International Scholarly Research Network
ISRN Pharmacology
Volume 2012, Article ID 984786, 6 pages
doi:10.5402/2012/984786

Review Article

Biomarkers in Alzheimer's Disease: A Review

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Received 3 April 2012; Accepted 20 May 2012

Academic Editors: R. Couture and M. Tohda

Alzheimer's disease is the most common form of dementia affecting millions of individuals worldwide. It is currently diagnosed only via clinical assessments and confirmed by postmortem brain pathology. The development of validated biomarkers for Alzheimer's disease is essential to improve diagnosis and accelerate the development of new therapies. Biochemical and neuroimaging markers could facilitate diagnosis, predict AD progression from a pre-AD state of mild cognitive impairment (MCI), and be used to monitor efficacies of disease-modifying therapies. Cerebrospinal fluid (CSF) levels of Aβ40, Aβ42, total tau, and phosphorylated tau have diagnostic values in AD. Measurements of the above CSF markers in combination are useful in predicting the risk of progression from MCI to AD. New potential biomarkers are emerging, and CSF or plasma marker profiles may eventually become part of the clinician's toolkit for accurate AD diagnosis and management. These biomarkers along with clinical assessment, neuropsychological testing, and neuroimaging could achieve a much higher diagnostic accuracy for AD and related disorders in the future.

1. Background

Alzheimer's disease (AD) is a neurological disorder and is the most prevalent form of age-related dementia in the modern society [1]. With increasing life expectancy, dementia is a growing socioeconomic and medical problem. Many factors have been linked to the incidence of AD, including age, gender (females are more likely to be affected), genetic factors, head injury, and Down's syndrome. It is estimated that, by 2050, the number of people aged 80 years or older will approach 370 million worldwide and that 50 percent of those aged 85 years or older will be afflicted with AD [2]. The diagnosis of AD is made by postmortem analysis of brains of patients with dementia.

Intracellular neurofibrillary tangles (NFT) containing hyperphosphorylated tau protein and apolipoprotein E and extracellular senile (neuritic) plaques containing many proteins, including β-amyloid (Aβ), α-synuclein, ubiquitin, apolipoprotein E, presenilins, and alpha antichymotrypsin, are considered pathological hallmarks of AD. Lewy bodies are present in the brains of about 60% of AD cases [3]. The pathogenic process of AD probably starts decades before clinical onset of the disease. During this preclinical period, there is a gradual neuronal loss. The first symptoms, most often impaired episodic memory, appear at a certain threshold. This clinical phase is often designated as mild cognitive impairment (MCI) [4]. To date, a definitive diagnosis of AD can only be made with both a clinical diagnosis and a postmortem histopathological examination of the brain. A clinical diagnosis of AD is based on medical records, physical and neurological examination, laboratory tests, neuroimaging, and neuropsychological evaluation. Diagnosis can be made with an accuracy of over 90%. However, neurodegeneration in AD is estimated to start 20 to 30 years before the first clinical symptoms become apparent. Treatment strategies might be most effective before pathological changes spread throughout the brain. Thus, an early diagnosis with reliable biomarkers is essential to distinguish between AD, mild cognitive impairment (MCI), and other dementia types [5]. Therefore, this paper discusses

various biomarkers which might prove to be significant diagnostic tools in AD.

2. Cerebrospinal Fluid Biomarkers

The cerebrospinal fluid (CSF) is a possible source of biomarkers of neurological diseases because CSF is in direct contact with the brain, and the molecular composition of CSF can reflect biochemical changes in the brain [6]. CSF biomarkers have been developed in parallel with imaging markers using MRI (magnetic resonance imaging) or PET (positron emission tomography) scans. CSF biomarkers Aβ 42, T-tau, and P-tau are useful for diagnostics of developed AD as well as in early stage AD [7]. The amyloid cascade hypothesis posits that the extracellular amyloid plaque consisting of aggregated beta- amyloid peptide (Aβ), peptide is generated from two proteolytic cleavages of the amyloid precursor protein (APP), damages brain regions and precipitates AD symptoms. Aβ generation from APP occurs when β- site APP cleaving enzyme (BACE-1) [8] cleaves the ectodomain of APP to first generate a membrane bound C-terminal fragment, another subsequent cleavage by γ- secretase activity further generates Aβ 40 and Aβ 42 [6]. Excess of amounts of free Zn and Cu, Fe, and Al and complement protein enhance the aggregation of Aβ. The aggregated form of Aβ participates in the formation of senile plaques [3]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play important roles in the initiation and promotion of neurodegeneration in the brains of patients with AD. Free radicals released during autooxidation of β-amyloid lead to neuronal damage. Mitochondria may be one of the most sensitive primary targets of oxidative stress in adult neurons. This may be due to the fact that mitochondrial DNA (mtDNA) does not encode for any repair enzymes, and, unlike nuclear DNA, it is not shielded by protective histones. In addition, mtDNA is in close proximity to the site where free radicals are generated during oxidative phosphorylation [9]. Alpha-ketoglutarate dehydrogenase complex (KGDHC), a mitochondrial enzyme, is decreased in brains of AD patients. Cu/Zn superoxide dismutase (SOD) and hemoxygenase-1 (HO-1), markers of oxidative stress, were elevated in aged transgenic mice [10].

Presenilin is a multi- transmembrane domain protein that associates with other proteins nicastrin, Aph-1, and Pen-2 to form the γ-secretase complex [11]. Mutations in presenilin-I gene have been found in about 50% of familial AD, whereas mutations in presenilin-II have been observed in less than 1% of familial AD. APP interacts specifically and transcellularly with either presenilin I or presenilin II. This complex is incorporated into intracellular vesicles which fuse with multivesicular bodies that contain proteases. β-amyloid is then produced by proteolysis of APP and released by the usual intracellular traffic between the lysosomal compartment and the plasma membrane into the extracellular spaces where it forms senile (neuritic) plaques [3]. Genetic predispositions to early-onset AD include mutations in APP and the presenilins, and all of which increase Aβ 40/Aβ 42 ratio [12]. Aβ40 and Aβ42 peptides found in amyloid plaques could form synapse-damaging oligomers. In the CSF, Aβ40, Aβ42, and other minor forms of peptides generated from APP (e.g., Aβ37 and Aβ38) could be detected and measured by immunochemical methods (such as ELISA) or liquid chromatography-mass spectrometry [6].

It has been suggested that Aβ concentration can serve as a good predictor of AD, since reduced levels in CSF have been reported in asymptomatic healthy elderly, who go on to develop AD, 1-2 years after followup. Recent reports suggest that soluble Aβ oligomers are rather synaptotoxic and causative for AD compared to insoluble, aggregated forms of Aβ. No correlation has been found between plaque load and degree of dementia. Some patients with assumed AD show no plaques while cognitive healthy elderly have senile plaques at autopsy. However, one cannot exclude a relationship to preclinical manifestations of AD. It is assumed that the formation of plaques is a downstream event of the generation of more toxic and soluble forms of Aβ. The reduction of Aβ1–42 in CSF could result from the formation of oligomers, which are not detected by Aβ1–42 ELISA [5]. Fukumoto et al. established a novel ELISA system that quantifies Aβ1–42 oligomers. They reported an inverse correlation between oligomers in the CSF and severity of dementia. However, measurement of Aβ oligomers in CSF is limited by its low concentrations and must still be validated as an effective biomarker [5].

NFTs (neurofibrillary tangles), on the other hand, are intracellular filamentous aggregates of the microtubule binding protein tau. In AD, tau is present in the somatodendritic compartment of neurons in an aggregated, filamentous form and is hyperphosphorylated at Ser/Thr and Pro epitopes. Different phosphorylation epitopes, such as threonine 181, 231, or serine 235, can be detected by different ELISAs. The initiating pathogenic event changes tau from a soluble, microtubule-stabilizing protein into its insoluble, aggregated form [13]. The hyperphosphorylated tau that undergoes an alteration in conformation cannot bind, and thus stabilize, microtubules, which in some way leads to neuronal dysfunction by disruption of axonal transport. Increased "free" tau in the cell body may aggregate, leading to tangle formation and neuronal death [13]. Several kinases and phosphatases have been implicated in the hyperphosphorylation event, particularly cdk5 and Gsk3β [13]. MCI converting to AD can be discriminated from stable MCI with 90% sensitivity and 100% specificity, indicating that T-tau is a good predictive marker for incipient AD. The concentration of P-tau181 is increased in AD and yields a sensitivity of 80% and specificity of 92% in discriminating AD from healthy controls. Thus, P-tau reveals a higher specificity than T-tau for diagnosing AD compared to other types of dementias. In addition, MCI patients, who convert to AD, have higher P-tau levels compared to patients with stable MCI. It has also been shown that cognitive decline and tangle pathology in individuals with MCI correlates with CSF P-tau concentrations. It seems likely that P-tau is not simply a marker for neuronal degeneration, but rather a more specific marker for AD by reflecting the phosphorylation states of tau and ultimately the formation of neurofibrillary tangles in the brain [5].

The apoE gene exists as three variants: ApoE2, -3, and -4. Persons who are homozygous for the apolipoprotein E

(APOE), e4 allele, develop AD 10 to 20 years earlier than those who have e2 or e3 alleles. Persons who are heterozygous for e4 develop AD 5 to 10 years earlier than those who have e2 or e3 alleles [3]. e4 allele binds to neurofibrillary tangles and β- amyloid [14]. The presence of e4 allele could be an important risk factor for AD. Proteasomes regulate certain transcriptional factors by splicing inactive peptide fragments on to active ones. They also play a crucial role in the degradation of ubiquitin-conjugated abnormal proteins. Increased levels of free and conjugated ubiquitin are found in CSF of patients with MCI progressing to AD [4]. Ubiquitin is covalently associated with insoluble neurofibrillary material of neurofibrillary tangles and senile plaques. A defect in ubiquitin conjugate enzymes or a mutation in ubiquitin (Ub) could also impair removal of unwanted proteins via proteasome [15]. The role of proteasome inhibition has been proposed for degeneration of neurons in AD brain, and Aβ is one of the factors that could inhibit proteasome activity [16]. Lipid per oxidation is an early event in AD, and the measurement of CSF F2-isoprostane levels may, in combination with other parameters, serve to predict AD [17]. Hypercholesterolemia may be a risk factor in the development of AD. High dietary cholesterol increases Aβ accumulation and thereby accelerates AD-related pathology in animals [18].

Alzheimer's disease is characterized by upregulation of the brain's innate immune responses, resulting in inflammatory processes that orchestrate cytokine and cellular responses and culminate in neuronal injury and destruction. A variety of immunological agents and other factors had been described in Alzheimer's brain, including interleukin-6, transforming growth factor β1, interferon α, and interleukins-2 and -3, heparin binding growth-associated molecule, nitric oxide synthase, macrophage-colony stimulating factor, interleukin-8 receptor B, monocyte chemoattractant protein-1, the beta-chemokine receptors CCR3 and CCR5 and macrophage inflammatory protein-1β (MCP-1), fibroblast growth factor-9, vascular endothelial growth factor, and the interferon γ-inducible chemokine IP-10 [19]. Some of the inflammatory biomarkers in CSF in AD patients are as follows: S100B is significantly elevated in CSF of patients with mild or moderate Alzheimer's disease [20]. Transforming growth factor β (TGF β) is an important astrocyte-derived cytokine that manifests both proinflammatory and anti-inflammatory properties which is induced by IL-1. Brain tissue levels of TGFβ and TGF β mRNA are increased in Alzheimer's disease [21]. Interleukin-6 (IL-6) is a major proinflammatory cytokine that can be induced by both IL-1 and S100B. IL-1 and IL-6 promote neuronal expression of neurofilaments and of tau protein and of the Aβ precursor protein. They also promote astrocytic expression of the Aβ plaque associated molecule α1-antichymotrypsin [22]. Macrophage colony-stimulating factor (M-CSF) is released by neurons in response to Aβ protein. M-CSF dramatically augments Aβ-induced microglial production of IL-1, IL-6, and nitric oxide in AD [23]. In AD, M-CSF immunoreactivity is found in neurons adjacent to Aβ deposits and M-CSF levels are increased in cerebrospinal fluid [24].

A definitive diagnosis of AD requires both clinically demonstrated dementia and amyloid plaques and tangles at autopsy, a molecular marker in peripheral tissue (e.g., skin, blood, and saliva) with high sensitivity and specificity, detectable soon after the onset of symptoms, could be important for enhancing the accuracy of clinical diagnosis and screening AD drug therapies. Several studies have suggested that AD may indeed have systemic manifestations caused by molecular, biophysical changes early in disease progression. Recent evidence in human patients and animal models supports the hypothesis that early dysfunction in the brains of Alzheimer's disease (AD) patients involves inflammatory signaling pathways [25]. The cognitive impairment of AD patients increased with changes in two inflammatory signals: lower plasma TNF-α levels and higher levels of IL-1β [26]. PKC- (protein kinase C-) mediated α-secretase activation is responsible for TNF-α generation. The deficits of PKC isozymes have been found in AD brain tissues and skin fibroblasts [27], as have deficits of PKC-mediated phosphorylation of MAPK (mitogen activated protein kinase). AD skin fibroblast cell lines have identified K+ channels that are sensitive to Aβ (1–42) interaction, changes in BK-mediated calcium mobilization via the IP3 (Inositol triphosphate) receptor, and changes in MAPK phosphorylation [27]. BK is a potent inflammatory mediator that is produced in both brain and peripheral cells (e.g., skin fibroblasts) under pathophysiological conditions such as trauma, stroke, ischemia, and asthma. Via the G-protein-coupled B2 BK receptor (BK2bR), BK activates the phospholipase C/phospholipid-Ca^{+2}/PKC cascade that, in turn, interacts with the Ras/Raf/MAPK kinase/MAPK signaling pathway, ultimately causing Erk1/2 phosphorylation [25]. Erk1 and Erk2 were previously reported to be activated in response to Aβ stimulation of the MAPK signaling pathways [25]. Therefore, a systemic pathophysiologic view of AD is consistent with recent observations that amyloid and tau metabolic pathways are ubiquitous in the human body and are manifest in blood, saliva, skin, and extrabrain tissues. Among the peripheral tissues, the superiority of skin fibroblasts over peripheral blood lymphocytes was recently discussed in a gene expression study for familial AD cases. Blood lymphocytes were found to be more susceptible to variation introduced by external stimuli such as fever, infections, and drug treatment [25].

Another inflammatory signal constitutes increased levels of C3a anaphylatoxin des-Arg and C4a anaphylatoxin des-Arg are found in the CSF of patients with MCI progressing to AD [4] C3a and C4a are part of the complement system implicated in the inflammatory processes of AD. β-amyloid directly activates the complement cascade by binding to C1q, which can produce the anaphylactic peptides C3a, C4a, and C5a [28]. A candidate cytokine related biomarker, that is, a phosphorylated C-terminal fragment of osteopontin, is found to be increased in the CSF of patients with MCI progressing to AD [4] Osteopontin is a pleiotropic integrin-binding protein and proinflammatory cytokine with functions in cell mediated immunity, inflammation, tissue repair, and cell survival. It has been identified as the most prominent cytokine-encoding gene expressed within multiple sclerosis lesions [29].

3. Plasma Biomarkers

CSF collection is invasive and unlikely to become a routine procedure in geriatric clinics. Finding peripheral biomarkers for AD is therefore of great interest. However, the levels of tau in the plasma are too low for any useful analysis. Aβ levels, while detectable, are also at least a magnitude lower. Earlier studies did not reveal significant diagnostic values for plasma Aβ peptides [30, 31]. Unlike changes in the CSF, reports of changes in Aβ levels in AD and pre-AD are rather inconsistent [32–34], and plasma levels do not necessarily reflect that in the brain [35]. In spite of these difficulties, several recent reports have now increased the confidence that plasma Aβ may be of diagnostic value. The Rotterdam Study was one of the largest prospective population-based cohort studies on the incidence and risk factors for age-related diseases, unique both in terms of its size and long-term followup. Van Oijen and colleagues found in this cohort an association between high Aβ40 and low Aβ42 levels and risk for AD dementia [36]. Another study, which compared plasma Aβ42 levels of 146 sporadic AD patients, 89 subjects with MCI, and 89 age-matched controls found that a reduction in Aβ42 is predictive for AD, and specifically, a transition from a normal state of cognition or MCI to AD [37].

A recent report also indicated that while plasma Aβ42 level alone may not be good enough as a biomarker, it is increased in early AD and changes in its levels could indicate a transition from MCI to AD [28]. In another cohort with long-term followup, the plasma Aβ42/Aβ40 ratio was shown to be a useful biomarker for identifying cognitively normal elderly white subjects at risk for developing MCI or AD [38]. The above studies have changed the outlook of plasma Aβ as an AD biomarker from an earlier perceived status of being "not very useful" to at least "moderately promising."

More studies are clearly warranted, as accurate and precise measurements of plasma Aβ are riddled with uncertainties and confounding factors. The nonspecific binding capacity of Aβ42 to proteins in the plasma is notorious. These bindings could mask detectable epitopes, and the degree of this masking could vary with metabolic conditions that differ from one subject to another. One other major uncertainty in the detection and measurements of plasma Aβ is the nature of the species measured by the antibodies used—whether they are monomeric, oligomeric, or both [39]. Standardisation of studies using well-characterised antibodies with known Aβ species specificities would help. A promising possibility along this line of thought is that risks of MCI to AD could be further tiered by, for example, the ratio of oligomeric to monomeric Aβ.

4. Anatomical Markers

Anatomical markers of AD are cerebral atrophy and macroscopic vascular alterations. Brains from AD patients are characterized by a severe atrophy leading to dilation of the ventricular system and a widening of cortical sulci [40]. In the early stages of the disease, the atrophy process affects mainly medial temporal areas including the hippocampal formation. The atrophy can be used as a marker of disease progression in clinical trials [4, 41]. In most of the Alzheimer's patients, amyloid proteins accumulate in the periphery of blood vessels leading to cerebral amyloid angiopathy (CAA) [42].

Positron emission tomography (PET), a noninvasive neuroimaging technique, allows for quantification and three-dimensional measures of distinct physiological variables, such as glucose metabolism, cerebral blood flow, and neurotransmitter and receptor function [43]. Regional deficits in cerebral glucose metabolism (CMRGlu) in the parietotemporal region, assessed with [^{18}F] 2-fluoro-2-deoxy-D-glucose (FDG) as tracer, have consistently been reported in AD. PET radiopharmaceuticals that bind to Aβ allowed the detection of amyloid deposits in the brain of AD patients [43]. The fact that the metabolic impairment correlates to deficits in neuropsychological domains and increases with progression of the disease suggests that PET may also provide a sensitive means to assess disease progression and severity [43]. Functional imaging allows measurement of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) activities, nicotinic, muscarinic receptor binding, and vesicular acetylcholine transporter in normal subjects and AD patients. These cholinergic surrogate markers are suggested to be more sensitive to early changes in brain than cerebral glucose metabolism [43].

Some of the other neuroimaging techniques include multiphoton microscopy and diffusion tensor imaging. Multiphoton microscopy is able to detect amyloid deposition labeled with specific fluorophores such as Thioflavin S or Thioflavine T derivative [44]. Near-infrared (NIR) imaging is another *in vivo* imaging technique that allows quantification of the cerebral amyloidosis in transgenic mice [45].

Diffusion tensor imaging (DTI) is based on the principle that water molecules are constantly in motion. Diffusion signals capture microstructural properties of white matter that cannot otherwise be captured on traditional structural MRI scans. Studies of the aging brain using DTI have generally found decreased fractional anisotropy (FA) and increased mean diffusivity (MD) in frontal white matter, the anterior cingulum, the fornix, and the corpus callosum [46]. These changes in anisotropy and diffusivity are generally attributed to fiber degeneration and demyelination with increasing age. Diffusion imaging studies of MCI and AD patients have observed decreased anisotropy throughout the brain but most notably in the temporal lobes. A large body of research has indicated that the medial temporal lobes (MTL), and in particular the hippocampus and entorhinal cortex, are the first to deteriorate in the course of AD. Several studies have used DTI to investigate the MTL in particular in MCI and AD. Fellgiebel and colleagues observed decreased FA and increased MD in the left hippocampus in AD patients compared to controls. Mielke et al. noted in AD patients decreased FA in the fornix and cingulum, the two major fiber bundles that connect the limbic lobes to the rest of the brain. They also observed less dramatic changes in individuals with MCI, suggesting that these microstructural alterations likely vary along a spectrum from MCI to AD [46].

Recently, the FDA approved the drug Amyvid (Florbetapir F 18 Injection) for the imaging of amyloid using positron emission tomography (PET) in adults being evaluated for Alzheimer's disease (AD) and other cognitive decline. According to Val Lowe, M.D., Professor of radiology at Mayo Clinic in Rochester, MN, Florbetapir F 18 is only one of a handful of PET drugs that have ever been approved for use in the USA [47].

Overview of the current literature provides an initial indication that treatment effects might indeed be reflected at the biomarker level. In several cases, biomarker studies led to unexpected results that opened up new questions; the answers to these questions will probably enhance our understanding of the pathophysiology of AD in the future. Further studies on core candidate markers will probably show that some presumed pathological mechanisms of marker regulation and expression are more differentiated and complex than currently supposed. Specific medium-term tasks in biomarker research include validation of the markers in autopsy-confirmed patient groups, determination of the benefit of biomarkers in the risk stratification of clinical study populations by using medico-economic models, and the controlled application of biomarkers in primary care. The aim should be to have early diagnostic markers ready in clinical practice when disease modifying treatments become available, so that those patients who would benefit from these strategies can be identified and treated in time. To this end, there is a need for thorough and rigorous codevelopment of biologic marker candidates with various functions and roles during all stages of drug development. This can only be achieved through planned synergistic collaboration between academic and industrial research partners. Biomarker research in neurodegenerative disorders is a fascinating and fast developing area; however, much can still be learned by more matured interdisciplinary fields such as oncology, immunology, and cardiovascular research.

There is clearly a growing interest among clinicians and basic scientists to tap on each other's expertise in the area of ageing neurobiology research. Such collaborations between geriatricians, neuroimaging specialists and neuropsychiatrists as well as molecular and cellular neurobiologists are being fostered. Streamlining research initiatives in a way that would maximise subject resources, data acquisition, and multifaceted analyses should be of high priority. The prospect of seeing how the above CSF and plasma biomarkers correlate with the clinical findings, stratified ethnically, is an exciting one.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

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