau over time.

In order to obtain the most information from early phase biomarker studies, it may be useful to measure combinations of biomarkers. This could include markers in both plasma and CSF. Although, as mentioned above, plasma A β is not diagnostically altered in AD, levels of plasma A β may be decreased by drugs that affect the production of A β from APP, such as gamma secretase inhibitors. This could be used to establish whether changes in plasma A β are predictive of changes in CSF A β or other biomarkers and helps to provide pharmacodynamic data on the study drug. One recent study of a γ -secretase inhibitor found that plasma A β levels decreased after administration of the drug, whereas CSF levels showed only a trend to decrease [37]. Predictions from early phase studies could be extrapolated to measures of plasma biomarkers in a larger-scale trial.

In pivotal studies aimed at modifying the course of Alzheimer's Disease, clinical outcome measures are essential to calibrate a biomarker signal. Brain imaging, in particular structural measures obtained from MRI, is the most widely applicable biomarker that could be collected in a multicenter study. It would be difficult to collect CSF except in a subset of patients, although plasma or urine could be obtained from all study participants. Recent experience in attempts to modify amyloid by immunization against A β have illustrated some of the promise of biomarkers as well as pitfalls. A multicenter clinical trial of active immunization in AD was prematurely stopped because encephalitis, sometimes severe, occurred in 8% of participants. A number of study participants had serial brain MRIs and a subset of these also had serial CSF studies. Although there was a trend towards slowing of cognitive decline in patients with high antibody titers compared to those who did not raise antibodies, biomarkers were difficult to interpret. Brain volume measured from MRI scans showed a decreased total brain volume in patients with high antibody titers, and a decrease in serial levels of CSF tau [36]. Although a serial decrease in CSF tau is consistent with slowing of neurodegeneration, this coincided with increasing atrophy on MRI, and the interpretation of the significance of these changes is difficult in the setting of an interrupted clinical trial.

5. Conclusions

Much progress has been made in studying candidate biomarkers, identifying markers of disease mechanisms, and initiating proteomic searches for novel markers related to AD. At present, biomarkers in CSF show promise as diagnostic aids, although the optimal studies to determine their usage in practice have not been done. CSF tau, phospho-tau, and A β 42 are examples of biomarkers that meet many of the criteria proposed by an expert working group, against which newer biomarkers can be calibrated. Treatment of AD is a greater challenge than diagnosis, and it is in this arena that biomarkers have much to offer in the development of disease-modifying drugs.

Disclosure

Dr. Galasko serves on the advisory boards of Elan Pharmaceuticals, Wyeth Pharmaceuticals, Eisai-Pfizer, and Eli Lilly.

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