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Diagnostic Methods and Biomarkers for Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most frequently occurring and intensively investigated neurodegenerative disorder, which is associated with extracellular senile plaques and intracellular neurofibrillary tangles. In this review, AD related diagnostic strategies and the potential biomarkers of AD will be discussed. Several proteomics methods were developed for disease diagnosis, such as ELISA, MALDI-TOF, SELDI-TOF, and 2 D-electrophoresis. Imaging technologies, such as MRI and PET analyses, are also important, since they could directly show the changes in the brain, associated with dementia progression. MRI technologies might estimate the presence and degree of neurodegeneration by identification and quantification of atrophy. PET could reflect the metabolic changes in the brain by various radioactive molecules (tracers). Along with neuropsycoanalysis of behavioral changes, the progression of dementia can be characterized with biochemical changes in brain metabolisms, in addition to fluctuations in many inflammatory mediators in the cerebral spinal fluid (CSF), blood and in other bodily fluids. These biochemical changes in the brain and other body fluids can be initiated before the appearance of AD symptoms. There is no specific marker for AD along with other dementia, but the combination of different markers may predict the disease progression more accurately. Monitoring the changes in their levels in brain, CSF, blood and body fluids with biomarkers in early disease stages might improve the diagnosis and therapies. Several molecules were established as successful biomarkers for AD diagnosis. Ratio of Abeta42/40 became an important AD marker, which could reflect the disease-associated changes in the blood plasma and CSF. Additional markers were available in blood, such as apolipoprotein E or inflammatory molecules. In CSF, the Abeta42, Tau or phospho-tau could be the most successful biomarker for AD progression. Several new biomarkers and diagnostic approaches were developed for differentiating AD from other forms of dementia. It should be important to predict the AD progression prior to the development of clinical symptoms. Above all, the improvement of above strategies, especially with diverse biomarkers, should support the precise diagnosis of AD, greatly enhancing both AD therapies and preventative measures.

Keywords: Alzheimer's diseases, Diagnosis, Biomarkers, Amyloid beta

Alzheimer Disease

Alzheimer Disease (AD) is the most common form of neurodegenerative dementia, especially in the elderly. AD represents the majority (approximately 70%) of all dementia cases[1,2]. Several additional types of dementia can be distinguished, such as vascular dementia (VD), dementia with Lewy bodies (DLB), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS) and Creutzfeldt-Jakob disease (CJD)[2,3]. In 2006, number of AD patients was estimated to be approximately ~25 million[4,5], and it was expected to increase due to the demographic changes and the extended lifespan[6-8]. In 2000, 4.5 million AD patients were reported in US, and this number was predicted to reach the 11-16 million by 2050[9,10]. Based on the statistics of Alzheimer's Association (2010), approximately 5.3 million AD patients were counted in US, which were older than 65 years of age, and only a few AD patients (approximately 200,000) were under 65 years of age[10]. A Canadian study revealed that the prevalence of AD was increased with the age (1.0-2.4% after 65 years, 6.9% after 74 years, and 26-35% among the people over 85 years of age)[10-12]. Korean statistic data from 2003 showed that the percentage of individuals over 65 years of age was 8.7%, and it was estimated to grow approximately up to 13.2% of total population by 2020. Korea was noted as one of the fastest aging

countries. In 2003, the number of demented elderly patients was approximately 300,000, which increased to 400,000 in 2009. This number was predicted to rise up to 6-700,000 by 2020 and up to 2 million by 2050[13-15].

In the 20th century, the life expectancy and the size of total population became higher. In the US, the lifetime increased from 49 years to more than 76 years, which resulted in a rise in the number of dementia[16]. AD was suggested as the third most expensive disease to treat in US, and the estimated cost of disease treatment was more than 100 billion USD between 2000 and 2006. The medical costs for one patient with AD (who is in her early stages of disease) could reach the ~20,000 USD per year[17-19]. The Blessed Dementia Rating Scale predicted the annual growth in direct medical costs to be ~1,411 USD, and the annual unpaid care giving costs could reach ~2, 718 USD per person[4,19]. In Korea, the medical costs for dementia patients are also increasing. Due to the data of Korean National Health and Insurance Corporation, the dementia-related medical costs increased from 56 billion KRW (2002) to 326.8 billion KRW (2007)[15].

The clinical diagnosis of dementia is based on the history of patient and informant's collateral (who can be a family member or caregiver), and on physical examination. Guidelines of AD diagnosis should mostly focus on mild to moderate stages, because the treatment might be more successful before the clinical symptoms appear[12,20]. Mild cognitive impairment (MCI) is important in AD diagnosis, since it can be defined as a "grey zone" between dementia and normality[21]. MCI is a cognitive decline syndrome with higher memory deficiency than that expected for an individual. MCI depends on the age and education, but it might not be associated with the daily activity. While some MCI patients can remain stable, other individuals with memory complaints (amnestic MCI) might have higher risks for dementia. Additional factors, such as cholirogenic dysfunctions, white matter lesions, cerebral infarctions, extracellular amyloid deposition, intracellular NFT formation might increase the risk of AD progression from MCI[22]. Since MCI is clinically heterogeneous, it can trigger to problems in the disease diagnosis and in the prediction of clinical progression[23,24]. There are a large number of undiagnosed and untreated dementia cases in early stages, and several patients might be overlooked. Moreover, early diagnosis and treatment is essential for the further development of AD therapeutics and human healthcare services[25,26].

Symptoms and Stages of AD

AD can be associated with neuronal loss and decreased synaptic activity, resulting in extracellular senile plaques, intracellular neurofibrillary tangles. These abnormalities could induce neurodegenerative cascades, resulting in brain mass reduction. From the first clinical diagnosis, the disease duration is usually 8-10 years, and the patients finally die[27]. Three major stages of AD can be distinguished as follows: In the early stages, memory and language problems can appear because of the loss of sensory and motor functions of the neurons. Personality changes, such as depression and aggression might be present in the very early AD. In second stage, memory decline and language impairments can be more prominent, which can be associated with paraphasia. The personality might be maintained, but patients could have greater tendency of confusion, and they need assistance in several activities. In the final stage, which usually appears 8-10 years after the first diagnosis, the full range of symptoms of mental illness has been developed. Patients with late AD might be unable to move, eat or speak. In addition, they might be more sensitive to other diseases, such as pneumonia or urinary tract infections[28].

Pathology of AD

Similarly to the other forms of dementia, AD is associated with neuronal death and brain destruction. The brain of a normal adult individual contains approximately 100 billon neurons with 100 trillion synapses. Significant reduction of synapses has been observed in AD, resulting in neuronal death and brain mass reduction[10]. AD diagnosis is based on the identification of morphological abnormalities in brain cells and tissues. AD is characterized by two key features: intracellular neurofibrillary tangles (NFT), and extracellular amyloid plaques or senile plaques (Figure 1)[6]. The presence of plaques and NFTs can result neuronal apoptosis through a variety of mechanisms, such as stress or inflammation[29]. NFTs are abnormal fibrous inclusions inside the brain cells, resulted by the hyperphosphorylation of Tau protein[3,6]. Amyloid plaques have been described as the most important features of AD neuropathology. The major components of these plaques are the 40-42 bp long amyloid beta (Abeta) peptides[5]. Abeta peptide has a "U" shape topology, and it has two parallel beta strands which are connected by a turn. The misfolded Abeta peptides have high ability to aggregate into oligomers or fibrils, and they could accumulate in the brain tissue. Abeta fibrils are resistant to protease digestion[30-33]. Abeta peptides are the products of proteolytic cleavage of large (770AA) amyloid precursor protein (APP). APP protein contains the

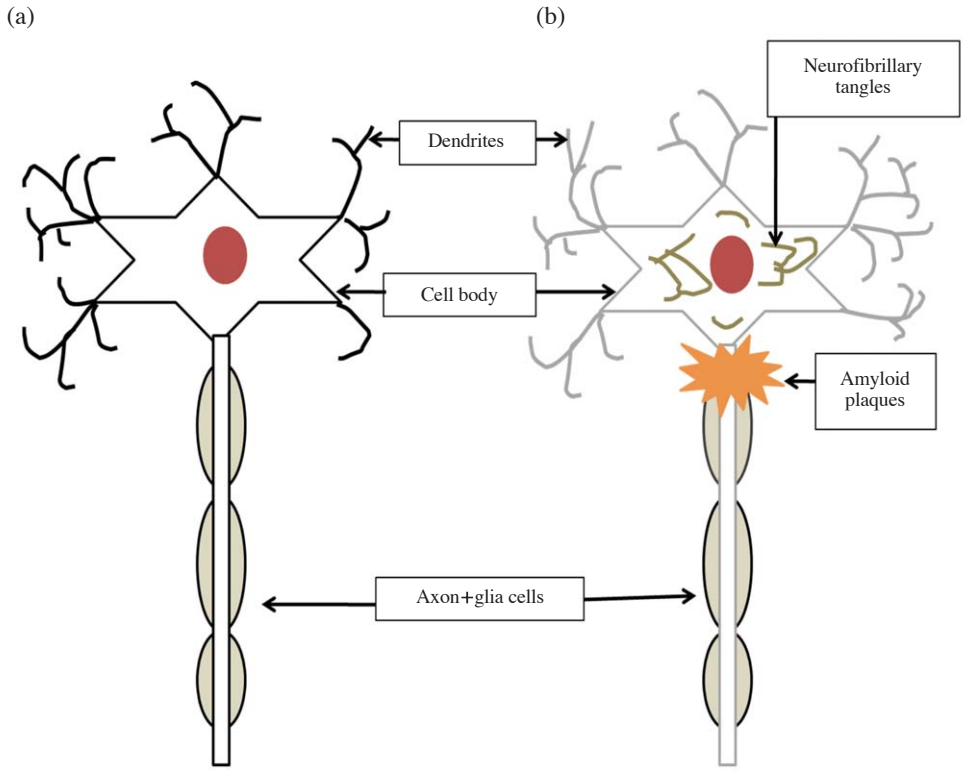

Figure 1. Differences between a (a) healthy nerve cell and (b) the nerve cell with AD. Amyloid plaques are located outside of the nerve cells. They contain the aggregated and abnormally folded Amyloid beta (Abeta) peptide. Intracellular neurofibrillary tangles are located inside the nerve cells. These tangles are resulted from the abnormal phosphorylation of Tau protein.

cleavage sites of 3 enzymes, called alpha, beta, and gamma secretases. Abeta peptides are resulted by the beta- and gamma secretase cleavage. Alpha secretase attacks the APP within the Abeta sequence, and it could reduce the Abeta formation and accumulation in the brain. Stimulating the activity of alpha secretase and/or blocking the beta and gamma secretases could inhibit the Abeta formation, and improve the therapies[34-37].

Diagnosis and Biomarkers of AD

Diagnostic process of dementia has three major components: the clinical diagnosis, the search for disease causes, and the identification of other contributing factors. Diagnosis should have 6 important steps: taking the clinical history of patient, making interviews with the family members or caregivers, physical analysis, brief cognitive- and laboratory tests, and structural brain imaging[27,38,39]. Diagnosis of AD diagnosis improved a lot in recent years, since the new technologies provide more accurate and rapid disease detection. However, the differential analysis of various dementia types, such as AD, VD, FTD, DLB, is a current issue in the diagnosis. MCI may increase the risk for neuropathology and AD. Identification of MCI can be helpful in the early diagnosis and treatment of dementia, but in several patients, MCI might not be associated with AD progression[40-44]. Development of early diagnosis should be important because the therapies for AD might be most effective in early stages of disorder. Biomarkers are essential in the disease diagnosis[40] (Table 1), because they can reflect the AD-specific changes *in vivo*[42]. The Ronald and Nancy Reagan Institute of the Alzheimer's Association and National Institute on Aging Working Group on Biological Markers of Alzheimer's Disease (1998) proposed the criteria for the ideal biomarkers, which could clearly reflect the disease progression[40,45]. First of all, biomarkers should be specific for the brain, measurements with them must be quatitative, and reflect the neuropathology. Ideal sensitivity and specificity of disease markers should be over 80%. Changes, associated with the disorder, should be detected by the marker in the early disease phase, since the treatments must be started as early as possible. In addition, they must be cost-effectivene, simple and reproduceable[40,45]. Using neuroimaging methods from the blood and CSF markers could

Table 1. Examples for AD biomarkers[36,37,40,45].

Marker	Presence			Diagnosis method	Role in AD progression
	Blood	CSF	Other		
Abeta peptide (42AA)	+	+		ELISA	Senile plaques
Tau protein (phosphor-Tau)		+		ELISA, imaging	Neurofibrillary tangles
ApoE E4 allele (gene and protein)	+	+		ELISA, genotyping	Neurotoxicity
APP		+		ELISA	Parent molecule of Abeta
DNA, genes	+			PCR, sequencing	Mutations, increase the risk for AD
Isoprostanes	+	+	Urine	GC/MS	Oxidation, nitritation (lipids, DNA, RNA)
Homocysteine	+			ELISA	Possible risk factor for dementia
ACT (alpha-1-Antichymotripsin)	+	+	serum	ELISA	Component os senile plaque, possible risk factor for dementia
IL6 receptor complex		+	serum	ELISA	Inflammatory mechanisms related to AD
C1q	+	+	serum	ELISA	Enhances the Abita aggregation

reduce the time of diagnostic process and the sample size. Finding ideal AD biomarkers is important, because early detection could improve the therapies. Diagnosis with the perfect biomarker should avoid the false positive- and negative result, and it has to detect only true positive and true negative results. There is no specific biomarker for AD, hence, a set of biomarkers should be used to confirm the presence or absence of the disorder[45]. Biomarkers in the body fluids, such as cerebrospinal fluid (CSF) or blood (plasma) can be mostly used in research settings. Several assays have been developed for detection of changes in the marker levels[6,37,38].

Common Proteomic Methods in AD Diagnosis

2D Electrophoresis

2 dimensional PAGE is a widely used proteomic technology, designed for the separation and identification of different proteins from mixtures. The first step of this process is the isoelectric focusing (IEF), which separates the proteins, according to their charge (isoelectric points). After IEF, the proteins should be separated by SDS-PAGE electrophoresis, according to their size. This process results a one dimensional map, where each spot would represent one protein. Several proteomic AD markers can be analyzed simultaneously by 2D PAGE, since this method could reflect the disease-related changes of each proteins[46,47].

Mass Spectrometry (MS)

Mass spectrometry (MS) is widely used in proteomic technology. Biomarkers of AD could be detected by MS from plasma or CSF. Three major modules of MS can be distinguished: the ion source, the mass analyzer and the detection unit. Two main approaches of MS are commonly used in the diagnosis: the matrix assisted laser desortion ionization (MALDI) and electrospray ionization (ESI)[48]. These technologies can generate unfragmented protonated molecules, and identify the properties of proteins, such as molecular mass, charge. In MALDI, single charged ions would be desorbed from solid phase, resulting in spectra interpretation. In ESI, the ions with variable charges would be desorbed from solution. ESI could produce multiple charged droplets, where the data could represent the different ionization stages[49-51]. SELDI is an alternative variation of MALDI, which is connected to a protein chip array or specific protein surface. The function of chip surface is the fractionation and enrichment of protein subpopulations[49].

Imaging

Magnetic Resonance Imaging

MRI is an important approach in clinical AD diagnosis, because it can characterize the structural lifetime changes in brain, especially in hippocampus. Discovery of new biomarkers which increase the sensitivity of such determinants could promote MRI as a central method in AD diagnosis[52,53].

Medial temporal lobe atrophy (MTA), can be a sensitive and important marker for dementia, including MCI and AD. Methods for atrophy identification should be rapid, simple, and must show the differences between AD and other types of dementia[54]. Studies have suggested that MRI can be a possible method to give additional information about the risk for AD onset[29,55,56]. A ten year follow-up study was performed by den Hejler *et al.* (2010), which measured the volume of hippocampus and the level of atrophy in dementia by MRI and neuropsychological tests. Dementia could be associated with reduced hippocampal volume and increased degree of atrophy. These findings suggested that atrophy and decline in hippocampal volume could be used as an early marker for dementia[57]. Quantitative

MRI studies have suggested that atrophy in hippocampus might be present before the development of clinical symptoms. These results supported the importance of brain imaging in MCI and AD patients[55]. Identification the features of MCI and its prediction of conversion into AD should be an important goal in AD diagnosis[52]. Structural MRI analyses should specifically detect and quantify the features, associated with AD pathology. It also should reflect clearly the differences between healthy controls and the AD patients. MRI screening should show high consistency and reproducibility across multiple independent cohorts, and correlate with clinical and invasive measures of cell pathology[29].

The first structural MRI devices analyzed only one specific brain segment, which was called region of interest (ROI). These methods have some disadvantages, such as low accuracy, and the ability of testing-retesting might be limited[29,58,59]. Development of a semi-automated MRI process should be important, since the quantification of ROI became more challenging. Automated methods could be used in various approaches of brain scanning[29,60]. Development of structural MRI softwares could automatically parcellate the brain into different anatomic regions, and it would quantify the degree of MCI or athropy[58].

MRI technologies have been accepted not only in clinical diagnosis, but also in monitoring the success of treatment. These technologies are based on changes in T1 and T2 contrast relaxation time. T2 relaxation time differences were not significant between AD patients and healthy controls, but T1rho contrast mechanism (spin lattice relaxation time) was successfully used for identification and characterization of different cancers. Studies were performed to find association between T1rho and AD, which compared the brain of normal individuals and AD patients. These measurements suggested that T1rho MRI could be promising method in AD diagnosis[61,62].

Positron Emission Tomography (PET)

Positron Emission Tomography (PET) is an imaging and measuring technology, which is using positron labeled molecules. Several biochemical processes can be monitored by PET, and their changes might predict the disease progression. PET can provide an accurate early differential diagnosis of AD[63]. The more accurate identification of Abeta levels with PET might offer new routes in AD research. Several types of radiopharmaceuticals (tracers) can be used for PET imaging, and several tracers are currently under development. F18 and C11 molecules are the most well-known PET tracers, which could be used in clinical settings[30,64]. For *in vivo* labeling, the most well-known C11 tracer is the [^{11}C]2-(4′-methylamino phenyl)-6-hydroxybenzothiazole or Pittsburgh Compound B (PIB), which could be useful for CSF imaging[30,65]. [^{11}C]BF-227[66] and [^{11}C]SB-13[67] are also widely used tracers. [^{18}F]DDNP or FDDNP could bind to abnormal brain proteins, especially to Abeta plaques. FDDNP might be a possible tracer for the detection of Abeta, NFT or prion protein assembly[30,68-70]. A well-known F18 chemical, [^{18}F]BAY94-9172 could bind to the same site of amyloid plaques, as does the PIB with similar affinity[71]. [^{18}F]DG can provide a sensitive analysis of neurophysiological effects, by monitoring the cerebral glucose metabolic rate[72]. Table 2 introduces the PET tracers, which could be used for AD diagnosis.

Blood Based Biomarkers

Blood diagnostic methods are currently under development. Investigations and analyses of plasma and serum markers are important goals in AD diagnosis, since a specific multi-marker profile might indicate the risk for dementia[69].

Genetic Markers

Genome wide association studies (GWAS) are currently used for testing the putative genetic loci, associated with AD. Analysis of genetic variations can provide better AD diagnosis[76]. Apolipoprotein E (*APOE*) gene E4 allele (Codon 112 and 158: Arg) can be associated with late onset AD (LOAD). E4 allele is not deterministic for AD, but it could increase the risk for AD and support AD diagnosis[77,78]. Mutations in amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) can be involved in the onset of familial or sporadic early onset AD (EOAD)[79].

Proteomic Markers

Abeta peptide can be monitored in CSF and blood plasma[37]. Scheuner *et al.* (1996) studied the relationship between AD mutations and blood Abeta42 concentrations[80]. APP protein was described as a key molecule in AD progression, by producing Abeta peptide. After the alternative splicing of *APP* transcriptome, at least three main APP isoforms (150 kDa, 130 kDa, 110 kDa) can be distinguished. Alterations in APP metabolism can be clinically significant in early stages of AD. Decreased ratio of high molecular weight APP to low molecular weight APP was measured in the plasma of AD and MCI patients, compared to normal controls[81].

APOE, a receptor ligand, plays an important role in lipid metabolism, brain regeneration, and immune modulation[82]. *In vitro* and *in vivo* studies revealed that APOE E4 allele might inhibit the Abeta clearance, and recycling. APOE protein with E4 allele can bind to Abeta peptides, and form fibrils with them. Amyloid

Table 2. PET tracers, which could be used in AD diagnosis.

Chemical	Characterization, focused on Amyloid imaging	References
[^{11}C]PiB	PiB-is a potential radiotracer to Amyloid plaques PiB could provide quantitative information on amlyoid uptake in living subjects	65
[^{18}F]-FDDNP	FDDNP binds to the Abeta and to the Tau aggregates. Successful in detecting senile plaques and NFTs Clearance time is slow FDDNP is able to cross the blood brain barrier and cell membranes, because it is lipophilic Helpful in differential analyses of dementia	65
[^{18}F]-FDG	FDG can show the *in vivo* the changes in the brain glucose metabolism FDG is a specific tracer for monitoring the AD progression	71, 73
[^{11}C]BF-227	This tracer cab bind to Abeta plaques with high affinity Acculates in the neocortex MCI converters could be clearly distinguished from non-converters by C11 BF-227	72
[^{11}C] SB13	Useful in fibrillar Abeta imaging Similar to the [^{11}C]PiB Useful in AD and amnestic MCI diagnosis	67
[^{18}F]-BAY94-9172	This molecule is promising in the early diagnosis Similar to the [^{11}C]PiB With [^{18}F]-BAY94-9172, amyloid deposition might be detected early This traces could be helpful in prevention and therapeutic strategies	64
^{18}F-AV-45 (Flobetapir)	FAV 45 might be a possible tracer in monitoring the glucose hypometabolism in neurodegenerative dementia	74
^{18}F-flutemetamol	F18-flumetamol might be a potential tracer for monitoring the Abeta amyloidosis. Similar to PiB Flumetamol provides good discrimination between the healthy controls and AD patients	75

fibrils can accumulate in lysosomes. Since these fibrils might be resistant to lysosomal digestion, this process may trigger to the destruction of lysosomes, to the release of proteases and cathepsins, followed by apoptosis or necrosis[82,83]. Neurotoxicity of APOE could be independent from Abeta, but the interaction between APOE E4 and Tau protein might enhance the Tau phosphorylation leading to NFT formation in the nerve cells. *In vivo* and *in vitro* studies have shown that APOE E4 allele could inhibit the neuronal growth and microtubule stabilization[84-87].

Neuronal inflammation might be involved in the onset of dementia. Interleukins (IL) and tumor necrosis factor alpha (TNF-alpha) could appear inside the senile plaques. Increased IL1 expression has been detected in microglia cells, which were located near to the senile plaques. IL1 was defined as a key molecule in initiation and propagation of neuroinflammatory changes[88]. After injury and infection IL-1 beta expression can increase. Levels of IL1 beta were higher in the serum of AD patients, comparing to the controls. Forlenza *et al*. (2009) found significant increase of IL1 beta levels in the serum of AD patients (n=58; c: 3.78+−0.81 pg/mL), comparing to the controls (n: 31; c: 1.20+−0.60 pg/mL). In MCI, intermediate IL1beta levels have been detected (n=74; c: 2.61 8 0.53 pg/mL), and it was not significantly different in controls[89]. IL6 could act as multifunctional cytokine with pro- and anti-inflammatory effects. Normally, the expression of IL6 is down-regulated, but in several disorders, including in AD, significant increase was found in the blood IL6 level. IL-6 has been monitored in THP-1 cells, by Abeta induction, and its level could increase rapidly in the early stages[90,91]. IL10 is a microglia-expressed anti-inflammatory cytokine, and its expression can also be increased in AD patients. Additionally, Il1 beta, IL6 and IL10 might be potential genetic risk factors for AD[92].

Other Markers

Reactive oxygen-radicals (ROs) can be involved in different kinds of disorders, including AD[87]. In AD brain, the degree of protein and DNA oxidation has been increased[93]. Antioxidant molecules might protect against ROS, and they could prevent or delay the effect of oxidative damage. Enzymatic (superoxide dismutase or catalase), or non-enzymatic (vitamins, phenol, bilirubin) molecules could be produced against the free radicals. Foy *et al*. (1999) tested the differences of antioxidant levels between healthy controls and AD or

VD patients. Levels of vitamin A, C, E and beta-carotine were significantly decreased in AD patients, comparing to the healthy individuals[94-96].

Since beta and gamma secretases are located on cholesterol-rich membrane rafts, hypercholesterolemia might be a potential early risk factor for AD. Cholesterol can be important in AD pathogenesis. In the serum and brain of AD patients, correlation was found between total cholesterol and the degree of APP cleavage, Abeta production and amyloid deposition[97,98]. Significant interaction was found between the APOE E4 allele, cholesterol levels and AD[99,100].

Homocysteine is the part of DNA methylation pathway, which can be involved epigenetic processes. Methionine transforms to S-adenosylmethionine (SAM) by ATP hydrolysis. SAM can add a methyl group to DNA, proteins or lipids. The removal of methyl- and adenosyl group from SAM results in S-adenosyl-homocysteine and homocysteine formation, respectively. Vitamin B12 and folate are needed for this reaction. Increased homocystein concentration in blood might be associated with reduced B12 and folate levels. Elevated levels of homocysteine could predict different disorders, such as cardiovascular problems, osteoporosis, pregnancy complications or dementia[101].

Vascular risk factors might be associated with several diseases, such as atherosclerosis, cognitive decline or AD. Increased platelet activation could predict the onset of dementia, especially in stroke survivors[102]. Stellos *et al.* (2010) studied the association between cognitive decline and expression of fibrinogen receptor, GPIIb-IIIa glycoprotein, P-selectin on circulating platelets. Inflammation mediates the platelet activation in vascular cells, leading to cerebrovascular dysfunctions and dementia. Abeta peptides were found in platelets, and this study suggested that platelet activation might be a successful marker in AD diagnosis[103,104].

Blood Diagnostic Methods

Tau and phospho-Tau are typical CSF markers, but due to their instability in blood, they might not be useful in the diagnosis in plasma. Plasma Abeta40 and Abeta42 have been established as successful markers for AD and MCI. Their levels can be measured by sandwich ELISA. Difficulties might be possible Abeta diagnosis from blood, since Abeta levels are low in plasma. False positive data can also be resulted by the lipoproteins or Fc binding proteins[105]. ELISA kit was developed by Roche Diagnostic, where the microtiter plates were coated with 6E10 antibody, and two capturing antibodies could be used: R226 for Abeta42 and R209 for Abeta40[106]. Platelet activation might be analyzed by whole-blood cytometry, with specific antibodies. After the binding of monoclonal antibodies, the intensity of fluorescence signal might indicate the platelet protein expression[107].

CSF Biomarkers

Biomarkers in CSF

Cerebrospinal fluid (CSF) has direct contact with the extracellular region of brain. The biochemical changes in CSF might predict the disease progression. Since AD is a neurodegenerative disorder, the CSF proteins could be promising markers for early diagnosis. Phospho-Tau, and Abeta have been established as the most important CSF markers[108]. These biomarkers may provide great accuracy in the early AD diagnosis[109].

APP protein is the most well-known precursor molecule in AD pathogenesis. APP protein carries the cleavage site of three enzymes: alpha, beta and gamma secretase. Normally, the alpha secretase cleavage results in a benign alpha-soluble peptide (alpha sAPP). In patients, the initial beta secretase (BACE) cleavage results in a beta-sAPP, and beta-sAPP would be cleaved by gamma secretase[110]. Beta- and gamma secretase could result three forms of Abeta peptide: Abeta42, Abeta40 and Abeta38. The role of Abeta38 in AD progression is currently not fully understood yet[111]. In healthy individuals, (normal metabolism) Abeta42 is secreted to CSF as a soluble protein, and decreased Abeta42 levels and Abeta42/Abeta40 ratio were observed in the CSF of AD MCI patients. Studies have suggested that the reduced levels of CSF Abeta might be associated with the amyloid plaque aggregation and with the decreased Abeta clearance. The levels of Abeta42 and the ratio of CSF Abeta42/Abeta40 were lower in AD and MCI patients, which suggested that they could be a promising AD marker[112,113]. Levels of extracellular chaperons, which promoted the macrophage Abeta-uptake could also be reduced in the CSF of AD patients[114].

CSF Tau is an alternative indicator of neuronal injury. The phosphorylated Tau might be more specific than normal Tau, but both of their level could be increased in the CSF of AD patients. This rise might be related to the release of Tau protein from the injured neurons, and its diffusion into the CSF. Increased CSF Tau levels were established as successful indicator of several disorders, such as Creuzfeldt-Jakob Disease (CJD), or stroke[115].

Levels of fibrinogen gamma-A chain were increased in CSF of AD patients[116]. Ubiquitins or neurofilament (NF) proteins might be potential CSF marker candidates. Increased NF proteins were reported in the CSF of AD patients. Combination of NFs with other CSF

markers, such as Tau, could be a promising strategy in AD diagnosis[117]. Retinol binding protein (RBP) and haptoglobin precursor allele 1 levels might decrease in the CSF of AD and MCI patients[118]. CSF ubiquitin levels have been elevated, but serum ubiquitin levels might be dropped in AD patients. CSF/serum ubiquitin ratio was shown different levels in AD patients and healthy controls[119].

Isoprostanes are the final products of non-enzymatic lipid peroxidation. Postmortem studies revealed that F2 isoprostane levels could be increased in CSF of MCI and AD patients. Isoprostanes have been suggested as accurate markers for disease identification[115,120].

Several markers or candidate markers have been discovered in CSF, but their clinical use might be limited by several issues. The sample collection, transportation and storage could be problematic, and the routine diagnosis might be costy[115]. Imaging of CSF amyloid markers with PiB could be useful in preclinical stages, but it might not be able to distinguish the disease stages. It is still unclear if the marker sensitivity would correlate with the natural AD progression[121].

CSF Diagnostic Methods

PET-PIB is a widely used for CSF biomarker detection hence it was described as a successful tracer for Abeta42 measurement. PET analysis indicated the Abeta42 drop in the CSF of early AD patients, and it remained low for those suffering from more progressed forms of disease. The amount of Tau in CSF could also be detected by PIB-PET. Measurement of CSF Abeta42 and CSF-tau/Abeta42 could be useful for early AD diagnosis and treatment[122].

CSF Abeta and Tau levels could be quantified by sandwich ELISA assays[123]. To determine the CSF Tau levels, a specific ELISA kit was designed by Innotest Company (Gent, Belgium), where microtiter plate has been coated by a monoclonal antibody (antihuman Tau, AT120). Two different Tau-specific monoclonal antibodies, H57 and BT2, can be used for Tau epitope identification[124]. CSF Abeta levels could be measured by a sandwich-ELISA method, BAN-50 antibody can be used as capturing antibody, and two HRP conjugated antibodies would identify the different Abeta forms (BA27 for Abeta40 and BC05 for Abeta 42)[125,126].

Trends in AD Diagnosis

In the amyloid plaques, Abeta42 can co-aggregate with Abeta40. Abeta40 starts at Asp1 and terminates at Val40, while Abeta42 contains two additional hydrophobic amino acids (Ile41 and Ala42) at the C terminal. Measuring the levels of Abeta oligomers (x-42), tTau and pTau in the CSF could be changed in AD patients. CSF markers might improve the differential diagnosis of neurodegenerative disorders. Currently, the differential diagnosis and the estimation of dementia severity is not accurate enough[127,128].

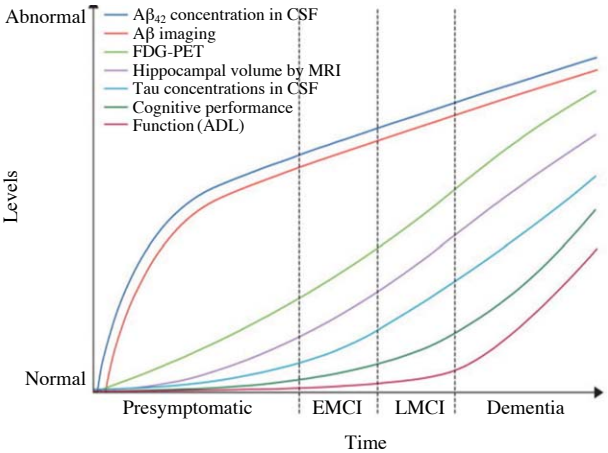

Figure 2. A hypothetical sequence of AD progression, initiated by amyloid deposition in the brain, and followed by neurodegeneration, designed by Ronald Petersen (2010). Amyloid deposition can result neuronal degeneration, leading to MCI and dementia, respectively. These measurements indicated the increasing degree of abnormality (EMCI-early MCI; LMCI: late MCI; ADL: Activity of Daily Living) This graph was adapted and reprinted with the permission of Lancet Neurology.

Abeta42 has more capacity to aggregate than Abeta40. Two forms of Abeta aggreges could be distinguished in plaques: they can form insoluble fibrils or small oligomers. Studies on neuroblastoma (LAN5) cells suggested that the Abeta oligomers can be more toxic than the fibrillar Abeta[129]. Fukumoto *et al.* (2010) developed an ELISA method, which is specific for high molecular weight (HMW) Abeta. BAN 50 antibody was used in this assay. These findings revealed that diagnosis with the assembled Abeta42 levels might be more successful than with its monomeric forms. The BAN 50 - ELISA system might be a possible strategy in the differential analysis of AD and MCI patients, and it could estimate the severity of disorder. In addition, BAN50-ELISA could be used in high throughput screening for modulators of Abeta assembly[128].

From 2004, the North American Alzheimer's Disease Neuroimaging Initiative (ADNI; California, Berkeley) has been analyzing the AD prediction and progression with the combination of different biomarkers and imaging technologies (MRI, PET) (http://www.adni-info.org/index)[130]. Similar projects were investigated in

Table 3. These values show the biomarker changes in CSF, related to AD, measured by XMAP immunoassay. As expected, the concentration of Tau and P-Tau increased and the concentration of Abeta decreased in the CSF of AD patients. The data showed that the level of AD markers changed dramatically in the first stage of the disorder (after 0-2,5 years), indicating that the diagnosis of these markers could be more effective in the early stages of dementia[135] (AAIC 2011 presentation).

	Concentration in CSF (ng/L)		
	Abeta	P-TAU	T-TAU
Controls	~700 (650-750)-	~60 (50-70)	~300 (250-350)
MCI to AD between 0-2,5 years	~325 (300-350)	~97.5 (90-105)	~850 (750-950)
MCI to AD between 2,5-5 years	~325 (300-350)	~87.5 (80-95)	~700 (600-800)
MCI to AD between 5-10 years	~340 (310-370)	~ 67.5 (60-75)	~450 (350-550)

Europe, Japan and Australia. Petersen (2010) designed a theoretical framework to model the disease-related cascade mechanism. This model introduced the potential mechanisms of AD progression, started with Abeta deposition, followed by additional pathological symptoms, leading to cell death. Further verification might be needed, but this model could be a useful in the development of imaging technologies and in the AD biomarker research. The ADNI assessed complementary roles for amyloid imaging (C^{11} PIB). Hippocampal volume of patients was measured by MRI, which demonstrated that AD cascade was the initial with the amyloid deposition, followed by Abeta induced atrophy in the hippocampus and memory problems. Biomarker changes in the brain have been monitored at the Mayo Clinic. The combination of risk factor genes, imaging technologies, and biomarkers could provide a promising field in AD prediction (Figure 2)[131].

Amyloid assembly in the brain can trigger neuronal degeneration, resulting in AD progression. Understanding the relationship between the protein aggregates and cognitive decline is a current trend in AD research. Abeta peptide is a key molecule in AD progression, which could exist in different forms, such as monomers, low molecular weight oligomers, high molecular weight oligomers or in fibrils. Studies are needed to determine, which form of Abeta would be the most toxic to the neurons. Studies on transgenic mice have suggested that oligomeric, but non-fibrillar amyloid aggregates might have stronger effects on memory decline and neuronal dysfunctions. Cheng *et al*. examined an APP mutation, called "Arctic APP" (E22G), which could be strongly associated with Abeta production, aggregation and plaque deposition. Blocking and reversing the Abeta aggregation and fibril formation could be a promising therapeutic approach for AD treatment[132]. Lesne *et al*. (2008)[133] and Cheng *et al*. (2007)[132] examined the Abeta*56 oligomer, and they detected impairment without plaques. These findings suggested that Abeta*56 aggregates could be associated with memory decline in the early disease stages. Abeta plaque deposition and fibril formation can increase with the age and it could be associated with decreased Abeta *56 levels in the brain. This data suggested, that reducing the amyloid burden might be less effective without decreasing the Amyloid*56. Reduction of big Abeta aggregates might be a promising therapy in the future[132-134].

In 2005, Olsson *et al*. developed a new xMAP technology, which was a multiparametric, bead-based assay. A flow cytometry method was performed, where they measured and quantified the Abeta 1-42 (MAb4D7A3, 21F12MAb and MAb3D6 antibodies), total Tau (AT120 and HT7 antibodies) and phospho-Tau (AT270 antibody) by using fluorescent microspheres. These microspheres were covered with specific antibodies (one microsphere with one kind of antibody), and labeled with a specific concentration of fluorescent dye, with a precise concentration ratio of red and orange fluorochromes. The first laser should be used for the classification of these beads by excitation at 635 nm. Microsphere sets can be combined within one method. One bead should be covered with only one antibody, and the signals from analytes in the mixture must be unequivocally identified. The third fluorochrome was the streptavidin-labeled phycoerythrin (PE). These fluorochromes have been used for quantification of molecular reaction which occurred on the surface of microsphere. PE and SV generated a green fluorescence signal, followed by PE excitation by the second laser at 532 nm. Multiple CSF markers can be used (different Abeta peptides, alpha-synuclein) in this analysis, and the changes of their levels might be detected before the clinical symptoms appear. This method could be a useful and promising approach in the differential diagnosis of dementia, and it might reduce the time and the sample size[135]. Table 3 shows data from XMAP determinations.

Recent studies have suggested that oligomeric, but non-fibrillar Abeta42 might have high neurotoxic capacity, by inducing the neuro-inflammation and block neuronal functions. Level of soluble Abeta oligomer aggregates can be elevated in CSF. Future investigations are needed to determine which oligomer mechanism can be mostly involved in the neuronal dysfunc-

tions. Diagnosis of Abeta oligomers might also improve drug development[136]. Rapid, specific methods are needed for the quantitative detection of Abeta oligomer aggregates, but they are currently not available. A promising, fluorescent dye-labeled peptide probe (PG46) has been designed, which can bind to Abeta oligomers, followed by the generation of fluorescent signals[137]. Small Heat shock proteins might also be potential therapeutic candidates against the toxic oligomers, but their protective mechanisms are currently not well understood. The neuron cultures of HspB1 deficient mice might be more sensitive to toxic oligomers, comparing to the wild-type controls. This data suggested that the cytoprotective HspB1 warrants can be great therapeutic approaches in the future[138].

Biomarkers in the peripheral region could enhance the efficacy of AD diagnosis. Saliva was frequently used to monitor the changes of several hormone levels, including testosterone, insulin, and cortisol. Salivary acetylcholinestherase changes can be detected, and they were validated as promising markers for AD[139]. Saliva is a biological fluid, produced by the salivary glands, and mucosa. APP and Abeta might be expressed in the epithelial cells, which could produce the saliva. Hormone levels in saliva could reflect the Abeta changes in CSF[140]. Oasais Diagnostic Company developed saliva tests for disease diagnosis and drug testing, such as the simple and immunochromathographic VeroFy test. It was applied against several disorders, such as diabetes, breast cancer. Due to the latest updates, this technology might be useful for AD and PD detection in the future[141].

Conclusions and Future Insights in Diagnosis

AD is one of the major health problems nowadays, and its diagnosis might be problematic. Biomarkers for neurodegenerative disorders are essential in early diagnosis, since they can be involved in monitoring the disease progression and in treatment optimization[124]. AD diagnosis was developed to a high degree in the recent years, but the differential diagnosis of the various neurodegenerative disorders is still not accurate enough. Identification and classification of different neurodegenerative disorders might be difficult, especially in the early stages[107]. MCI can be important in the early diagnosis of AD and other kinds of dementia, since the MCI-related clinical changes might increase the risk for AD progression. MCI could represent a transitionary stage, or "grey-zone" between healthy individuals (normal aging) and patients with dementia[23,24].

Development of AD diagnosis could provide more accurate early detection. Further research is needed to enhance the precision of the current diagnostic tools. Since the therapies are more efficient in the very early AD stages, specific markers should be recognized, which predict the disease progression before the clinical symptoms appear[2,142]. Diagnostic development could be enhanced by discovery of additional biomarkers. An increasing set of biomarkers are available nowadays, but most of them are not ready for clinical use yet. However, studying these markers might be a promising goal of future research. Combination of imaging technologies with genetic and proteomic diagnostic methods might provide more accurate prediction of AD progression, and it could also improve the treatment and prevention. The goals of biomarker-development are the reduction of sample size, the process duration, and the improvement of efficiency. Treatment in early stages of AD might postpone the clinical symptoms of dementia, and slow down the disease progression [2,115,142].

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