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Review

Peripheral Biomarkers of Alzheimer's Disease

Tapan K. Khan* and Daniel L. Alkon
Blanchette Rockefeller Neurosciences Institute, Morgantown, WV, USA

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Abstract. Currently available diagnostic tests have moved the field closer to early diagnosis of Alzheimer's disease (AD); however, a definitive diagnosis is made only with the development of clinical dementia and the presence of amyloid plaques and neurofibrillary tangles at autopsy. An ideal antemortem AD biomarker should satisfy the following criteria: the ability to diagnose AD with high sensitivity and specificity as confirmed by the gold standard of autopsy validation; the ability to detect early-stage disease and track the progression of AD; and monitor therapeutic efficacy. AD biomarker technologies currently under development include *in vivo* brain imaging with PET and MRI (i.e., imaging of amyloid plaques, biochemical assays in cerebrospinal fluid (CSF) and peripheral tissues. CSF biomarkers have received increased attention in the past decade. However, it is unclear whether these biomarkers are capable of early diagnosis of AD, prior to Aβ accumulation, or whether they can differentiate between AD and non-AD dementias. In addition, CSF biomarkers may not lend themselves to diagnostic screening of elderly patients, given the invasiveness of lumbar puncture, inter-laboratory variability in techniques and sample handling, and the circadian fluctuation of CSF components. Although commonly viewed as an abnormality of the brain, AD is a systemic disease with associated dysfunction in metabolic, oxidative, inflammatory, and biochemical pathways in peripheral tissues, such as the skin and blood cells. This has led researchers to investigate and develop assays of peripheral AD biomarkers (a few with high sensitivity and specificity) that require minimally invasive skin or blood samples.

Keywords: Alzheimer's disease biomarkers, blood cell-based biomarkers, fibroblast-based biomarkers, lipidomic biomarkers, metabolic biomarkers, peripheral biomarkers, proteomic biomarkers.

Alzheimer's disease (AD) is the most common form of dementia in the elderly, representing approximately 65% of all dementias in this population. AD affects approximately 3% of the total population aged 65–74 years, 10% aged 75–84 years, and 33% aged >85 years. In the United States alone, 5.5 million people suffer from this irreversible neurodegenerative disorder. According to the World Alzheimer's report, approximately 40 million people worldwide are living with dementia, with an estimated cost of $604 billion in 2010. The mean life expectancy after a clinical diagnosis of AD is approximately 7 years, with only 3% of individuals living longer than 14 years after diagnosis. A recent ten-country wide survey of 10,000-adult sponsored by GE Healthcare found three quarters of people would want to know whether they have a particular neurological disorder, even in the absence of a cure (S. Lawrence, Fierce Medical Device August 19, 2014; http://www.fiercemedicaldevices.com). More interestingly, the same survey found 81% of the respondents would want to know whether their loved one has neurological disease. Most people in the survey think that diagnosis should be funded either by government or private health insurance companies. More than half of them responded that they would be willing to pay by themselves, including in most populous countries like China (83%) and India (71%). All of this information reinforce the urgency of early diagnosis of AD.

*Correspondence to: Tapan K. Khan, Blanchette Rockefeller Neurosciences Institute, 8 Medical Center Drive, Morgantown, WV 26505, USA. Tel.: +1 304 293 0934; Fax: +1 304 293 3675; E-mail: tkhan@brni.org.

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The hallmarks of AD include memory loss, deposition of amyloid-β (Aβ) plaques, development of neurofibrillary tangles containing hyperphosphorylated protein tau (p-tau), neuronal degeneration, abnormal loss of neuronal networks, and synaptic loss. Currently, the diagnosis of AD is based on neuropsychological tests and exclusion of other age-related dementias. Disease progression and increasing severity of symptoms can support a diagnosis of AD, but definitive diagnosis is only possible at autopsy, with the presence of characteristic pathologic brain lesions, amyloid plaques and neurofibrillary tangles, in the brain. Although early treatment of AD might slow disease progression, the ability to diagnose AD in its earliest stages is currently limited. This clinical need has fueled the search for AD biomarkers that can not only accurately diagnose early-stage AD, but also differentiate AD from non-AD dementias (frontotemporal dementia, Lewy body dementia, vascular dementia, tauopathy, etc.), assess risk of AD in combination with other known risk factors, facilitate identification and screening of potential therapeutic agents, track prodromal stages of the AD, guide therapeutic decision-making, and monitor therapeutic efficacy.

RISK FACTORS FOR ALZHEIMER'S DISEASE

Three genes have been linked to early-onset AD (familial AD): amyloid-β protein precursor (AβPP) on chromosome 21, presenilin-1 (PS1) on chromosome 14, and presenilin-2 (PS2) on chromosome 1. Individuals with mutations in these genes have higher levels of Aβ than those who do not carry the mutations. By contrast, sporadic AD is a multifactorial, heterogeneous degenerative disease resulting from the combined effects of genetics, age, and other non-genetic risk factors such as diet and lifestyle. The incidence of sporadic AD within a particular family with a history of AD is always higher than that within families with no history of the disease, with the risk of AD approximately 2-fold higher for first-degree relatives of family members with AD. Others have found that the likelihood of developing AD is higher for monozygotic twins than for dizygotic twins if one twin already has AD [1]. Based on these observations, there is clearly a genetic component of sporadic AD.

Yet only a few low-penetrance genes have been linked to sporadic AD, including apolipoprotein E4 (APOE4) [2] and sortilin-related receptor (SORL1) [3]. More than 1,000 articles have been published on candidate AD genetic susceptibility factors. Only the presence of the APOE ε4 allele has an established link to increased risk of sporadic AD; heterozygotes have an approximately 3-fold higher risk of sporadic AD and homozygotes have a 15-fold higher risk. However, the presence of the APOE ε4 allele alone is unable to predict AD, indicating that other factors are involved. SORL1, also known as LR11 (lipoprotein receptor), is a neuronal apolipoprotein E receptor that is expressed at significantly lower levels in the brain tissue of AD patients [3, 4], but other studies do not consistently support that genetic variations in the SORL1 gene increase the risk of AD [5].

Age is the strongest risk factor for sporadic AD. Epidemiological studies have found that <1% of individuals aged 60–65 years have AD, and the prevalence increases exponentially to 24%–33% of individuals aged 85 years. Non-genetic risk factors for sporadic AD include brain injury, vascular disease, hypertension, high cholesterol, atherosclerosis, coronary heart disease, obesity, and diabetes, as well as an inactive lifestyle and poor diet.

CHALLENGES IN DIAGNOSING ALZHEIMER'S DISEASE

In addition to being a heterogeneous, genetically complex neurological disorder, the neurodegenerative processes underlying sporadic AD probably begin several years before the clinical signs and symptoms are recognized, and it is believed that AD progresses to the advanced stage through multiple prodromal stages over a period of approximately two decades [6]. In addition, AD often develops in combination with other neurological disorders of old age, including age-related decline in cognitive function, or mild neurocognitive disorder, making a definitive diagnosis of AD very difficult.

Sustained prodromal stages

AD has a gradual onset with continual deterioration in cognitive ability, progressing through a pre-symptomatic stage to mild cognitive impairment (MCI) to mild AD to severe AD. The clinical manifestation of the disease is preceded by a long prodromal stage, during which neuropathological lesions form. Clinical diagnosis of AD is unreliable, particularly during early stages of the disease. Our own autopsy-validated caveat study showed that in the early stages of AD (disease duration $\leq$4 years from the date of the

first documented symptom of dementia the date of clinical diagnosis) clinical diagnosis was correct in 50% of cases, compared with 83% of cases with >4 years of disease duration [7]. In a similar autopsy-validated study, two clinical diagnoses were made, one early in disease progression, and another much later [8]. The overall clinical accuracy reported for the first clinical diagnosis was approximately 60%, compared with 81% for the later clinical diagnosis.

Overlapping clinical features

Even when the best-trained specialists conduct clinical neuropsychological assessments, they may be unable to diagnose AD when it presents with other dementias, such as Lewy body dementia, vascular dementia, frontotemporal dementia, and tauopathy. In an autopsy-confirmed cohort study, 33% of patients with AD also presented with vascular dementia, Lewy body dementia, and tauopathy (unpublished result of Blanchette Rockefeller Neurosciences Institute). Thus, clinical diagnosis alone may not accurately distinguish AD from non-AD dementias.

Multiple molecular etiologies

AD is a multi-factorial, genetically complex and heterogeneous disease with two distinct categories namely, the early onset familial AD with well defined genetic causes, and the late onset sporadic AD (LOAD). Only a few genes, such as AβPP, PS1, and PS2 have been identified to cause familial AD. However, similar genes have not been identified for LOAD that accounts for >95% of AD cases. A few low penetrance genes and risk factor genes have been identified such as: APOE4 [2], SOLR1 [3], and those on the AlzGene data base (http://www.alzgene.org) for LOAD. Genome-wide association studies using the AlzGene database identified 32 genes as risk factors for sporadic AD, including SORL1, CLU, PICALM, and CR1. As described above, SORL1 encodes an apolipoprotein E receptor. Other non-genetic risk factors have been implicated such as age, head trauma, etc., from AD epidemiological studies. The incidence of inheritance of LOAD is also very high (58–80%, from different studies). Therefore, a critical unsolved problem for AD research is to identify the genetic causes of the non-familial or LOAD form of the disease and the motivation for identifying the multi-factorial genetic contribution is very clear. It is established that LOAD arises as a consequence of a combination of genetic variations, environmental risk factors, and aging (epigenetic).

BIOMARKERS OF AD

The ideal biomarker for AD should have a sensitivity >85% for detecting AD, and a specificity >75% for differentiating other non-AD dementias, according to the National Institute on Aging consensus criteria. Because sporadic AD is often not diagnosed until later stages when cognitive deficits become clinically significant, in the past two decades, researchers have focused on the identification of biological markers that can provide an earlier diagnosis of AD or assess the risk of developing AD. An ideal antemortem AD biomarker should have the following criteria: (i) ability to detect fundamental features of AD neuropathology that can be validated at autopsy; (ii) ability to differentiate AD from non-AD dementias; (iii) ability to detect early stages of AD and differentiate the stages of AD progression to guide therapy; (iv) highly reliable, easy to perform, and inexpensive; and (v) use minimally invasive sample collection, such as from peripheral tissues, without requirement for lumbar puncture or other invasive sampling procedures. In addition to postmortem pathologic changes in brain, there are several biomarkers currently being investigated for the diagnosis of AD, including markers in the CSF, PET and MRI neuroimaging markers, and markers detected in peripheral tissues such as blood and skin (Table 1).

Peripheral biomarkers

Although AD is commonly regarded as a disease of the brain, it is now recognized that AD is a systemic disease that affects peripheral tissues outside the central nervous system, from the earliest stages of the disease. Amyloid pathogenesis and tau metabolic pathways are not limited to the brain, but are ubiquitous in the human body and found in blood, saliva, skin, and other peripheral tissues [9]. For example, primary human skin fibroblasts of symptomatic and presymptomatic patients carrying the Swedish familial AD mutation produce excess Aβ protein [10–13]. AD-specific Aβ deposition has also been noted in the human lens [14], as well as AD-related abnormalities in blood cells [15–17] and Aβ deposition in blood vessels, skin, subcutaneous tissue, and intestine of AD patients [18]. Aβ also forms deposits in the skin of AD patients, which causes measurable abnormalities in fibroblast biology [10]. The implication is that peripheral biomarkers for AD may provide less invasive and inexpensive sample sources for AD diagnostic testing, particularly compared with CSF-based tests. Blood plasma, blood cells, skin fibroblasts, and peripheral

Table 1
Biomarkers of Alzheimer's disease

Central nervous system biomarkers	
Brain tissue	Neurofibrillary tangles
(at autopsy)	Amyloid plaques
	Brain atrophy/decreased brain volume
Cerebrospinal Fluid	$A\beta_{1-42}$
	Total tau
	p-tau-181
Neuronal imaging	MRI
	fMRI
	^{11}C-PiB PET
	^{18}FDG PET
	^{99m}Tc-HMPAO SPECT
Peripheral biomarkers	
Plasma	*Aβ peptides*: $A\beta_{1-40}$, $A\beta_{1-42}$
	Tau proteins: tau, p-tau-181
	Inflammatory proteins: CRP, antichymotrypsin, macroglobulin, interleukins, TNF-α, complement factors, homocysteine
	Others: Clusterin, APOE, SAP
	Metabolism: lipidomics; proteomics
Blood cells	*Signaling molecules*: Aβ, AβPP, β-secretase, α-secretase, GSK-3, PKC
Skin fibroblasts	*Signaling molecules*: GSK-3, PKC and Erk1/2
	Enzymes: GFAP, S-100b, glutamine synthetase
	Metabolism/oxidative damage: 8-hydroxyguanoside, 4-hydroxynonenal, SOD, isoprostanes, nitrotyrosine, NO-metabolites, prostaglandins, 24S-hydroxycholesterol, heme-oxygenase 1, kallikrein-like bradykinin, cholesterol sulfate

Aβ, amyloid-β; p-tau-181, phosphorylated tau at threonine 181; MRI, magnetic resonance imaging; fMRI, functional MRI; PET, positron emission tomography; ^{11}C-PiB, [^{11}C]-Pittsburgh Compound;^{18}FDG, [^{18}F]-fluoro-2-deoxy-D-glucose; SPECT, single-photon emission computed tomography; ^{99m}Tc-HMPAO, hexamethylpropylene amine oxime; CRP, C-reactive protein; TNF-α, tumor necrosis factor α; AβPP, amyloid-β protein precursor; GSK-3, glycogen synthase kinase-3; PKC, protein kinase C; Erk1/2, extracellular signal-related kinases 1 and 2; GFAP, glial fibrillary acidic protein; SOD, superoxide dismutase; NO, nitric oxide.

blood vessels hold considerable promise as peripheral tissue sample sources for AD biomarker assays (Table 2). Gasparini et al. [9] provided a rationale for the use of peripheral biomarkers for testing pathophysiological hypotheses and diagnosis, and several biomarkers in the blood and skin have shown considerable promise for diagnosing AD [7, 9, 19–21].

Metabolomics to identify AD biomarkers

Metabolomics is defined as global metabolic profiling using a combination of proteomic, lipidomic, and/or genomic/transcriptomic approaches. Identification of new biomarkers of AD using metabolomics has received enormous attention in recent years. Because metabolomics detect end point perturbations in the proteome, genome, and lipid profile caused by disorders, they are much more relevant to the development of drug efficacy tests and pharmacodynamic analyses compared to other approaches. Two metabolomic approaches are commonly used for developing new AD biomarkers: lipidomics and proteomics. Blood-based AD metabolic biomarkers are more attractive for use in diagnostic tests because sample collection is easy, and the tests are relatively non-invasive and less time-consuming; however, metabolic biomarker-based tests have limited sensitivity and specificity.

Lipidomic AD biomarkers

Lipidomics is the analysis of lipid and lipid derivatives in biological fluids, such as blood plasma and serum. There are several convincing reasons to take a lipidomic approach to identify AD biomarkers. First, AD results from abnormality in the brain, which is the most lipid-rich organ in the human body. Second, the lipid transporter protein APOE4 is a known risk factor of late-onset AD. Third, in the liver of AD patients, the expression level of peroxisomal D-bifunctional protein, which catalyzes the conversion of tetracosahexaenoic acid into DHA, is selectively reduced [22]. In addition, peroxisomal dysfunction in AD contributes to glycerophospholipid deficits [23]. Results from studies of animal models of AD have also provided fundamental information on lipid dysregulation during various stages of AD. For example, the levels of docosahexaenoyl (22 : 6), cholesterol ester, ethanolamine plasmalogens, and sphingomyelins were markedly increased in AβPP/tau

Table 2
Peripheral tissue biomarkers

Tissue	Biomarker	Comments	References
Skin fibroblasts	Electrophysiological *K*+ channel dysfunction	Need to be validated	[59]
Skin fibroblasts	Dysfunctional MAPK Signaling (increased Erk1/2 phosphorylation in response to bradykinin)	Need to be validated	[67]
Skin fibroblasts	Differential stimulus-elicited phosphorylation of Erk1/2 (relatively higher Erk1 compared with Erk2	High specificity and sensitivity	[7], [60]
Skin fibroblasts	Fibroblasts network morphology assay	High specificity and sensitivity. Need to be validated by other laboratory.	[97]
Skin fibroblasts	Reduced levels of PKCε	High specificity and sensitivity. Need to be validated by other laboratory.	[21]
Skin fibroblasts	Unfolded P53 expression at the basal level	Moderate specificity and sensitivity	[70], [98]
Eye lenses	Cytosolic Aβ deposition	Moderate specificity and sensitivity	[14]
White blood cells	Increase in GSK-3. Abnormality in protein conformation.	No difference between AD and MCI	[54]
Red blood cells	Conformation changes in PKC	Need to be validated	[53]
Plasma and serum	Decreased $A\beta_{1-40}$ and $A\beta_{1-42}$ by immunoassay	No difference between AD and controls	[20]
Plasma	Lipidomics and proteomics. Set of blood plasma protein measures by multiplex platform	Need to be validated	[25]
Plasma inflammatory molecules	Proteomics antibody array	Not promising after validation by other laboratory	[41]
Blood plasma components	Flow cytometry-based immunoassay	Need to be validated	[44]

AD, Alzheimer's disease; Aβ, amyloid-β; GSK-3, glycogen synthase kinase 3; MAPK, mitogen-activated protein kinase; MCI, mild cognitive impairment.

Table 3
Lipids identified as biomarkers in blood plasma of AD patients

Lipid identified	Method of detection	Comments	References
Phophatidyl inositol ↓; Dioleoylphosphatidic acid↑; Phosphatidyl choline C38 : 4↓	LC-MS	Cell membrane integrity may be sensitive for detecting preclinical AD.	[25]
Desmosterol/cholesterol ratio↓	LC-MS and GC-MS. The difference between AD and control cases was statistically significant.	There were several overlaps. Sensitivity in males was much lower than for females.	[26]
Ratios of specific ceramide/sphingomyelin with the same fatty acid chain ↑	Shotgun lipidomics MS	Low specificity and sensitivity. Genotype-specific differences within AD group.	[27]
Lipid peroxidation indicator: isoprostane 8, 12-iso-iP$F_{2\alpha}$-VI ↑	GC-MS	Significant increase in both AD and MCI groups compared with controls ($p < 0.001$)	[28]

AD, Alzheimer's disease; GC, gas chromatography; LC, Liquid chromatography; MS, mass spectroscopy; MCI, mild cognitive impairment.

mice compared to controls [24]. Extensively studied lipidomic biomarkers of AD include abnormal glycerophospholipids (due to abnormality in integrity of cell membranes) [25], lower desmosterol [26], higher ceramide/sphingomyelin ratios [27], and abnormal lipid peroxidation [28] (Table 3). Lipidomics will continue to identify relevant biomarkers of AD for early-stage disease detection, risk assessment, and monitoring of drug efficacy.

Proteomic AD biomarkers

Assays that detect blood-based biomarkers are easily applicable to the general care setting, as they only require a routine blood draw, and can be used to monitor disease progression or treatment efficacy with multiple blood draws over time. Plasma $A\beta_{1-42}$ has been proposed as potential diagnostic biomarker for AD, with changes in $A\beta_{1-42}$ as a marker of disease progression, for some time. Unfortunately, the majority

of cross-sectional studies of plasma $A\beta_{1-42}$ concentrations in humans have not revealed any differences between individuals with or without AD. This is also the case in animals; mouse studies have shown inconsistent trends in $A\beta_{1-42}$ levels across AD models and controls. However, longitudinal studies in humans of changes in plasma $A\beta_{1-42}$ levels over time have produced some promising results [29–31], making this assay similar to those used to detect prostate-specific antigen for prostate cancer. Nevertheless, while a decrease in CSF $A\beta_{1-42}$ levels correlates well with AD and disease progression [32–33], changes in human plasma $A\beta_{1-42}$ remain inconsistent [20], particularly for sporadic AD. Another issue is that some studies show an increase or same in plasma $A\beta_{1-42}$ level in normal aging humans without dementia [34–37].

Inflammation occurs in the brain of AD patients at both preclinical and clinical stages of the disease, possibly even before Aβ and tau changes [38–40]. There is some evidence that activation of microglia produces cytokines, chemokines, and inflammatory growth factors in the brain and blood plasma. One study reported that a set of 18 inflammatory biomarkers can distinguish patients with AD from those with MCI with an accuracy of 90% [41]. However, attempts to reproduce these findings by other laboratories found a diagnostic accuracy of only 60%–70% [42, 43]. Other important serum inflammatory markers being investigated as potential AD biomarkers include C-reactive protein, antichymotrypsin, macroglobulin, interleukins, and homocystine. Using multiplex technology, a recent study found 10 plasma proteins that are strongly associated with disease severity and disease progression [44]. To improve the accuracy of the study, some unusual stringent conditions were applied for data analysis. The most important blood-based AD biomarkers identified by proteomics are summarized in Table 4. There are few proteins that are consistently up- or down regulated in all studies [41, 44–47].

There are several reasons for failure of blood serum based AD biomarkers. Firstly, the integrity of blood-brain barrier (BBB) in AD is not extensively studied. The degree of crossing analytes (proteins/peptides) is limited with the degree of loss of BBB integrity. AD is a slow heterogeneous progressive disease and that may affect the BBB integrity differently. Secondly, brain proteins/peptides crossed by the BBB may be degraded or metabolized in blood. Thirdly, the levels of fluctuation of proteins/peptides concentration depend on physical state of the patients (sleep cycle, food intake, etc). Fourthly, and most importantly, interference of other old age conditions such as blood pressure, blood glucose levels, concentration of inflammatory molecules, etc, may hamper the diagnosis.

Cell-Based AD Biomarkers

AD is an irreversible progressive dementia with long prodromal stages. New diagnostic criteria for AD proposed by various consensus groups describe the appearance of AD dementia occurring in several stages: pre-dementia, MCI due to AD, pre-symptomatic AD (asymptomatic AD), and clinical dementia due to AD [48–50]. Molecular signaling alterations may occur in early stages, long before synaptic loss and neuronal degeneration, with clinical symptoms appearing much later. There are several advantages to studying alterations in cellular systems as potential biomarkers for AD. First, alterations in AD-specific molecular signaling signatures may better distinguish between non-AD dementia and AD. Second, very early detection (detection much earlier than the appearance of clinical symptoms) of defective signal transduction mechanisms may open up new avenues for effective drug discovery. Alterations in two AD-specific cellular systems have been intensely studied as potential biomarkers of AD: blood cells and cultured skin fibroblasts.

Blood cell-based AD biomarkers

Abnormalities due to AD pathology have been described in platelets, red blood cells, and white blood cells. Protein kinase C (PKC) has a well-established function in memory and synapse formation, and PKC signaling pathways are disrupted in patients with AD and in animal models of AD [51]. Decreased PKC levels, activity, and cellular localization of PKC have been noted in the brains of AD patients [52]. PKC conformations in red blood cells, measured by a specialized fluorescence spectrum, are different in samples from patients with or without AD [53]. In early-stage AD, glycogen synthase kinase-3 (GSK-3) has been found to be high in white blood cells [54]. Several important AD-related Aβ-processing abnormalities have been described in platelets derived from AD patients compared to normal age-matched control (AC) cases. These abnormalities included increased β-secretase and decreased α-secretase activities [55], increased Aβ levels [55], and low AβPP isoform ratios (120–130 kDa to 110 kDa) in AD compared to controls [55, 56]. Results of blood cell-based AD biomarker studies are summarized in Table 5.

Table 4
Proteomics-based peripheral biomarkers of AD patients

Protein identified	Method of detection	Comments	References
All ↓ in AD: TNF-α; PDGF-BB; M-CSF; G-CSF; CCL5; CCL7; CCL15; EGF; GDNF; IL-1α; IL-3 All ↑ in AD: Ang-2; ICAM-1; CCL18; CXCL8; IGFBP-6; IL-11; Trail-R4	Proteomics antibody array. Patient groups: Non-AD dementia ($n=11$); AD ($n=86$); MCI ($n=47$); Control ($n=21$); Rheumatoid arthritis ($n=16$)	Immune-responsive analytes and cytokines. High accuracy for detecting AD.	[41]
All ↓ in AD: APOC3; TTR; ICAM-1; RANTES; Cystatin. All ↑ in AD: PEDF; CC4; A1AcidG; Clusterin	Flow cytometry-based immunoassay. Patient groups: AD ($n=476$); Control ($n=452$); MCI ($n=220$)	One of the largest multi-center validation studies. Predicted conversion of MCI to AD with an accuracy of 87%.	[44]
All ↓ in AD: IL-17; EGFPR All ↑ in AD: Insulin-like growth factor binding protein 2; pancreatic polypeptide; Ang-2; Cortisol; Beta-2-microglobulin	Multiplex immunoassay. Patient groups: AD ($n=207$); Control ($n=754$)	Fold change between AD and control groups was not high.	[47]
Clusterin ↑ in AD	LC-MS Total subjects ($n=744$)	Has a role in atrophy in AD pathogenesis. Significantly ($p<0.001$) associated with the rate of progression of AD.	[45]
All ↓ in AD: Creatine MB; G-CSF; S-100B; IL-10; IL-1ra; Prostatic acid phosphatase; C-reactive protein; TNF-α; Stem cell factor; MIP1α. All ↑ in AD: Thromboprotein; Alpha-2-macroglobulin; Tenascin; TNF-β; Beta-2-microglobulin; Eotaxin; Pancreatic polypeptide; von Willebrand factor; IL-15; VCAM-1; IL-8; IGFBP2; Fas ligand; Prolactin Resistin.	Multiplex Immunoassay Patient groups: AD ($n=197$); Control ($n=203$)	Specific algorithm in data analysis provided high specificity and sensitivity.	[46]
Aβ ↓ in AD	$A\beta_{1-40}$ and $A\beta_{1-42}$ by immunoassay	No significant difference between AD and controls	[20]

AD, Alzheimer's disease; Aβ, amyloid-β; G-CSF, Ang-2, angiopoietin-2; APOC3, apolipoprotein C3; CCL, chemokine containing a C-C motif; CXCL, chemokine containing a C-X-C motif; EGF, epidermal growth factor; G-CSF, granulocyte-colony stimulating factor; GDNF, glial-derived neurotrophic factor; ICAM-1, intercellular adhesion molecule-1; IGFBP2, insulin-like growth factor binding protein 2; IL, interleukin; IL-1ra, Interleukin 1 receptor antagonist; MCI, mild cognitive impairment; M-CSF, macrophage-colony stimulating factor; MIP1α, macrophage inflammatory protein 1-α; PDGF-BB, platelet-derived growth factor BB; PEDF, pigment epithelium-derived factor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF-α, tumor necrosis factor-α; TNF-β, tumor necrosis factor-β; TRAIL-R4, TNF-related apoptosis-inducing ligand receptor-4; TTR, transthyretin type receptor; VCAM-1, vascular cell adhesion molecule 1.

Skin fibroblast-based AD biomarkers

During development, the ectoderm differentiates into skin, the sense organs, and components of the early nervous system. A great deal of evidence supports the notion of a "brain-skin axis" in which biochemical changes in the brain are mirrored in ectoderm-derived peripheral tissues such as the skin [57, 58]. Consistent with the amyloid hypothesis of AD pathogenesis, it has been shown that Aβ secretion is elevated in the skin fibroblasts of patients with familial AD compared with unaffected patients [11, 12], and that Aβ treatment of cultured normal skin fibroblasts stimulates an AD phenotype [59, 60]. Several recent publications have described that the basic pathogenic mechanism of amyloidogenesis is similar in brain and skin fibroblasts [82].

Table 5
Peripheral blood cell-based biomarkers of AD patients

Type of blood cells	Molecular abnormality in AD	Comments	References
White blood cells	↑ GSK-3	Patient groups: AD (60); MCI ($n=33$); Control (20) Overlap with control, MCI, and AD MCI	[54]
Red blood cells	Alteration of PKC conformation	Patient groups: AD ($n=33$); Control ($n=25$) Distinguished between AD and PD	[53]
Platelets	β-secretase activity ↑; α-secretase activities ↓; Aβ ↑; AβPP isoform ratios (120-130 kDa to 110 kDa) ↓.	Patient groups: AD ($n=31$); Control ($n=10$) Some overlap between AD and controls	[55]

AD, Alzheimer's disease; Aβ, amyloid-β; AβPP, amyloid-β protein precursor; GSK-3, glycogen synthase kinase 3; MCI, mild cognitive impairment.

Table 6
Cellular signaling pathway abnormalities in skin fibroblasts from patients with AD

Affected pathway	Molecular abnormality in AD	References
AD-linked gene expression	AβPP, PS1, PS2 in familial AD	[6]
PKC isozyme activity	Defective PKC isozymes in familial and sporadic AD	[19], [64], [65], [66]
Folate binding	Enhanced folate binding	[74]
MAPK signaling	Defective tau protein serine phosphorylation	[68]
Erk1/2 signaling	Dysfunctional stimulus-activated signaling cascade	[67], [69]
Extracellular matrix	Differences in ECM production and bFGF response in sporadic and familial AD	[73]
p53 activity	Altered conformation of p53 Decreased sensitivity to p53-dependent apoptosis	[70]
Cholesterol processing	Altered cholesterol ester cycle	[71]

AD, Alzheimer's disease; AβPP, amyloid-β protein precursor; bFGF, basic fibroblast growth factor; ECM, extracellular matrix; MAP, mitogen-activated protein kinase; PKC, protein kinase C; PS1, presenillin-1; PS2, presenillin-2.

Other abnormalities have been noted in the skin fibroblasts of patients with AD (Table 6). These include deficiencies in DNA repair and abnormalities in Ca^{2+} homeostasis [61–63], defects in PKC isozymes in patients with familial or sporadic AD [19, 64–66], altered gene expression in patients with familial AD [6], MAP kinase signaling pathway abnormalities [67–69], conformational modifications of the p53 protein [70], altered cholesterol processing [71], differences in extracellular matrix ECM components [72, 73], and abnormal folate binding [74]. Based on these observed abnormalities, several groups are trying to identify and validate peripheral diagnostic biomarkers of AD using skin fibroblast samples (Table 6). Fibroblast-based biomarkers of AD under investigation include K+ channels [59, 75], PKC isozymes [19, 21], Ca^{2+} signaling components [76], MAP kinase Erk1/2 phosphorylation [67], bradykinin-induced phosphorylation of Erk1 and Erk2 [7, 60], mitochondrial function, anti-oxidative pathway components, and bradykinin activity. In the medical literature, there are several examples of the use of skin fibroblasts to assay metabolic abnormalities linked to neurological disease, such as Refsum disease [77] and Lesch-Nyhan syndrome [78]. Skin biopsies have been used to diagnose neurometabolic and neurodegenerative diseases [79, 80]. Fibroblasts based diagnostic laboratory tests are common for several in-born metabolic and neurodegenerative diseases with specific genetic causes (Table 7).

The advantages of skin fibroblast-based diagnostic assays include simple, inexpensive sample collection that can be performed in the primary care setting, and multiple samples can be taken over time to track disease or treatment efficacy. Technically, it is easy to culture fibroblasts from skin biopsies without contamination of other cell types; cell-cell contact pathologies can be assessed in adhering fibroblasts but not in non-adhering blood cells or saliva; the cultured fibroblast population is homogeneous and can generate a greater signal to noise ratio than mixed tissue samples; and the proliferative nature of primary fibroblasts allows repeat experiments with cells from a low number passages. The superiority of skin fibroblasts over peripheral blood lymphocytes for AD bioassays was discussed [6]. The analysis of RNA quality from lymphocytes and fibroblasts from same patients suggests that blood samples are more susceptible to external

Table 7
Fibroblasts based diagnostic laboratory tests

Disease name	Disease characteristics	Biochemical/analytic method	References
Fucosidosis	In-born metabolic disease	Deficiencies in α-L-Fucosidase	[99]
			[a]Mayo Medical Laboratories, Mayo Clinic, Test Unit Code 8815.
Abnormality in Fatty acid metabolism	Fatty Acid Metabolism (mitochondrial beta-oxidation) and Carnitine Homeostasis	Fatty acid oxidation probe assay	[100]
			[a]Mayo Medical Laboratories, Mayo Clinic, Test Number 81927.
Pompe disease	Reduced α-Glucosidase enzyme activity	Skin fibroblasts or muscle biopsy is the diagnostic gold standard	[101]
			[a]Mayo Medical Laboratories, Mayo Clinic, Test Unit Code 89897
Niemann-Pick C disease	A special kind of chronic neurodegenerative disease with a typical lysosomal lipid storage disorder	Diagnosis requires living skin fibroblasts to demonstrate accumulation of un-esterified cholesterol by staining with filipin.	[102][b], [103]
Tuberculosis	Mycobacterium tuberculosis	Pyrazinamide susceptibility in lung fibroblasts	[104]

[a]Mayo Medical Laboratory. [b]The NP-C Guidelines Working Group.

conditions such as acute stimuli, nutritional status, fever, infections, and drug treatment.

Minimally invasive punch biopsied skin fibroblast-based assay do have some limitation. The lag time between biopsy and test results; it takes several weeks to complete due to the slow growth of skin fibroblasts in culture. The opportunities afforded by a simple diagnostic skin test for AD continue to drive innovations to overcome these technical challenges, however, and the development of skin fibroblast-based assays for AD remains an active area of research.

Erk1/2 signaling cascade in skin fibroblasts

Based on a study that described bradykinin-induced abnormalities of Erk1 and Erk2 phosphorylation in cultured skin fibroblasts from AD patients in comparison to control cases [67], we pursued Erk1/2 as a potential diagnostic biomarker for AD. In work at our Institute, we found that the extracellular signal-regulated kinases (Erk1 and Erk2) are phosphorylated differentially in cultured skin fibroblasts from the Coriell Cell repository from patients with or without AD in response to the inflammatory agonist bradykinin in combination with serum growth factors [7, 69]. By conducting an internally controlled comparison of stimulus-elicited changes in Erk1 and Erk2 phosphorylation, we were able to produce an autopsy-validated AD Index that accurately distinguished fibroblasts of AD from fibroblasts of normal controls and from non-AD dementias [7, 69]. The accuracy of Erk1 and Erk2 AD Index values was inversely correlated with disease duration, suggesting maximal efficacy of the AD Index bioassay in early diagnosis. The Erk1/2 biomarker accurately distinguished AD from non-AD dementia within the first 4 years of disease symptoms. Finally, we also demonstrated that when the AD Index agrees with a clinical diagnosis of AD, there is a high probability of accuracy based on autopsy validation. For autopsy-confirmed AD cases, the performance of the Erk1/2 AD-index was remarkably high (96% accuracy for the Erk1/2 biomarker, and 88% accuracy for clinical diagnosis). In the absence of autopsy validation (i.e., clinical diagnosis only), the accuracy of the Erk1/2 biomarker for diagnosis of AD was 82%. The accuracy of clinical diagnosis (67%) was quite low compared with the Erk1/2 biomarker (100%) for patients who had mixed AD dementias. The specificity of the Erk1/2 biomarker for AD was also quite high, ruling out AD for a subgroup of healthy controls (no cancer, heart disease, arthritis, stroke, or family history of AD and a Mini-Mental State Examination score of >27) [7].

Thus, as an AD biomarker, skin fibroblast Erk1/2 phosphorylation could have important clinical utility for increasing diagnostic certainty, particularly in the early phase of the AD progression. We have also used this biomarker to evaluate the effects of PKC activators bryostatin and its synthetic analog, picolog, on cultured fibroblasts treated with Aβ [60]. The pathophysiologic relevance of this peripheral biomarker was tested by

examining $A\beta_{1-42}$–induced changes in Erk1/2 signaling [7].

Ca^{2+} imaging in Alzheimer's Disease skin fibroblasts

Disturbed Ca^{2+} homeostasis in the brain is a hallmark of AD. Aβ stimulates the sustained activation of Ca^{2+}-permeable receptor channels, resulting the elevated Ca^{2+} in cytoplasm. Damage by oxidative trace and Aβ triggering the internal Ca^{2+} reaches to the high level and that might exhausts the buffering capacity of total internal Ca^{2+} pool, and then it starts the Ca^{2+}-mediated Ca^{2+} release particularly from mitochondria and endoplasmic reticulum. In familial AD, PS1 and PS2 mutations can promote the formation of passive Ca^{2+} leak channels in the endoplasmic reticulum, which increases Ca^{2+} levels and further implicates defective Ca^{2+} signaling in the pathogenesis of AD [81]. A newly discovered gene, CALHM1, related to cytosolic Ca^{2+} concentration and Aβ level has also been reported and found to be defective in AD patients [82].

Altered Ca^{2+} homeostasis in AD brains is also manifested in peripheral tissues. Peterson et al. [83] published the first report of decreased Ca^{2+} uptake by human skin fibroblasts from AD patients compared with AC cases. The same group also found that though the Ca^{2+} uptake by fibroblasts decreased with aging, and uptake was decreased further in AD fibroblasts, total cell Ca^{2+} was increased in fibroblasts from aged and AD patients compared with young control cases [62, 63]. Their findings suggested that the level of free Ca^{2+} may also be abnormal, as well as the concentration of cytosolic free Ca^{2+}. Cytosolic free Ca^{2+} in skin fibroblasts from AD patients and ACs could be elevated by various drug treatments, such as 3, 4-diaminopyridine, serum, N-formyl-methionyl-leucyl-phenylalanine, and bradykinin. Treatment increased cytosolic free Ca^{2+} transiently, with the rate of the increase slower and the magnitude of the rise less pronounced in cells from AC and AD patients when compared to young controls [84]. Altered Ca^{2+} homeostasis might also contribute to mitochondrial oxidative processes, such as glucose and glutamine oxidation, which were found to be depressed in cells from normal aged individuals and even lower in AD patients. In support of this notion, a separate study found that mitochondria of cultured skin fibroblasts from skin samples taken at autopsy from patients with histopathologically confirmed that AD showed a decreased uptake of Ca^{2+} and increased sensitivity to free radicals [85]. Inspired by the above studies [86–87], investigated the possibility of developing a peripheral diagnostic biomarker for AD based on abnormal Ca^{2+} processing; however, they found that cytoplasmic ionic Ca^{2+} levels were neither pathologically relevant in AD nor of diagnostic value.

After this setback, researchers began looking at alternate approaches to Ca^{2+} as a diagnostic AD biomarker. One group used specific stimulation of Ca^{2+} abnormalities in fibroblasts from AD patients to clarify differential responses from normal cells. The *K*+channel blocker TEA increases intracellular Ca^{2+} in normal skin fibroblasts; the response to TEA is low in cells from sporadic AD patients as well as in cells from a few familial AD cases [59]. Bradykinin at low doses is well known to induce intracellular Ca^{2+} release through IP3 generation [88]. It also activates phospholipase C and elicits enhanced Ca^{2+} signaling in AD fibroblasts [76]. Based on these findings, standard Ca^{2+} fluorescence imaging techniques were used to measure the Ca^{2+} response in skin fibroblasts after stimulation with TEA or bradykinin. The biochemical response was reported as the ratio of percent response after TEA stimulation and the percent response after bradykinin stimulation [87, 88]. A "proof of concept" study of the Ca^{2+} biomarker assay was conducted to validate the ratio measurement, and a cut-off value of 1.8 (% response to TEA challenge/% response to BK challenge) was established [76, 88] (Neurologic Inc. unpublished data). Values $\geq$1.8 would be considered negative for AD and values <1.8 would be indicative of positive test for AD. The overall agreement of the Ca^{2+} ratio with clinical diagnosis of AD was 62% between in AD, non-AD dementia, and AC cases. Despite the low sensitivity, there was a low false-positive rate (Neurologic Inc. unpublished data).

In a separate study, the absence of a TEA-induced increase in Ca^{2+} in fibroblasts was unable to distinguish control cases from AD [89], although the study was conducted with a small number of samples and with different methodology than other studies. Several factors may contribute to the observed variability between studies of Ca^{2+}-based bioassays for AD, including (i) cell cycle dependence for the AD Ca^{2+} response, (ii) skin fibroblast morphology and, (iii) cellular motility and cytoskeleton dynamics. A cell-cycle-dependent abnormally depressed Ca^{2+} response of skin fibroblasts from two independent AD families was observed after stimulation by 100 nM bradykinin, 100 nM vasopressin, or 10% fetal calf serum (FCS) in Ca^{2+}-free condition compared with control fibroblasts at 48 hours after plating [90]. The study also found that on the 7th day after plating, the abnormal calcium

response was no longer evident. When fibroblasts were arrested in S phase, they showed a significantly lower calcium peak after bradykinin stimulation. Ca^{2+} response also changes due to different cellular morphologies of fibroblasts. TEA-induced intracellular Ca^{2+} responses were not observed in AD skin fibroblasts compared with control cases across studies [89, 91]; this is likely due to differences in plating techniques and cell morphology, with cellular monolayers used by Matsuyama et al. [91], and single-cell imaging used by Failli et al. [89] and Etcheberrigaray et al. [92].

Deficit of PKCε in AD

In AD transgenic mice, activation of PKCε was found to prevent synaptotoxic Aβ-oligomer elevation, PKCε deficits, early synaptic loss, cognitive deficits, and amyloid plaque formation [93]. Activation of PKCε facilitated degradation of Aβ via the endothelin converting enzyme [94], activation of α-secretase to generate the synaptogenic non-toxic sAβPPα [95], and reduction of GSK3-β activity [96], thereby decreasing hyperphosphorylation of tau. Deficits of PKCε in AD human brains implicated to contribute to early AD pathology, including loss of synapses. Beside neurons skin fibroblasts also express PKCε. Skin fibroblast samples from AD patients also demonstrated a deficit in PKCε compared to controls and an AD-specific change in the toxic Aβ-oligomer (amylospheroids, ASPD) effects on PKCε [21]. This assay assessed the concentration of PKCε in skin fibroblasts before and after treatment with 500 nM toxic ASPDs derived from soluble oligomeric $Aβ_{42}$ using enzyme-linked immunosorbent assay (ELISA). The quantitative output of this assay was the rate of change of PKCε levels in skin fibroblast of AD and AC cases with increasing concentration of externally added toxic ASPDs.

Fibroblasts network morphology assay

The formation of skin fibroblasts network was altered in AD cases [97]. This assay quantified the difference of dynamics of AD, AC, and non-AD dementia fibroblasts' network formation in three dimensional culture systems. Cultured skin fibroblasts were platted on a 1.8 mm layer of three dimensional matrix, and images of aggregation were captured with an inverted microscope every other hour for up to 48 hours. The outcome was quantified as the aggregate area size divided by the number of aggregates (area/N_a). The population of aggregates was expected to be lower in AD cases than AC fibroblasts. In actual assay, AD fibroblasts showed large, isolated aggregates. Aggregates were smaller and more numerous for AC and non-AD dementia fibroblasts after 48 hours of plating. The area/N_a output clearly distinguished among AD, AC, and non-AD dementia fibroblasts. The accuracy, sensitivity, and specificity were very high.

Other fibroblast-based biomarkers for Alzheimer's Disease

Transcription and conformation of p53, an apoptosis mediator protein, is altered by Aβ peptide in cells from patients with early stage AD, supporting the use of p53 as a peripheral biomarker of AD [70, 98]. Though p53 may prove to have similar sensitivity and specificity of CSF biomarkers for diagnosing early-stage AD, the main drawback of this approach is the effect of normal aging on p53. Conformation changes in p53 may be related to aging, though these changes are not consistently observed [99].

Multi-factorial approaches to fibroblast-based biomarker discovery

It is clear that AD is not linked to mutation of single gene or dysfunction of a single protein. Sporadic AD is a genetically heterogeneous disease, resulting from many interrelated mutations occurring in multiple genes that combine with the effects of age and other non-genetic risk factors. Though several genes have been linked to familial AD, such as AβPP, PS1, and PS2; by contrast, only APOE4 and to some extent SORL1 genes have been associated with sporadic AD.

CONCLUSIONS

Amyloid pathogenesis and tau metabolic pathways are not limited to the brain, but are ubiquitous in the human body and found in blood, skin, saliva, and other peripheral tissues such as eye lenses. For example, primary human skin fibroblasts of symptomatic and presymptomatic patients carrying the Swedish familial AD mutation produce excess Aβ protein. Factors that have systemic impact, such as genetics, hypoxia, ischemia, and metabolic dysfunction could, therefore, be critically important in the etiology of AD. These studies provided further support for the systemic expression of AD pathophysiology while symptomatic expression is restricted to the brain.

Given its multifactorial nature, the diagnosis of sporadic AD is challenging, and early diagnosis is impossible. There is a clear need for the development of a simple, inexpensive, minimally invasive test for AD to diagnose the disease, ideally at the earliest stages, to predict the likelihood of developing AD,

and to monitor disease progression and therapeutic efficacy. Discovery and development of candidate AD biomarkers may also lead to the identification of new therapeutic targets and approaches. Use of neuroimaging and CSF biomarkers hold promise. However, in contrast to a few of the peripheral biomarkers mentioned above (e.g. the AD Index, PKC epsilon deficits and morphology assays in skin fibroblasts) that have shown high sensitivity and specificity, the CSF and neuroimaging biomarkers continue to face challenges related to invasiveness of sample collection, cost, interlaboratory variation and ability to distinguish AD from non-AD dementias. Combinations of AD biomarkers into a molecular signature or index may prove to be more accurate than any single biomarker. The future focus for AD biomarker research will include (i) improvements in diagnostic specificity; (ii) improved ability to differentiate AD from non-AD dementias and MCI; (iii) improved identification of different AD phenotypes; (iv) the capacity to monitor prodromal stages of AD; and (v) development of tests for early-stage disease.

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