From proteomics to biomarker discovery in Alzheimer’s disease

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Abstract

Alzheimer’s disease (AD) is the most common form of dementia in the elderly. AD is an invariably fatal neurodegenerative disorder with no effective treatment or definitive antemortem diagnostic test.Littleis known about the changes in the brain preceding or accompanying initiation of the disease. Understanding the biological processes, which occur during AD onset and/or progression, will improve the diagnosis and treatment of the disease. As we will discuss in this review article, using high-throughput cDNA microarray we identified candidate genes whose expression is altered in the brain of cases at risk for AD dementia. However, it is possible that the use of the cDNA microarray technology alone may underestimate post-transcriptional modifications and therefore provides only a partial view of the biological problem of interest. As such, the combination of cDNA and protein arrays may provide a more global picture of the biological processes being studied. Based on this hypothesis, we initiated a series of high-throughput proteomic studies and found that the expressions of proteins involved in synaptic plasticity are selectively altered in the brain of cases at high risk to develop AD dementia (mild cognitive impairment; MCI). This is consistent with our cDNA microarray evidence showing that the expression of a-type synapsins is selectively altered in the brain of MCI cases. Collectively, these studies support the feasibility and usefulness of high-throughput cDNA microarray and proteomics techniques to study the sequential changes of distinctive gene expression patterns in the brain as a function of the progression of AD dementia.

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1. The use of high-throughput technology in search of molecular indexes of early AD dementia

Critical questions in understanding the classic neuropathological features of AD (amyloid plaques and neurofibrillary tangles (NFT)) are if their occurrence precedes, is in synchrony with, or follows the earliest and mildest signs of cognitive decline. Indeed, frequently used diagnostic approaches to AD reflect more the progression of neuropathology and minimize the cognitive symptoms of the disease, especially when the deficits are mild or moderate. For example, Haroutunian et al. [31] found that cortical AD neuropathology (e.g., neuritic plaque pathology) increases significantly when the very first signs of dementia become detectable. With the exception of the visual cortex (VC), relative to cognitively intact subjects, AD neuropathology is already detectable in cerebral cortex regions (e.g., superior temporal gyrus (STG), entorhinal cortex (EC), inferior temporal gyrus) and in the hippocampal formation of early AD dementia cases. In some of these brain regions, AD neuropathology continues systematically with increasing severity of dementia [2]. The similarity of neuritic plaque pathology in the different neocortical regions in the early stages argues against a clear focus of initial neocortical abnormalities with subsequent recruitment of other regions as a function of increasing dementia. Moreover, increases in neuritic amyloid plaque density are reflected by similar increases in the levels of β-amyloid (Aβ)-peptide [34], adding biochemical precision to the conclusions drawn from the neuropathological studies [61]. However, while AD neuritic Aβ plaque neuropathology might significantly influence the severity of dementia during early AD, other factors, such as NFT pathology, which appear at relatively later AD dementia stages [31], may also contribute to the dementia progression. This information is of high interest and suggests that specific neuropathological features may influence AD dementia at specific stages of the disease. However, little is known about the molecular mechanism underlying the clinical progression of AD. To yield information relevant to the understanding of the molecular mechanisms involved in the onset and progression of clinical AD dementia, we initiated a series of high-throughput array studies (cDNA and proteomics) exploring the sequence of changes in gene expression in the brain of cases at high risk to develop AD (MCI) relative to normal neurological control cases, and eventually, during the conversion from MCI through the early stages of AD dementia.

2. Proteomics and the search for novel indexes of AD clinical progression

Proteomics has emerged in the last few years as a multidisciplinary and technology-driven science that focuses on proteomes: the complex of proteins expressed in a biological system, their structures, interactions, and post-translational modifications. In particular, proteomics examines changes in protein levels and other protein alterations that result from or foster specific diseases, or are induced by various external factors, such as toxic agents. The disciplines that study protein structure, function, and level are called structural, functional, and expression proteomics, respectively. Expression proteomics, although itself under development, is the most advanced and widely used proteomic technology today. When we speak of proteomics we usually mean expression proteomics, as is the case of changes in steady-state levels of brain proteins in certain conditions (e.g., in neurodegenerative disorders). Although the term proteomics is only a few years old, its roots go back to the 1980s when the usual methods of protein identification were immunoblotting and co-migration with known purified proteins in one-dimensional gel electrophoresis technique [65]. As we will discuss below, the development of proteomics can be attributed primarily to the refinements in mass spectrometry (MS), improvements in computer and software sciences, and the flood of data now available from genomic sequencing of many organisms. The future promises the release of much more information. As a consequence, several recent studies have used this new technology to investigate common health concerns, including but not limited to, aging [12,13], bacterial infections [68], cancer [6,11,17,20,30,39,42,50,55,58,60,63], osteoporosis [41], atherosclerosis [16,21,44], and neuropathic and inflammatory pain [2,27]. As detailed below, protein alterations in brain of early (frank) AD dementia or in subjects at high risk for developing dementia have been
investigated and the research continues to proliferate [8,10,14,40,53,57,66]. As further discussed in the following sections, using cDNA microarray [53], and most recently proteomic technologies, we identified a series of novel gene products (biomarkers) whose abnormal expression in the brain may play an important role in the progression, and possibly, the onset of AD dementia.

3. cDNA microarrays and the search for novel biological indexes of AD clinical progression

In high-throughput cDNA microarray studies in our laboratory, we screened 6794 (Incyte VI human microchips) human genes in the brains of cases characterized by moderate dementia, assessed by clinical dementia rating (CDR) 2 [33,35]. We found 32 genes (25 known and 7 EST) with >1.8 altered expression in the superior temporal gyrus relative to cases characterized by normal cognitive status [33]. Among others, we found down-regulation of the synaptic vesicle protein synapsin IIa, which normally plays an important role in synaptic vesicle turnover and neurotransmitter release [29,32]. We decided to continue our investigation by assessing the changes in protein expression of the known splice variants I, II, and III of the a- and b-type synapsin isoforms [32] by Western blot analysis. We examined brain regions at high risk (EC) or relatively unaffected (VC) during AD or in normal neurological control cases. Interestingly, relative to age matched control cases characterized by normal cognitive status (CDR 0), a >2-fold decrease of the splice variants I, II, and III of the a-type synapsin isoform was found in the EC of MCI cases (CDR 0.5–1) as compared to cognitively normal individuals [45].

While the mechanisms leading to the selective altered expression of a-type synapsin in the EC of MCI cases is unknown [33], independent studies in our lab found that excitotoxicity, but not β-amyloid (Aβ1–42) toxicity, recapitulates the selective altered expression of a-type synapsin in cortico-hippocampal neurons, in vitro [47] (Ho and Pasinetti, unpublished observations). Finally, because recent evidence suggests that MCI clinically progresses to greater dementia severity at rates dependent on the level of cognitive impairment, and that MCI cases often have the neuropathologic features of AD [33], our studies provide a novel biological basis for a better understanding of the mechanisms underlying the conversion from MCI to frank AD dementia. Collectively, our cDNA microarray studies strengthen the utility of our systematic approach of exploring molecular events in the brain of MCI cases and the subsequent development of independent studies to further clarify underlying mechanisms of clinical progression from MCI to AD dementia.

4. Identification of novel candidate proteins whose expression in the brain is altered in MCI cases relative to normal neurological controls

As discussed above, we note that cDNA microarray techniques primarily provide a snapshot of steady state mRNA levels at one specific time under certain conditions, potentially underestimating the occurrence of post-transcriptional and translational modifications of target gene expression in the brain. In high-throughput cDNA microarray studies in our laboratory, we screened 6794 (Incyte VI human microchips) human genes in the brains of cases characterized by moderate dementia, assessed by clinical dementia rating (CDR) 2 [33,35]. We found 32 genes (25 known and 7 EST) with >1.8 altered expression in the superior temporal gyrus relative to cases characterized by normal cognitive status [33]. Among others, we found down-regulation of the synaptic vesicle protein synapsin IIa, which normally plays an important role in synaptic vesicle turnover and neurotransmitter release [29,32]. We decided to continue our investigation by assessing the changes in protein expression of the known splice variants I, II, and III of the a- and b-type synapsin isoforms [32] by Western blot analysis. We examined brain regions at high risk (EC) or relatively unaffected (VC) during AD or in normal neurological control cases. Interestingly, relative to age matched control cases characterized by normal cognitive status (CDR 0), a >2-fold decrease of the splice variants I, II, and III of the a-type synapsin isoform was found in the EC of MCI cases (CDR 0.5–1) as compared to cognitively normal individuals [45].

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These results provide novel molecular evidence for the importance of selective altered gene expression during the earliest detectable stage of AD dementia. In view of the evidence that synapsin family members are important in the regulation of neurotransmitter release, our cDNA microarray studies suggest that the altered regulation of selected synapsin-mediated neurotransmitter release may be involved in the early phase of AD cognitive decline. While previous evidence suggested that MCI is characterized by neuronal loss (e.g., in the EC) and qualified neuropathologically for diagnosis of frank AD [45], we note that the MCI cases used in our cDNA microarray studies did not meet neuropathological AD criteria [33]. Most importantly, we also found that the expression of the pre-synaptic plasma membrane protein synaptophysin was unaffected in the EC of MCI cases, suggesting that the decreased expression of the a-type synapsin occurred in absence of structural loss of synapses. Thus, as summarized in Scheme 1, our working hypothesis regarding potential mechanisms involved in the conversion from normal cognitive status to MCI and eventually to AD dementia is that a potential selective and specific malfunction of synapses that are morphologically intact (e.g., altered synaptic vesicle release-cycling) might underlie the initial cognitive decline in the MCI cases. However, we also note that a recent study indicated a minor decline of synaptophysin expression in the frontal cortex of cases characterized by MCI and also mild dementia (CDR 0.5–1) as compared to cognitively normal individuals [45].

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products of interest. While in independent cDNA array studies we continued the characterization of 25 known candidate gene products whose expression is selectively altered in the brain of early AD [53], we initiated PowerBlot proteomic (BD Transduction Laboratories, Lexington, KY) studies that employed Western blot techniques for large-scale application and allowed the screening of 750 independent proteins in a single assay. In ongoing studies in the lab we identified 50 candidate protein species whose content is consistently and reproducibly altered (>2.0-fold) in the EC of MCI cases (n = 6) relative to cases normal cognitive controls (CDR 0) (n = 5).

The expression level of these 50 candidate index proteins of early AD was further explored using miniaturized PowerBlot immunoarrays employing a customized cocktail of 50 antibodies raised against our candidate index protein species. As shown in Fig. 1, 23 differentially regulated proteins (>2-fold; P < 0.05) were identified and primarily related to clusters of specific biological variations. Five clusters were found to have patterns that correlated primarily with variations of proteins involved in the following: (1) synaptic functions/neurotransmitter related, (2) cytoskeleton/cell adhesion, (3) cell cycle, (4) apoptosis, and (5) transcription/translation.

To perform an independent validation of the PowerBlot data, we used traditional Western immunoblot techniques to evaluate the level of expression of individual MCI-related protein species across a larger cohort of MCI cases and cognitively normal controls (n = 7–8 per CDR group). Consistent with the PowerBlot evidence, we confirmed a >3-fold decrease in the expression of the synaptic protein tomosyn in the EC MCI cases (Fig. 2A) relative to normal cognitive control cases (Fig. 2B). Tomosyn plays a major role in the mechanisms associated with neurotransmitter vesicle pool refilling in a calcium-dependent manner [69]. This result strengthens the hypothesis that a functional alteration of synaptic functions may be at the basis of cognitive alterations in MCI.

5. Consistencies between cDNA and PowerBlot proteomic arrays

Our cDNA microarray studies discussed above demonstrate that the expression of selected gene products whose activity play an important role in synaptic activities [38] are differentially regulated in the brain of MCI cases. In particular, as discussed in Pasinetti [52], we found that the expression of gene products belonging to synaptic functions/neurotransmitter related included synapsin II [29,32], N-ethylmaleimide-sensitive factor [71], tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activating protein [37] is altered in the brain of AD. Consistent with this evidence, the PowerBlot proteomic immunoblot studies showed alterations in the expression of three additional proteins involved in synaptic vesicle/neurotransmitter related functions (tomosyn [46], Rabaptin-5 [49], and vti-1b [3]) in the brain of MCI cases relative to neurological control cases. Although the identification of altered expressed gene products in our cDNA and PowerBlot array studies may be influenced by a random bias in the selection of target cDNA or the protein assayed, including the possibility of lack of representation...
of pertinent gene products in both arrays, the present studies demonstrate that the two technologies can complement each other in the search of molecular markers of early AD.

6. Brain vs. body fluids: the search for novel biomarkers of AD dementia using surface-enhanced laser desorption ionization (SELDI)–mass spectrometry (MS) ProteinChip technology

As compared to the brain, body fluids have the advantage of being more easily accessible for the identification of potential biomarkers that might reflect AD dementia progression. Procuring serum/plasma (and to some extent, CSF) is a non-invasive procedure and is thus more likely to be of clinical use. However, the identification of a biomarker in these samples requires that the protein be secreted at high enough levels to be identified. Moreover, circulating factors may be secreted by multiple cell types from different organs and may therefore lack specificity for the pathophysiological event being studied in the brain. For example, a complicating factor for biomarker studies in serum/plasma is that levels of albumin, transferrin, and IgG are likely to be more abundant than the levels of the potential biomarker and may interfere with biomarker detection. While the brain may still represent a starting point in the search for novel biomarkers of early clinical AD dementia, ongoing SELDI–MS studies in the lab are exploring variation in protein expression patterns in cerebral spinal fluid (CSF) during the transition from normal cognitive functioning to MCI through frank AD dementia.

SELDI–MS technology is a system that enables rapid protein profiling, identification, and characterization from crude biological samples [25]. In particular, this system uses ProteinChip Arrays that contain chemically (cationic, anionic, hydrophobic, hydrophobic, etc.) or biochemically (e.g., antibody, receptor, DNA, etc.) treated surfaces [60] (Fig. 3). Crude biological extracts (e.g., brain tissue extract, CSF, serum/plasma) are applied to the ProteinChip Arrays, which selectively capture subclasses of proteins with specific physical or biochemical characteristics based on protein interactions with the ProteinChip Array surfaces, as shown for detection of Aβ-peptides (Fig. 4). Using this technology, the molecular size and quantity of individual proteins absorbed on each chip are then directly assessed by a time of flight mass spectrometer [60]. This generates a quantitative protein mass profile of the proteins bound to each of the ProteinChip Array surfaces. While control samples are always run in parallel with the experimental samples, the resulting profiles can be compared directly using a number of integrated software features to highlight relevant changes in the patterns of underlying protein expression. The SELDI technology is highly sensitive; the lower limit of detection is 10 fmol [54]. In addition, protein analysis using the SELDI technology is quantitative and highly reproducible. As such, SELDI technology presently available in our laboratories is more amenable to high-throughput proteomic procedures and presents a viable platform for the discovery and validation of potential biomarkers of early AD dementia. For protein identification
purposes, the same chip platform used for biomarker discovery and validation can easily be incorporated into strategies to facilitate rapid target protein purification. Since purified protein can be digested on-chip with proteases, the same chip platform will be useful for generating peptide maps that can be compared to a peptide database for protein identification. Recent evidence confirmed the utility of the SELDI technology in monitoring proteins and protein profiles for diagnostic applications including staging of disease and disease progression [4,24,54,69]. Most excitingly, a recent study successfully employed the SELDI–MS proteomic technology to identify biomarkers in the circulating cells that predicted HIV-1 associated cognitive impairment with a sensitivity of 100% and a specificity of 75% [43].

7. SELDI–MS proteomics in the search for AD biomarkers in biological fluids

The goal of biomarker discovery studies is to identify protein profiles, possibly in accessible biological fluids, which can ultimately be used to develop a rapid, sensitive, and specific high-throughput diagnostic assay. While we are aware that 2D electrophoresis can effectively resolve a large number of proteins, we are also aware of its limited

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**Fig. 3.** Chemical and biological surfaces for SELDI–MS protein expression profiling and protein interaction assays using ProteinChips technology.

**Fig. 4.** Schematic representation of Aβ detection system using PS10 antibody ProteinChips by SELDI–MS protein expression analysis.
reproducibility rendering it difficult for use in biomarker discovery and validation studies involving multiple cases and multiple disease stages. The most likely scenario for studies using 2D gel technology would require the identification of the protein, the production of a high-quality antibody to the protein, and then the development of an ELISA or other sensitive immunoassay. This may be acceptable if a diagnostic test with 95% accuracy would be involved. However, because of the heterogeneity expected in our samples, e.g., MCI vs. normal cognitive cases, it is conceivable that multiple biomarkers will be required to accurately discriminate among cases characterized by different dementia stages and normal cognitive status. The advantage that SELDI ProteinChip technology has over 2D electrophoresis is that the same chip platform used to identify the biomarker can also be used to develop a rapid, sensitive, and high-throughput assay. Protein profiling using SELDI technology offers a novel means for identifying and eventually monitoring the onset and progression of AD clinical dementia. An extra advantage of using SELDI–MS technology is that it will not be essential to identify the proteins themselves in order to make a diagnosis. Moreover, as further discussed below, by utilizing “groups of biomarkers” we will be not constrained by the sensitivity or specificity of any single biomarker. Next we will discuss the feasibility of using SELDI–MS technology in the identification and characterization of potential prognostic, diagnostic, and possibly biomarkers antecedent of AD and/or MCI.

8. Biomarker discovery in Alzheimer’s disease

An ideal biological marker for AD may represent an essential mechanism of the disease, and its validity should be confirmed by the underlying neuropathology. The biomarker should be sensitive and specific for detecting AD, as well as reliable, accessible, and non-invasive (reviewed in Morris [48]). The development of reliable and valid biomarkers is important because AD is a highly prevalent disorders but remain under diagnosed and under treated. For example, prognostic biomarkers that serve as indicators of AD progression could be used as surrogate endpoints for therapeutic responses, possibly reducing the time, effort, and cost associated with drug trials that currently require large samples to be followed for long time periods to measure clinical and cognitive outcomes [26]. Moreover, diagnostic biomarkers would aid in AD recognition and enable patients to benefit from available symptomatic drug therapies. Perhaps, and most importantly, antecedent biomarkers could be used to identify those cases at high risk for developing AD (MCI). Not only could antecedent biomarkers inform research into the cause or causes of AD, they might also used to predict those individuals who might benefit most from the development of disease-modifying or preventive interventions.

8.1. Aβ content in biological fluids as a prognostic biomarker of AD

Amyloid plaque deposits in the AD brain consist mainly of aggregates of Aβ-peptide [19,26], a 4 kDa peptide derived from proteolytic processing of a large transmembrane amyloid precursor (APP). Two forms of Aβ-peptide, 1–40 and 1–42, of which 1–42 is believed to be the more toxic [22,64], constitute the primary components of the amyloid plaques. Numerous N- and C-terminal truncated forms have also been identified in clinical samples from patients with AD [1]. Precise mapping of the patterns of these various species of Aβ-peptides in tissue extracts and biological fluids would provide a classical example of a prognostic biomarker and eventually contribute to our understanding of AD pathogenesis. In the past, amyloidogenic Aβ-peptides have been analyzed by ELISA, which involves indirect chemical or radioactive detection methods [15,23,36,51]. More recently, the SELDI ProteinChip Aβ assay discussed above proved to be an advantageous approach for the simultaneous detection of multiple Aβ-peptide forms. For example, a monoclonal antibody raised against the N-terminal amino acid sequence 1–10 is covalently coupled to a pre-activated ProteinChip array to create a biologically active surface [7,18]. Moreover, samples of CSF and/or crude cellular or tissue extract can be added to the ProteinChip array, which captures multiple Aβ-peptide fragments that have a common N-terminal but differ at their C-terminals. This assay has been widely used in studies of AD pathogenesis [18,28,67,70], the role of β- and γ-secretase processing of APP [4,5,24], as well as in discovery studies exploring new drugs [5,18], and the potential development of a vaccine therapy for AD [67].

8.2. Protein “combination” as a diagnostic biomarker in AD

A combined increase in CSF levels of the Aβ1–42 peptide and tau proteins was recently proposed as a diagnostic indicator of AD [59]. However, this CSF characteristic does not have the necessary sensitivity and specificity for use as a routine clinical assay. As discussed above, a definitive diagnosis of AD can still only be made by a postmortem assessment of brain neuropathology. There is still demand for a simple biochemical test for early diagnosis of AD, a test that could distinguish AD or MCI patients from normal cognitive elderly subjects and distinguish between AD and other forms of dementia. A recent report is promising. For example, Carrette and colleagues [9], using a SELDI ProteinChip array technique, demonstrated a panel of five polypeptides with significantly altered expression levels (four were up-regulated and one was down-regulated) in CSF of nine AD patients as compared to CSF from 10 healthy control subjects; four of these biomarkers were further purified and identified by MS as cystatin-C, two β-2-microglobulin isoforms, a 4.8 kDa VGF polypeptide, and an unknown 7.7 kDa polypeptide. Using
the combination of these five markers, Carrette and colleagues correctly classified six of the nine AD patients and all 10 controls demonstrating a 100% specificity and 66% sensitivity in a small sample size [9].

The CSF Aβ concentration ratio (Aβ1–42/Aβ1–40) has also been proposed as a diagnostic tool for distinguishing patients with AD from normal control subjects and subjects with non-AD forms of dementia [62]. This assay has a diagnostic specificity of 88% and a sensitivity of 59%. Without a doubt, the five-polypeptide panel test, with a greater specificity and sensitivity, might be more promising as a diagnostic tool. In the future, if this evidence can be validated on a larger set of clinical samples, it could be used as a definitive antecedent biomarker for AD, or perhaps for assessing the probability, severity, and progression of AD as a prognostic biomarker.

8.3. Cytosolic Aβ deposition in eye lens as an AD diagnostic biomarker

Until recently, AD-associated accumulation of Aβ amyloid plaques had only been observed in the brain. However, Goldstein and colleagues [28] have discovered cytosolic Aβ-peptides in lens fiber cells from AD patients. The investigators first detected a high-molecular-weight protein and a low-molecular-weight (around 4 kDa) peptide in protein extracts of human lens. The protein and peptide were identified by Western immunoblotting as full-length APP and Aβ-peptide, respectively, and sequencing with electrospray ionization MS–MS tandem technology confirmed the identities. Tryptic analysis of the peptides of the 4 kDa band excised from the gel revealed a 12-residue sequence that is common to both the Aβ1–40 and Aβ1–42 peptides. The MS–MS tandem technology could therefore not distinguish between the two Aβ-peptide forms. Goldstein et al. [28] then used the SELDI ProteinChip-based Aβ assay to analyze the lens extracts and identified both forms of Aβ-peptides, neither of which was detectable in lens extracts from control subjects. The study tentatively suggests that Aβ can be detected in lens cells of AD cases where it might promote region-specific lens protein aggregation, extra-cerebral amyloid formation, and eventually cataracts.

8.4. Ongoing investigation of antecedent biomarkers in AD

Based on the encouraging cDNA/PowerBlot high-throughput array data discussed above, we have initiated a series of new studies to evaluate the potential of SELDI–MS technology as a means to identify novel protein biomarkers that may be used as indices of onset and eventually progression of early AD cognitive decline. In ongoing studies using SELDI–MS microchips for the detection of negatively charged or copper-binding proteins (Fig. 4), we found a novel protein biomarker whose levels are increased in the CSF of four early MCI cases relative to four normal cognitive control cases [35]. We are currently exploring the possibility of using this novel protein as a biomarker across the entire spectrum of clinical AD by assessing its regulation in CSF as a function of progression of clinical dementia (unpublished observations). Work is presently in progress in our laboratory to purify this protein and to determine its identity by amino acid sequencing as a means to understand its biological functions in normal and pathologic conditions.

9. Future perspectives

It is clear that the combination of high throughput cDNA and protein microarrays will have a significant effect on neurobiological research and on efforts to discover the mechanisms involved in neuropsychiatric diseases. Although these approaches are powerful, we note that they should be used to complement other traditional methods, such as those used in molecular biology and genetics. Most importantly they should be used with great care and rigor to avoid misidentification of false positives. The relatively youthful stage of proteomic techniques has left the field without a “gold standard” to guide investigators and enhance comparability of proteomics and biomarker discovery studies in the field of AD. A recent consensus panel (Antecedent Biomarkers in Alzheimer’s Disease, in Alzheimer’s Forum, 2003) suggested that an ideal biomarker for AD should detect a fundamental feature of AD neuropathology that needs to be validated in neuropathologically confirmed cases. This biomarker should have a diagnostic sensitivity >80% for detecting AD and a specificity of >80% for distinguishing other forms of dementia. Recommended steps to establish a biomarker include (1) confirmation by at least two independent studies conducted by qualified investigators with the results published in peer-reviewed journals, and (2) evidence supporting the utility of the biomarker in assessing a beneficial effect in AD disease-modifying therapy. The work has only just begun.

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