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Biomarkers in Alzheimer's disease: past, present and future

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Abstract

Epidemiological and molecular studies suggest that Alzheimer's disease (AD) has multiple etiologies including genetic mutations, genetic variations affecting susceptibility and environmental factors. These aspects can promote the formation and accumulation of insoluble amyloid- β and hyperphosphorylated tau. Since the disease is multifactorial and clinical diagnosis is highly exclusive, the need for a sensitive, specific and reliable biomarker is crucial. The concept of a biomarker implies sensitivity and specificity relative to the condition being considered. For clinical practice, AD diagnosis has been based on adherence to clinical criteria such as the NINCDS/ADRDA and DSM-IV. A more recent set of diagnostic criteria proposed incorporates imaging findings into the diagnosis of AD. In this article, we consider the most studied candidates or group of candidates for AD biomarkers, including pathological processes and proteins (amyloid- β , tau, oxidative stress, mitochondrial/metabolic changes and cell-cycle processes), or autoantibodies thereto, as well as genetic factors.

Keywords

Alzheimer's disease; amyloid- β ; biomarker

For clinical practice, the diagnosis of Alzheimer disease (AD) has been based on adherence to clinical criteria, such as the National Institute of Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA) and Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) [1]. A more recent set of diagnostic criteria proposed to incorporate imaging findings into the diagnosis of AD [2]. The concept of a biomarker implies sensitivity and specificity relative to the condition being considered [3]. Any potential biomarker should have a range of features to fulfill clinical expectations and it should be useful for detecting early stages of the disease.

The importance of biomarkers and diagnostic criteria with their inherent sensitivity and specificity is the context in which they are employed. Many small studies have published

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impressive results, usually based on populations where the prevalence of AD is very high. The positive predictive value, derived from Bayes' theorem, is sensitive to the underlying prevalence in the population. In a clinical setting, the prevalence of AD may be 60–70% of all dementia cases. In the East Boston study [4], the prevalence of AD over the age of 85 years was 47%. Assuming a biomarker with 95% sensitivity and specificity, the positive predictive value would be 94%.

However, dementia clinics tend not to see cognitively healthy subjects, therefore artificially inflating AD prevalence. In community-based studies, AD prevalence in populations over the age of 65 years, for example, may be approximately 10–15%. In a population with a 1% prevalence rate, the 95% sensitive and specific test yields a positive predictive value of 16%. Therefore, usage in low-prevalence populations requires essentially 100% specificity in order to be effective as a screening test [5].

Another consideration for biomarkers is their sensitivity to change. Neuroimaging protocols, such as the Alzheimer's Disease Neuroimaging Initiative (ADNI), are in progress [6–9]. One of the aims of ADNI is the determination of biomarkers for diagnosis, as well as those predictive of change, for example, from mild cognitive impairment (MCI) to AD. The search for these reliable biomarkers is, in part, a response to the recognition that screening tests for cognitive impairment, such as the Mini-Mental Status Examination [10], or even the Alzheimer's disease assessment scale (ADAS) [11], may not be sufficiently sensitive to change, particularly for measuring MCI to AD progression. With regard to the clinical diagnosis of AD, psychological diagnostic tools, such as the Mini-Mental Status Examination, indices of staging and progression have limited accuracy. Moreover, imaging studies are expensive and not routinely used. Thus, there is an increasing interest in biochemical biomarkers involving the analysis of genes or proteins in tissues, blood and body fluids, to allow for the diagnosis of symptomatically and clinically almost indistinct processes. The majority of research has focused on biomarker parameters in blood plasma or sera, which are relatively easily sampled, yet only few studies were well correlated with disease. Ray *et al.* proposed 18 proteins in blood plasma as biomarkers that may provide an earlier prediction of AD [12]. Another approach is to assay for peripheral biomarkers in the cerebrospinal fluid (CSF), with high specificity and sensitivity.

According to the amyloid- β ($A\beta$) hypothesis, brain amyloidosis accumulating $A\beta_{42}$ and shorter peptides, and in particular oligomeric $A\beta$ assemblies, is a leading cause of neurodegeneration in AD [13]. Analyzing for $A\beta$, as well as its immunological response, is a potential measure of disease. Not only $A\beta$, but all of the major genetic and protein elements deregulated in AD, such as amyloid precursor protein (APP), tau, presenilin 1/2 and ApoE, play roles in disease pathogenesis [14–16]. In this regard, transcriptional profiling of genes as a biomarker assay could potentially predict disease.

In AD, neurons have been shown to inappropriately enter the cell cycle without the ability to fully complete it [17]. The synchronous nature of the cell cycle is lost, and such mitotic aberration leads to neuronal dysfunction and death. As such, these cell-cycle mechanisms, acting either positively by stimulation or negatively through removal of inhibitory signals, provide promising molecular targets for pharmacological intervention as well as sources of potential biomarkers. Similarly, free radicals, free-radical generators and antioxidants also control the pathological process of neurodegeneration [18]. Increased mitochondria mass is a feature of the same neurons that demonstrate disease-related abnormalities, and undergo subsequent oxidative damage and cell death in AD [19]. Oxidative stress, at a point when the mitochondrial mass is highest, poses an elevated and chronic oxidative insult to the cell. Thus, oxidative stress parameters should also be considered as AD biomarkers.

Alzheimer's disease: pathogenesis

Alzheimer's disease is a progressive and insidious dementia that severely debilitates affected individuals and, ultimately, ends in their death. It affects up to 15% of people over the age of 65 years and nearly half of all individuals by the age of 85 years [20], and it is characterized by the selective loss of cortical neurons within the hippocampus and the temporal and frontal lobes. Two pathological lesions with parallel spatial distribution, namely the senile plaque and neurofibrillary tangle (NFT), are hallmarks of the disease and are largely associated with dementia. NFTs, which contain a highly phosphorylated form of the microtubule-associated protein tau, are the major intracellular pathology of AD, while senile plaques are extracellular and are primarily composed of A β . According to the A β hypothesis [13], brain amyloidoses with A β (42 and shorter peptides), particularly with oligomeric A β assemblies [21], are a leading cause of neurodegeneration in AD. As the disease produces a destruction of higher-order brain functions, its high prevalence is an increasingly serious global health dilemma and, as such, sensitive and reliable biomarkers are needed to execute early and accurate clinical diagnosis.

A β : pathological protein & marker of AD?

People with AD have an abundance of A β -containing senile plaques within the brain, and while this may or may not be a central driver of disease pathogenesis [22–24], this feature is of clinical importance in biomarker consideration. Notably, pathological formation and deposition of amyloid is a characteristic feature of other pathologies as well, including Down's syndrome, cerebral amyloid angiopathy, multiple myeloma, hemodialysis-associated amyloid disease, Creutzfeld–Jacob disease and familial amyloid polyneuropathies. In each case, a different amyloid protein is responsible for the pathology. Amyloid deposits consist of abnormally misfolded proteins that represent a hallmark of their associated disease and are a source of further toxic effects. For example, in familial amyloid polyneuropathy, the deposits consist of mutated transthyretin amyloid fibrils, particularly in the PNS [25], while in Creutzfeld–Jacob disease, the prion protein accumulates. In AD, the misfolded protein, A β , deposits in the CNS. Thus, A β is an important and integral feature of disease that has a potential use as a pathological indicator and biomarker, and understanding its origins, formations and neurochemistry could yield important discoveries that would be helpful in the diagnosis of AD.

Amyloid- β is derived from a larger precursor APP encoded on chromosome 21 [26]. Notably, Down's syndrome patients, who carry an extra copy of the *APP* gene due to the trisomy of chromosome 21, demonstrate A β deposition very early and often develop dementia by their mid-30s. Moreover, general mutations in this gene can give rise to the full spectrum of AD pathology. APP is a transmembrane cellular protein with a large extracellular spanning region and intracellular terminus. Physiological cleavage results in secretion of the extracellular domain, named secreted APP, which appears in the medium of cell culture studies as well as *in vivo* in the CSF. Interestingly, secreted APP has been reported to increase cell survival and adhesion, and prevent intracellular calcium accumulation and death of neurons [27,28]. However, among the final results of cleavage (first by β - and then by γ -secretases) are several variants of A β , which are prone to aggregate. Aggregating rapidly into oligomers and ultimately plaques, A β ₄₂ is toxic to neurons, and several mechanisms for its toxicity have been proposed. First, its physical presence has been shown to damage neurons. Moreover, aggregations of A β have been proven to be toxic to cell membranes and to elicit inflammatory responses from glial cells [29]. A β may also activate apoptosis or cause microtubule collapse, leading to tau protein hyperphosphorylation or depletion of presynaptic APP, resulting in a loss of synaptic transmission [28]. Intensive work thus continues in order to fully understand the process by which APP is cleaved to release A β fragments, how fragments accumulate to form plaques and how plaques themselves can influence the process of neurodegeneration.

A number of reports demonstrate that patients with AD have lower levels of serum anti-A β antibodies than healthy age-matched individuals [30,31]. However, other studies indicate that the level of anti-A β antibody may be much higher in AD as compared with controls. In addition, A β antibody titers were negatively correlated with cognitive status such that more cognitively impaired individuals tended to exhibit higher anti-A β titers [32]. The immunochemical measurement of plasma A β is influenced by several variables, such as the binding with other proteins [33,34], the high tendency for A β ₄₂ to aggregate [35] and by anti-A β -antibodies [36]. Thus, attention was focused on the potential of measuring autoantibodies directed against A β , both as a potential treatment for AD, as well as a reliable biomarker of AD. Naturally occurring antibodies against A β are found in the CSF and plasma of patients with AD, as well as in healthy control subjects [37]. Immunization of mice with A β ₄₂ and subsequent administration of these antibodies against A β into APP-overexpressing transgenic mice dramatically reduced amyloid plaque deposition, neuritic dystrophy and astrogliosis, most likely by enhancing A β ₄₂ clearance from the brain [38,39].

In biological fluids, antibodies and antigens are in a state of dynamic equilibrium between bound and unbound forms, which is concentration dependent. Although antibody titers against a particular antigen in a given disease state may be strongly elevated, only a fraction of the total amount is likely to be detectable via ELISA owing to interference by antigen-antibody complexes. Previously, the importance of measuring total amounts of antibody following antigen dissociation was demonstrated. This body of work provided an explanation for the discrepancies between the previous reports that measured nondissociated antibody levels. The novel methodology was deemed to be reliable in both animal and human studies [40,41].

Recently described antigen-dissociation techniques were applied to samples obtained as part of a population-based analysis of the prevalence of AD [42]. Serum antibodies to A β ₄₂ were measured before and after antigen dissociation by low pH. The levels of dissociated antibody in AD patients were always significantly different from controls, and levels of A β antibody after dissociation, but not nondissociated levels, correlated negatively with both duration of the disease and age in the AD patients. Moreover, the change in concentration of A β ₄₂ antibody from pre- to postdissociation (i.e., the dissociation Δ) directly reflected the progression of AD in terms of both time since diagnosis and age of the patients, with a lower dissociation Δ indicating a more advanced stage of AD [42]. These findings indicate the dissociated A β ₄₂ antibody level to be of useful diagnostic value in the beginning of the neurodegenerative process.

It may be suggested that during the long, preclinical, nonsymptomatic phase of AD, corresponding to the accumulation of its soluble form in the brain, the levels of A β ₄₂ are, on average, higher than normal [43]. The formation of amyloid plaques in the brain, as well as the progressive aggregation of A β ₄₂, lead to the decline of A β ₄₂ plasma levels to within normal limits, thus appearing no different from the population not at risk of AD. Moreover, at the stage of MCI, the levels of A β ₄₂ are still high [44], and later, plasma A β ₄₂ may decrease, as demonstrated by several studies that reported no difference of A β plasma levels between AD patients and controls [43]. According to this interpretation, plasma A β levels reflect A β metabolism in the brain not only in the preclinical phase of AD, but also in MCI. However, plasma levels taken while the disease is progressing may, in fact, display a low level of A β in the sera of a patient with advanced AD, mainly due to the long process of antibody production and amyloid binding to antigen. Evaluation of samples in our study, ultimately, demonstrated the basic premise of the aforementioned hypothesis – A β -antigen effectively masks the detection of A β autoantibody, but its dissociation increases and renders accurate the sensitivity of measurement. The fact that the effect is dependent on A β level and is more visible in affected AD individuals may be of exceptional value for further biomarker research.

Tau phosphorylation: a cause & marker of neuronal death?

The hyperphosphorylated form of the microtubule-associated protein tau is a major component of NFTs, and thought of as a central intracellular pathological protein in AD [23,45–47]. Hyperphosphorylation of tau is thought to alter normal microtubular dynamics in the neuron, a phenomena that might ultimately lead to neuronal dysfunction [48,49]. It is important to note that physiological hyperphosphorylation of tau, driven by cyclin-dependent kinases (CDKs), occurs when cells are mitotically active [50–53]. Of note, CDKs, such as CDK2 and CDK5, as well as Cdc-kinases and MAP2 kinases, which phosphorylate tau *in vitro* [54,55], are increased in AD in a topographical manner that completely overlaps with phosphorylated tau [56–58]. In addition, it was demonstrated that CDK7, an age-dependent CDK-activating kinase associated with phosphorylated tau in AD, may be essential to all other mitotic alterations, since CDK7 plays a crucial role as an activator of all the major CDK/cyclin substrates [59]. Nevertheless, CDKs are highly sensitive to regulation by growth factors. The reactivation of these mitogenic kinases in the AD neuron means that these neurons may be resensitized to growth factors [60]. In any case, the presence of an overaccumulation of hyperphosphorylated tau, in the form of NFTs, is a hallmark feature of AD and, as such, its use as a biomarker has potential, as recent studies indicate [61].

Genetic biomarkers: special attention to ApoE & ADNI studies

Over 160 mutations on three genetic loci have been identified to be responsible for most cases of familial early-onset, autosomal-dominant AD (with onset before the age 65 years) – chromosome 14, presenilin-1; chromosome 1, presenilin-2; chromosome 21, APP. These early-onset forms are responsible for less than 1–2% of cases.

The discovery of these genetic loci linked to AD has led to important insights into the disease pathogenesis. The *APP* gene product from chromosome 21 is cleaved by three enzymes known as the α -, β - and γ -secretases to produce amyloidogenic A β or nonamyloidogenic breakdown products. The activity of these alternative pathways of APP catabolism, as well as the rate of APP expression, is controlled by genotype as well as environment.

The mutations of human presenilin genes *PSEN1* and *PSEN2*, are linked to early onset AD. They are localized to chromosomes 1 and 14 [62]. Although the function of human presenilins is currently not fully understood, their homologs are involved in Notch processing and signaling. These two proteins have redundant roles in cell fate [63–65], cytoskeletal anchorage, cell division, early embryonic development [66] and tumorigenesis [67]. In addition, presenilins interact with centrosomes and kinetochores, thus giving them a significant role during chromosomal segregation and mitosis [66].

Presenilin overexpression also leads to G₁ arrest and this overexpression is potentiated by the *PSEN2* (N141I) mutation. Bearing in mind that presenilin expression arrests cells in the G₁ phase of the cell cycle, changes in the levels of cell-cycle inhibitory proteins p21, p27 and p53, and the cell-cycle regulatory proteins c-myc and pRb were examined in presenilin-transfected cell lysates. No increases or decreases were evident, suggesting that the presenilins mediate cell-cycle arrest by mechanisms other than simple changes in the steady-state levels of these cell-cycle-related proteins [68].

What is interesting is that presenilin over-expression, in the environment of the adult neuron, leads to oxidative stress, loss of calcium balance and also to greater vulnerability to apoptosis [69,70]. Indeed, AD-linked presenilins demonstrate greater apoptotic effects [71]. Neurons that are vulnerable to AD have exited from G₀ and entered G₁ but may be blocked from progressing into the S phase at the G₁/S boundary by the presenilin (and possibly APP) mutations. A G₁ blockage would allow cell-cycle regulatory proteins to accumulate and

produce the stasis as is found in AD; hence, these mutations yield a contracted time course of the basic pathophysiology in AD. Furthermore, the presenilin and APP mutations may lead to the upregulation of CDKs, which can have dual roles in both cell-cycle regulation as well as signaling cell death [72,73]. Indeed, the role of the cell-cycle inhibitory proteins, such as p16, p19 and p21 that accumulate in AD, can act to shunt possible apoptotic stimulation in early AD [72].

While presenilins may have relevance to both apoptotic and developmental mechanisms due to the inter-relationship of cell death, growth and differentiation, they are not the only genetically determined AD-related factor associated with the cell cycle. The most important genetic risk factor for sporadic AD is the *ApoE* $\epsilon 4$ allele. The *ApoE* $\epsilon 4$ allele is associated with a two- to threefold increased risk of getting the disease when one copy is present, and when there are two copies the risk is increased as much as 12-times (the *ApoE* gene is codominant). However, the *ApoE* $\epsilon 4$ allele is not necessary or sufficient for AD, and perhaps 50% of the inherited risk of getting the disease is currently unknown. The strong association of the *ApoE* genotype to AD is a potent indicator of the importance of lipid metabolism and diet in the pathogenesis of the disease. Among the genetic markers, the *ApoE* gene has been widely examined owing to its well-documented role in AD and vascular diseases. A number of reports on human longevity demonstrate that *ApoE* $\epsilon 4$ allele frequency is lower in older age groups than younger or middle-aged subjects. ApoE is a polymorphic glycoprotein that plays an essential part in the binding to receptors for the uptake of chylomicrons, very-low-density lipoprotein remnants and of low-density lipoprotein. The three major isoforms are ApoE $\epsilon 3$ (Cys112/Arg158), $\epsilon 4$ (Arg112/Arg158) and $\epsilon 2$ (Cys112/Cys158). *ApoE* polymorphism is an essential determinant in the interindividual variations of lipids in healthy subjects, in various populations. Its influence can be significant on the efficacy of nutritional or therapeutic interventions. The allele $\epsilon 4$ appears to be associated with an increased risk of premature atherosclerosis. *ApoE* polymorphism contributes to the lipid disorders in diabetes and obesity.

Studies have demonstrated that genetics play a role in the extracellular deposition of amyloid. Individuals afflicted with AD carrying the *ApoE* $\epsilon 4$ isoform have a greater number of A β plaques when compared with *ApoE* $\epsilon 3$ carriers, and inheritance of an *ApoE* $\epsilon 4$ allele increases the risk of AD when compared with *ApoE* $\epsilon 2$ and *ApoE* $\epsilon 3$ carriers [74]. The relationship of ApoE to AD is based on a meta-analysis from population-based studies. Farrer *et al.* examined the association between *ApoE* genotype and AD in relation to age, gender and various ethnic and racial groups [75]. A total of 40 research teams contributed data on *ApoE* genotype, gender, age at disease onset and ethnic background for 5930 patients who met criteria for probable or definite AD and 8607 controls without dementia who were recruited from clinical, community and brain bank sources. In this study, odds ratios (OR) and 95% confidence intervals for AD, adjusted for age and study, and stratified by major ethnic group (Caucasian, African-American, Hispanic and Japanese) and source, were computed for *ApoE* genotypes $\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 3$, $\epsilon 2/\epsilon 4$, $\epsilon 3/\epsilon 4$ and $\epsilon 4/\epsilon 4$ relative to the $\epsilon 3/\epsilon 3$ group. The influence of age and sex on the OR for each genotype was assessed using logistic regression procedures. Among Caucasian subjects from clinic- or autopsy-based studies, the risk of AD was significantly increased for people with genotypes $\epsilon 2/\epsilon 4$ (OR: 2.6; 95% CI: 1.6–4.0), $\epsilon 3/\epsilon 4$ (OR: 3.2; 95% CI: 2.8–3.8) and $\epsilon 4/\epsilon 4$ (OR: 14.9; 95% CI: 10.8–20.6); whereas, the ORs were decreased for people with genotypes $\epsilon 2/\epsilon 2$ (OR: 0.6; 95% CI: 0.2–2.0) and $\epsilon 2/\epsilon 3$ (OR: 0.6; 95% CI: 0.5–0.8). The *ApoE* $\epsilon 4$ –AD association was weaker among African-Americans and Hispanics, but there was significant heterogeneity in ORs among studies of African-Americans. In addition, the *ApoE* $\epsilon 4$ –AD association in Japanese subjects was stronger than in Caucasian subjects ($\epsilon 3/\epsilon 4$, OR: 5.6; 95% CI: 3.9–8.0; $\epsilon 4/\epsilon 4$, OR: 33.1; 95% CI: 13.6–80.5). The $\epsilon 2/\epsilon 3$ genotype appears equally protective across ethnic groups. Among Caucasians, *ApoE* genotype distributions are similar in groups of patients with AD whose diagnoses were determined clinically or by autopsy. In addition, the *ApoE* $\epsilon 4$ effect is evident at all ages between 40 and 90 years but diminishes after 70 years of

age, and the risk of AD associated with a given genotype varies between sexes. The general conclusion was that the *ApoE* ϵ 4 allele represents a major risk factor for AD in all ethnic groups studied, across all ages between 40 and 90 years, and in both men and women.

From a population perspective, it is possible to calculate the ‘attributable’ risk of a factor if one knows the frequency of the risk factor in the matched, nonaffected population and the relative risk or OR. For example, the attributable risk due to family history can be calculated as 26%. That is, in the group studied, 26% of all AD cases could be attributed to probable genetic factors. A similar calculation for *ApoE* ϵ 4 suggests that over 30% of the cases between 65 and 80 years of age may be attributed to the ϵ 4 allele. The degree to which family history and *ApoE* represent the same cases is unknown. If these risk factors are additive at the population level, family history/*ApoE* ϵ 4 would account for over 30% of the attributable risk for AD [76].

There are still many uncertainties regarding the epidemiology of AD. It is crucial that these weaknesses are addressed, particularly in longitudinal studies carried out on community samples or well-defined cohorts, in which the history of exposure to putative risk factors is obtained in detail before the development of AD.

Recently, Potkin *et al.*, using hippocampal atrophy as a quantitative phenotype in a genome-wide scan, identified candidate risk genes for sporadic AD [77].

Researchers performed a genome-wide association study on 381 participants in the previously mentioned ADNI study. Samples were genotyped using the Illumina Human610-Quad BeadChip (San Diego, CA, USA). A total of 516,645 unique single-nucleotide polymorphisms (SNPs) were included in the analysis following quality-control measures. Two analyses were completed – a standard case–control analysis and a novel approach using hippocampal atrophy measured on MRI as an objectively defined, quantitative phenotype. A general linear model was applied to identify SNPs for which there was an interaction between the genotype and diagnosis on the quantitative trait. The case–control analysis identified *ApoE* and a new risk gene, translocase of outer mitochondrial membrane (*TOMM*)40. *TOMM*40 risk alleles were approximately twice as frequent in AD subjects as controls. The quantitative trait analysis identified 21 genes or chromosomal areas with at least one SNP, which can be considered potential a ‘new’ candidate loci to explore in the etiology of sporadic AD. These candidates included *EFNA5*, *CAND1*, *MAGI2*, *ARSB* and *PRUNE2*, genes involved in the regulation of protein degradation, apoptosis, neuronal loss and neurodevelopment [77].

Therefore, genetic factors may be useful as part of the group of parameters, with certain implication on risk, to form the complex multifactorial biomarker.

Redox imbalance & AD

There is abundant evidence that oxidative stress and free-radical damage play an essential role in the pathogenesis of AD [78]. As free radicals, free-radical generators and antioxidants act as crucial control parameters of the cell cycle, and because energy is an obligate requirement for dividing cells, mitotically active cells exhibit the most mitochondrial proliferation and thus contain the highest levels of reactive oxidative species [79]. Notably, in AD, increases in the number of mitochondria are found in the same neurons that also exhibit cell cycle-related abnormalities and undergo subsequent oxidative damage and cell death [80]. While in a normally mitotic cell, mitochondrial replication is imperative for providing the energy required for cell division, in AD cells, the neuronal cell cycle is interrupted or dysfunctional and the neurons incur a ‘phase stasis’ characterized by excessive mitochondria. Such ‘excess’ mitochondria levels are potent sources of free radicals that cause homeostatic and redox imbalances, especially in those redox reactions involving calcium metabolism [81]. Thus, cell-

cycle dysfunction, when mitochondrial mass is highest, poses an elevated, and possibly chronic, oxidative assault upon the cell, far beyond the blunting capacity of endogenous antioxidants.

Importantly, imbalances in redox homeostasis are also played out via numerous signal transduction cascades that are intimately linked to cell-cycle control as well. Indeed, activation of p38 MAPK and ERK links tau phosphorylation, oxidative stress and cell cycle-related events in AD [82–84]. MEK, ERK1/2, cyclins, cdks and their inhibitors (i.e., p16INK4a family) and p21Ras are elevated early in AD and colocalize in pyramidal neurons with NFT [85]. Neuronal ERK is increased in AD, and phosphorylation of ERK, as well as phosphorylation of p38 and CREB, by NGF or EGF, is differentially modulated by oxidative and other stresses [86]. In support of this notion, compromised mitochondrial function was found to lead to increased cytosolic calcium and to the activation of MAPKs (ERK1/2) [87]. In addition, activated forms of ERK are found to be decreased in cells overexpressing heme oxygenase-1, indicating that tau and heme oxygenase-1 both serve overlapping protective roles in regulating oxidative stress [88]. Ultimately, the activation and accumulation of any number of these redox-related compounds may reveal a biochemical signature that could be used as potential biomarkers.

Cell-cycle biomarkers in AD

In AD, whole populations of nonstem cell neurons leave their quiescent state and re-enter the cell cycle. However, such neuronal re-entry into the cell cycle is noneffective and, ultimately, leads to neurodegeneration. It is perhaps ironic to discover that neurodegenerative diseases, where cell loss is a key feature, may provide clues to understanding the plasticity of the adult CNS. In AD in particular, there is accumulating evidence that susceptible neuronal populations seem to reactivate the cell cycle. This exit from a quiescent state is manifested in several ways, including the ectopic expression of cyclins along with their cognate CDKs and their inhibitors, recruitment of mitogenic signal transduction pathway components and the increased transcriptional activation of a variety of mitosis-related proteins. While the cause of this apparent neuronal re-entry into the cell cycle is not fully understood, the consequences for these terminally differentiated cells are disastrous and lead to oxidative stress, cytoskeletal abnormalities, mitochondrial dysfunction and, ultimately, neuronal death. The re-emergence into the cell cycle by neurons accounts for many of the cardinal features of AD. Mitotic alterations, including the recruitment of mitogenic factors and oxidative stress, may be part of the neurodegenerative process in AD [82,89,90].

Quiescence, cell division and differentiation are states central to the regulation of growth and development. Increased growth stimuli, such as extrinsic mitotic pressure, activate key factors for G₀ exit and G₁ progression, including the complex-forming CDKs (i.e., CDKs 4, 5, 6 and 7) and their cognate activating cyclins (i.e., cyclin D1, D3, E and B1). These complexes are able to phosphoregulate a wide variety of relevant substrates [91]. Together, they orchestrate DNA replication, cytoskeletal re-organization and cellular metabolism required for proliferation, development and cell-cycle progression. It has been argued that a number of the cell cycle-related phenomena found in AD can also occur as sequelae to other processes, such as apoptosis, trophic-deprivation and DNA repair [72,92,93].

As previously mentioned, A β is derived from the larger precursor APP, encoded on chromosome 21, and familial AD is linked to mutations in the *APP* gene. Of interest here is that APP is upregulated secondary to mitogenic stimulation and cell cycle-dependent changes can regulate APP metabolism. APP, through the stimulation of Ras-dependent MAPK cascade *in vivo*, is thus correlated with highly phosphorylated tau [27]. The early p21Ras expression pathway is activated during the post-translational modification of APP and tau phosphorylation, which precedes neurofibrillary degeneration and A β formation [94]. In

addition, the presence of p21, highly phosphorylated tau, Ki-67 and cell cycle-associated nuclear antigen protein may have a role in the production of abnormally phosphorylated tau, which, in turn, leads to the formation of cytoskeletal derangements in susceptible neurons [86]. These findings point to cell-cycle reactivation and the upstream ectopic expression of cell-cycle markers as critical, common and early events in AD pathogenesis [95,96].

Ledoux *et al.* found that APP is upregulated upon mononuclear cell activation with the cell mitogen phytohemagglutinin. APP may participate in the regulation of cell activation in peripheral mononuclear cells, which could contribute to a circulating pool of APP and A β . Similar to APP, A β is upregulated secondary to mitogenic stimulation [97]. Furthermore, APP metabolism is regulated by cell cycle-dependent changes [98] and has neurotrophic effects at low (nM) concentrations [99], consistent with its mitogenic activity *in vitro* [100]. Presumably, the effect of A β itself is mediated through MAPK [101] and, therefore, may play a direct role in the induction or propagation of cell cycle-mediated events in AD. Therefore, A β , along with oxidative stress and cell-cycle re-entry, may have common etiologies. However, it is worth noting that, while A β -mediated cell death, at least *in vitro*, is dependent on the presence of various cell cycle-related elements [73], *in vivo* analysis of the basal nucleus of Meynert and the locus ceruleus, where A β is rarely seen, found little or no topographical relationship between A β and the ectopic expression of cell-cycle markers in diseased brains [102]. Thus, A β may only become toxic *in vivo* when the neuronal cell-cycle machinery is activated or when levels exceed the body's ability to regulate its turnover.

The identity of the signals that lead the potential AD neuron to attempt to exit from a quiescent state and re-enter the cell cycle remains to be determined. However, a number of growth factors and mitogens are elevated in the AD brain and may drive cell-cycle re-entry. Resensitization to these exogenous or surface-derived signals can lead to the activation of the mitotic engine and drive cell proliferation, as seen in AD. Candidate growth factors include neurotrophic factors, NGF, TGF- β 1 [103], PDGF, EGF and basic FGF [104–106]. In addition, IGF-1, which has been shown to mediate transient site-selective increases in tau phosphorylation in primary cortical neurons [107], is involved in axonal growth and development, and can mediate the cytoskeletal reorganization that occurs during neurite outgrowth and, perhaps, in aberrant neuronal sprouting [108].

Periodically, new markers of cell-cycle entry are shown to be associated with either neuronal pathology in AD or present in those neurons that are vulnerable to changes that lead to AD. This accumulating evidence helps cement the early and possibly irreversible fate of these vulnerable neurons. It is fascinating that neurons that have been in G₀ phase for nearly 50–60 years make compensations, which include an attempted re-entry into the cell cycle among others. This ectopic cell-cycle activation is also seen in mitotically active cells during neoplastic transformation and neurogenesis [70]. The mechanisms of cell-cycle activation may differ at many levels, but most intriguing in AD is that this re-entry is incomplete, and results in stasis, dysfunction and death. Despite many suggestions that neuronal death in AD is the result of apoptosis, stereotypical cytopathological manifestations that define the terminal phases of apoptosis are not seen in AD, such as chromatin condensation, apoptotic bodies and blebbing. Moreover, caspases, such as caspase 6, which cleave APP and presenilins, are localized to the pathological lesions associated with AD. However, while upstream caspases such as caspases 8 and 9 are clearly found in association with the intraneuronal pathology of AD, downstream caspases, such as caspases 3, 6 and 7, are present only at control levels. Given that execution of apoptosis requires amplification of the caspase-mediated apoptotic signal, the results indicate that in AD there is a lack of effective apoptotic signal propagation to downstream caspase effectors. It was concluded that AD represents the first *in vivo* situation reported in which the initiation of apoptosis does not proceed to caspase-dependent cell death. This novel

phenomenon of apoptotic avoidance, termed abortive apoptosis or abortosis, may represent an exit from the caspase-induced apoptotic program that leads to neuronal survival in AD [109].

To date, there is no evidence that a nuclear division has been completed in these vulnerable neurons although there is circumstantial evidence of increased DNA in certain vulnerable neuronal populations [110]. However, this could equally be attributed to premature chromosome separation [111]. What seems to be plausible, given this scenario, is that these terminally differentiated neurons in the adult CNS are inherently restricted in their ability to divide. Hence, even though these neurons enter the cell cycle, they do not go beyond G₁ and stop short of actual division. Therefore, the neuron is arrested in an 'intermediate state' of the cell cycle [112]. Thus, a multitude of markers representing different phases of the cell cycle will likely be present and provide a basis for assay.

Conclusion

Alzheimer's disease is likely to result from the complex interplay of genetics, environment and aging that affect cellular metabolism, mechanistic pathways and stress response, resulting in abnormal protein deposition, mitochondrial dysfunction, and severe synaptic and neuronal loss. Unfortunately, the incidence of AD will only increase as our population ages, and there is a clear urgency for improvements in the early diagnosis of the disease that are thought to be crucial for the success of current and future therapies. The spectrum of biomarker candidates encompasses vast numbers of genetic indicators, protein and chemical changes, and brain morphology changes that can be seen with modern imaging techniques and, collectively, may provide significant and sensitive biomarkers that provide the earliest possible disease diagnosis and, therefore, an opportunity for promising treatment strategies.

Future perspective

While amyloid and amyloid-related compounds may be useful biomarkers in the early diagnosis of AD, the multitude of other characteristic features of AD presented here may also be appropriate. Genetic mutations, for example, play a role in a subset of AD patients (often with early disease onset and more severe disease progression) and genetic analysis could thus play a role in disease diagnosis. Likewise, oxidative damage to various proteins, nucleic acids, and other cellular compounds, likely arising from mitochondrial abnormalities, is found early in the disease and may provide certain biochemical signatures of disease as well, and cell-cycle reactivation markers and even components of the apoptotic cascade may produce detectable cell responses. Ultimately, specific assays for genetic, protein, and oxidative profiles, as well as those for A β and its immunological response, may serve as a relevant group of biomarkers that could be informative to individuals regarding risk of disease, as well as for indicators of the progression of disease. Correspondingly, new developments in treatment options are likely to become available.

Executive summary

Alzheimer's disease is a progressive, neurodegenerative disease with growing incidence & prevalence

- It affects up to 15% of people over the age of 65 years and nearly half of all individuals by the age of 85 years.
- High prevalence of Alzheimer's disease (AD) is an increasingly serious global health dilemma.

AD has a multifactorial etiology

- The occurrence of AD may be regulated by genetic mutations and genetic variations affecting susceptibility, as well as environmental factors.
- All known etiological factors can promote the formation and accumulation of insoluble amyloid- β (A β) and hyperphosphorylated tau.

Brain amyloidoses with A β (1–42 & shorter peptides), particularly with oligomeric A β assemblies, are a leading cause of neurodegeneration in AD

- A β deposits in the CNS in AD. A β is thus an important and integral feature of disease that has potential use as a pathological indicator and biomarker. The level of A β in the sera fails to fulfill the features of a reliable biomarker.
- Attention has focused on autoantibodies directed against A β both as a potential treatment for AD, as well as a reliable biomarker of AD.
- A β -antigen effectively masks the detection of A β autoantibody, but its dissociation increases and renders accurate the sensitivity of measurement and may be of exceptional value for further biomarker search.

Search for an AD biomarker is widely present in contemporary neuroscience

- For clinical practice, the diagnosis of AD has been based on adherence to clinical criteria, such as the NINCDS/ADRDA and DSM-IV.
- A more recent set of diagnostic criteria proposed to incorporate imaging findings into the diagnosis of AD.

There are new parameters being proposed as biomarkers in AD

- Cell-cycle mechanisms, acting either positively by stimulation or negatively through removal of inhibitory signals, provide promising molecular targets for pharmacological intervention, as well as sources of potential biomarkers.
- Free radicals, free-radical generators and antioxidants also control the pathological process of neurodegeneration in AD. Oxidative stress, at a point when the mitochondrial mass is highest, poses an elevated and chronic oxidative insult to the cell. Thus, oxidative stress parameters should also be considered as AD biomarkers.

Conclusion

- Since the clinical diagnosis of AD is currently based on qualitative, often subjective, psychometric assessments and more often than not by exclusion of other causes of dementia, the need for a sensitive, specific and reliable biomarker for disease is crucial. Any potential biomarker should have a range of features to fulfill clinical expectations and should be useful for detecting early stages of the disease.

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- of considerable interest

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