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Review Article

Biotechnology Section

Exploring Biomarkers for Alzheimer's Disease

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ABSTRACT

Alzheimer's Disease (AD) is one of the most common form of dementia occurring in elderly population worldwide. Currently Aβ42, tau and p-tau in the cerebrospinal fluid is estimated for confirmation of AD. CSF which is being used as the potent source for biomarker screening is obtained by invasive lumbar punctures. Thus, there is an urgent need of minimal invasive methods for identification of diagnostic markers for early detection of AD. Blood serum and plasma serves as an appropriate source, due to minimal discomfort to the patients, promoting frequent testing, better follow-up and better consent to clinical trials. Hence, the need of the hour demands discovery of diagnostic and prognostic patient specific signature biomarkers by using emerging technologies of mass spectrometry, microarrays and peptidomics. In this review we summarize the present scenario of AD biomarkers such as circulatory biomarkers, blood based amyloid markers, inflammatory markers and oxidative stress markers being investigated and also some of the potent biomarkers which might be able to predict early onset of Alzheimer's and delay cognitive impairment.

Keywords: Amyloid precursor protein, miRNA, Fluid biomarkers

INTRODUCTION

Alzheimer's Disease (AD) is one of the most prevalent dementia seen in elderly worldwide. According to the current reports, it is estimated that almost one new case of AD develops every 33 seconds, and almost a million new cases every year, with estimated prevalence of almost 13.8 million around the globe [1]. The main symptoms include memory loss, cognitive impairment, disorientation and psychiatric symptoms [2,3]. The preliminary diagnosis of AD is made by a combination of clinical criteria which includes a neurological examination, mental status tests and brain imaging [4]. However, based on the above clinical tests, the task of AD becomes difficult especially in patients having mild or early stages of AD. Hence, the need for biomarkers evolved which show strong indications of Alzheimer's disease and also provides conclusive diagnosis of early onset of AD. This also contributes to developing disease modifying therapies at early stage thus preserving normal brain function or delaying cognitive impairment.

Currently, presence of dementia is confirmed by analysing the Cerebrospinal Fluid (CSF) with established biomarkers like amyloid beta protein, tau protein and phospho-tau expression levels. CSF is known to act as a valuable source of biomarkers, since besides being in direct contact with the brain and spinal cord it provides a complete representation of various biochemical and metabolic profiles of the brain. However, this fluid is obtained by lumbar punctures in patients which are both invasive as well as painful for the patients, which makes the diagnosis difficult and also irreproducible. Hence, the need of the hour demands of new biological biomarkers which being less intrusive and easily obtainable, can also be more sensitive and specific [5]. This will further help in the diagnosis in the prodormal stage of dementia and would also lead to identification of conditions of patients with mild cognitive impairment so that the onset of dementia could be delayed [6].

1.1 Alzheimer's Disease in Younger Patients

AD was initially referred to as "presenile dementia" with its first patient being 51 years of age at the time of presentation. However, after studies conducted by Blessed and colleagues (1967) it was seen that the brains of patients suffering with senile dementia had quantitatively similar senile plaques and neurofibrillary tangles as seen in presenile AD patients [7].

Autosomal dominant familial AD is seen to be more commonly affecting younger individuals as compared to sporadic AD. Studies have shown that ApoE4 genotype can lead to a more aggressive clinical form of AD in younger patients. These patients tend to suffer from Logopenic Progressive Aphasia (LPA) often characterised by prolonged word finding pauses, anomia and impaired sentence processing. This point towards a cortical involvement in younger patients with early onset of AD [8].

Also, recent researchers have given evidences of an association between early onset of AD and Down's syndrome. This was concluded because of recognition of the role of Amyloid Precursor Protein's (APP) over production due to increased gene dosage with trisomy 21 [9], thus pointing towards a possibility that people with Down syndrome have an increased risk of developing younger onset dementia after age of 50 years [10].

1.2 Understanding Biomarkers

Biomarkers or "biological markers" are the category of medical signs which define a medical state from outside the patient and can be reproduced and measured accurately, unlike the medical symptoms which are mere indications of a patient's condition described and perceived by the patients themselves. According to the definition given by National Institutes of Health Biomarkers Definitions Working Group in 1998, "Biomarkers are evidence of any biological, pathogenic or pharmacogenomic response when administered to any therapeutic change" [11]. Biological markers are basically any kind of substances, structures or processes which could be measured in/outside the body and may influence any changes in the body and probable prevalence of any disease in the body [12].

For a possible potent biomarker for Alzheimer's disease, following criteria has been unanimously decided by researchers worldwide [13-16].

- Reflect aging of brain.
- Describe pathophysiological processes in brain.

Amyloid beta	Tau protein	Phosphorylated tau
Aβ plaque depositions are used widely to characterize AD. Secretases cleaves Aβ from large APP and processing of these amyloidgenic pathways produces these 42 amino acid peptides ($Aβ_{1-42}$) which end up as aggregates in brain. Analysis of CSF in AD patients shows a significant reduction of Aβ of about <500pg/ml in comparison to controls with 794±20 pg/ml of Aβ [17].	The intaneuronal inclusion of the microtubule associated protein tau serves as another established biomarker for AD. Tau protein which is known to increase gradually with age <300pg/ml (21-50 years) to almost <500 (>71 years) shows a significant exponential increase in AD patients of about >450 to >600 pg/ml (in patients of ages 51-70 years). Hence proving to be a good prognostic biomarker [18].	AD exhibits condition of tau protein being phosphorylated in almost 39 possible sites. Wherein, position 181 works as a definite biomarker in AD as compared to controls. The phosphorylation of Tau protein results in both lack of functions and also neuronal dysfunction. The other notable phosphorylated tau proteins include (phosphor-tau-199, -231, -235, -396 and -400 [19].

[Table/Fig-1]: Established biomarkers for Alzheimer's disease [17-19].

- Any pharmacological change should be reflected.
- Highly sensitive and specific.
- Reproducible results over time changes.
- Clear cut-off values with at least two-fold changes
- Easy collectible results and inexpensive tests.

1.3 Established CSF Biomarkers for Alzheimer's Disease

Considering previous researchers, three biomarkers have been internationally established and published worldwide, for diagnosis of Alzheimer's disease [Table/Fig-1] [17-19]. These biomarkers are obtained from CSF and these biomarkers collectively increase the validity for diagnosis by giving results which are sensitive to >95% and specific to >85% [20-23].

known genes through post-transcriptional gene silencing (RNAi) [24]. The dysregulation of miRNA expression in peripheral blood can serve as a potent source of diagnosis of Alzheimer's and other brain related disorders [25] [Table/Fig. 2] [26-46]. Schipper (2007) identified a number of downregulated miRNAs when compared to 16 sporadic AD patients with 16 controls using a microarray chip. These downregulated miRNA included miR-34a, miR-81b and let-7f [26]. The targets of these miRNAs were interestingly found to be part of p53, Notch and Bcl-2 pathways which are already known to be involved in AD pathogenesis.

Another interesting research carried out by Geekiyanage and Chan (2012) showed decreased levels of miR-137, miR-181c, miR-9 and miR-29a/b in neocortical regions of AD patients. Similar

miRNAs	Evidences in samples	References
miR-34a, miR-181b	Increased expression in PBMC	[26]
miR-9	Downregulated in serum	[27]
miR-112, miR-161, let-7d-3p, miR-5010-3p, hsa-miR-26a-5p, hsa-miR-1285-5p, and hsa-miR-151a-3p upregulated; miR-103a-3p, miR-107, miR-532-5p, miR-26b-5p, let-7f-5p	Downregulated in peripheral blood	[28]
miR-107	Downregulated in temporal cortex	[29,30]
hsa-let-7d-5p, hsa-let-7g-5p, hsa-miR-15b-5p, hsa-miR-142-3p, hsamiR-191-5p, hsa-miR-301a-3p and hsa-miR-545-3p	Differentially regulated in Plasma	[31]
miR-29	Downregulated in temporal cortex, cerebellum and serum	[32]
miR-27a-3p	Reduced expression in cerebrospinal fluid	[33]
miR-34	Upregulated in hippocampus	[34,35]
60 miRNAs including Let-7 family members	Differentially regulated in cerebrospinal fluid	[36]
miR-181	Downregulated in temporal cortex and serum	[37]
miR-146a, miR-155	Increased levels in cerebrospinal fluid and extracellular fluid	[38]
miR-106	Downregulated in temporal cortex	[39]
miR-9,miR-125b, miR-146a, miR-155	Increased levels in cerebrospinal fluid and extracellular fluid	[40]
miR-146a	"Selective" upregulation in temporal cortex and hippocampus	[41]
Let-7b	Increased levels in cerebrospinal fluid	[42]
miR-15a	Increased levels in plasma	[43]
miR-34c	Increased levels in plasma	[44]
miR-132 and miR-134 families	Upregulated in plasma	[45]
miR-29a/b, miR-181c, miR-9	Downregulated in serum	[46]

[Table/Fig-2]: Circulatory miRNAs associated with AD [26-46].

2. NEED FOR CIRCULATORY BIOMARKERS

As discussed previously, the CSF which is used for diagnosis for AD is both intrusive and invasive for patients. The main reason being, CSF fluid is obtained by lumbar punctures which cause nausea, severe backache and weakness in elderly people. Also maintaining track of patients for regular diagnosis becomes very difficult. Thus, it is essential to identify new biomarkers in other sources like serum, urine etc. which are cheaper, less invasive and easily collectible. The main advantages of using blood for diagnosis are, being easily obtainable a proper follow-up of the patients can be maintained over a period of time. Also analysing blood cells (e.g., peripheral blood mononuclear cells, lymphocytes, monocytes or platelets) can be more specifically related to AD pathologies.

2.1 "Circulatory" miRNAs

miRNAs belong to the class of non-coding RNA molecules of around 22 nucleotide length which regulate more than 60% of all

results were seen when the same follow-up study was performed on blood levels of AD patients (n=7) with controls (n=7), although the downregulation was at a lower level [47].

Villa et al., and Bekris et al., through their studies were able to demonstrate downregulation of miRNA 29b and 15a which regulated transcription factor Sp1 which is known to regulate expression of APP and tau which are known AD related genes, along with other target genes [48-51].

Researches suggest that a systematic increase in specific miRNAs may help in suppressing various cellular functions like redox defences and DNA repair mechanisms in brain and peripheral tissues supporting the role of miRNAs as potential therapeutic biomarkers for AD in future. Scientists worldwide have showed that an increase in specific miRNAs can regulate crucial cellular functions in brain and peripheral tissues. Thus the contribution of miRNAs in functions such as redox defences and DNA repair mechanisms advocate the potential of miRNAs as potential therapeutic biomarkers for AD in near future.

2.2 Blood based amyloid markers

Although the efficiency of Amyloid beta is a highly sensitive and specific biomarker from CSF for AD has already been established, new studies are being focussing on evaluating Aβ as a potential biomarker from blood serum as well. In a recent meta-analysis review performed by Koyama and colleagues conducted on 13 studies of 10,303 AD patients and controls, to monitor $A\beta_{1-42}$ and the ratio of $A\beta_{1-42}$/ $A\beta_{1-40}$ in plasma to predict AD, showed highly statistical and clinically significant decrease in $A\beta_{1-42}$ ratio to predict cognitive impairment. However, the results are still not conclusive enough, since the plasma levels are largely affected by factors like subject's age, lifestyle, laboratory conditions, assay variability, etc., [50].

Other studies have shown varied forms of amyloid beta, in blood plasma, to be crucial potential AD biomarkers for future research. Perez et al., (2012) showed the ratio of free to cell bound $A\beta_{1-17}$ in blood samples of Mild Cognitive Impairment (MCI) and age-matched control groups to be significantly varying, thus concluding $A\beta_{1-17}$ in blood plasma to be a highly sensitive and specific biomarker for AD [51].

Various platforms are being developed to measure Aβ levels in blood serum and plasma, such as ELISA developed by Araclon Biotech Ltd., to perform colorimetric tests to measure ratios of $A\beta_{1-40}$/ $A\beta_{1-42}$ in patients showing MCI [52]. Other tests like electroluminescence are being developed to further probe into possibility of Aβ in blood to serve as potential biomarkers in near future [53].

2.3 Inflammatory Markers

Neuroinflammatory variability involving astrocytes and activated microglia and the secreted mediators such as oxygen species, chemokines and cytokines was seen on various neuropathological studies conducted on AD brains [54]. The accumulation of such components, may lead to alterations in immune functions and transition of innate immune cells to aggravated proinflammatory cytokines tumour necrosis factor (TNF)-α leading to an increased rate of cognitive decline and also neuronal cell death in some cases [55,56]. In studies conducted by Laske et al., (2013) it was shown that TNF-receptor 1 can be a potent inflammatory biomarker for understanding AD patients better [57]. In another promising study a blood panel of 18 biomarkers (combination of cytokines, growth factors and binding proteins) allowed diagnosis of Alzheimer's and MCI with an accuracy of ~90% [58].

According to researches, an inflammatory response plays a crucial role in neurodegeneration during progression of AD. Both genetic as well as pathological studies have shown an overexpression of proinflammatory cytokine interleukin β (IL- β) in AD patients, as compared to controls. The role of complement receptor type 1 has also been seen in clearance of amyloid β [59]. Several studies show the association between AD and inflammatory biomarkers such as IL- 1β, IL- 2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-18, Interferon (IFN)-γ, Tumour Necrosis Factor (TNF)-α, Transforming Growth Factor (TGF)-β, and acute phase Reactant Protein c (CRP) [60-65].

Ceramides, sphingomyelins and sulfatides have also been seen to be integral part of neuronal functions and synthesis of bioactive metabolites related to AD [66]. Various studies have shown the serum levels of ceramides differing in AD patients, MCI patients and their respective controls. Since, high base ceramides levels might lead to increased risk of impairment in a normal cognitive brain and a significant decline in a cognitively impaired brain along with decline in the hippocampal volume [67]. Vascular Cell Adhesion Molecule I (VCAM-I), Intracellular Adhesion Molecule I (ICAM-I) and selectins also might serve as biomarkers of microvascular injuries, being in increased levels in plasma samples of patients with late onset AD suggesting endothelial dysfunction [68]. Increased apoptosis in CD4+ T cells and NK has also been proved in AD patients when compared to their controls, along with increased levels of B-cell lymphoma 2, caspases and antioxidant enzyme Superoxide Dismutase (SOD1) [69,70].

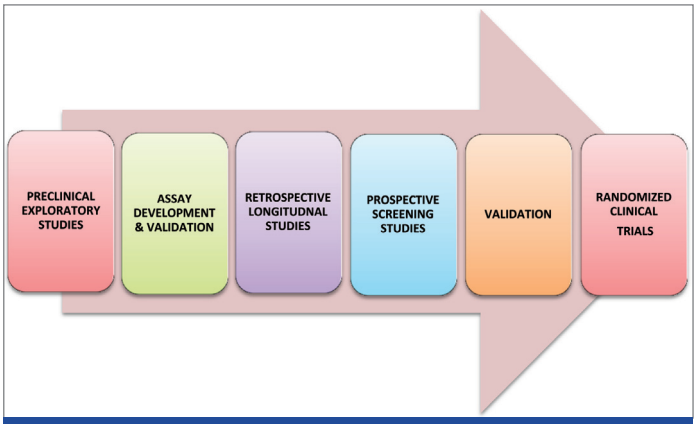
However, the accuracy of these inflammatory biomarkers especially cytokines as potential biomarkers still need to be validated because of a little inconsistency seen in parallel studies. Researchers think this is mainly due to difference in the type of samples being analysed i.e., cerebrospinal fluid, peripheral blood plasma, blood serum etc.

2.4 Biomarkers for oxidative stress

Oxidative stress is another dimension being explored for biomarkers in AD. The neurodegenerated parts of brain have been known to show increased Reactive Oxygen Species (ROS) levels. In such conditions, proteins undergo post-translation modifications, leading to formation of mixed disulphides, nitration of tyrosine residues, and formation of lipid peroxides [71]. Protein oxidation besides causing toxic cell damage also results in fragmentation and aggregation, leading to proteolysis [72]. AD and MCI patients both have been detected with increased protein aggregate levels in the forms of fibrils along with increased lipid formation [73]. The most common known markers for oxidative stress include protein glutathionylation, free fatty acid releases, DNA oxidation, iso and neuro prostane formation, 4-Hydroxy 2 trans Nonenal (HNE), lipid peroxidation and advanced glycation end products detection [74].

3. STRATEGIES FOR DEVELOPING PATIENT SPECIFIC BIOMARKER PROFILES

With increased knowledge of various pathways playing significant roles in AD and other factors, it has become clearly evident that one biomarker profile is not enough to identify differentially expressed proteins between AD patients and controls and provide conclusive diagnostic results. Hence, scientists now-a-days are focussing on developing methods to measure various biomarkers simultaneously on a single microarray chip or well.



[Table/Fig-3]: The process of biomarker discovery.

3.1 Stages of Biomarker Screening [Table/Fig-3]

3.1.1 Pre-exploratory Studies

This phase consists of approvals from Ethical committee so as to ensure proper enrolment of subjects in the study, collection of blood samples, transportation, storage and disposal of collected samples after usage. The ethical committee ensures that proper information is given to the subjects before sample collection. After the ethical approvals are taken, diseased and control samples are compared for generating hypothesis for detection of disease. Techniques such as microarray, mass spectrometry, immunohistochemistry, protein expression profiling and western blots are employed in this phase [75].

3.1.2 Assay Development and Validation

In this phase, clinical assays are developed to discriminate samples with/without disease. The collected samples consist of disease before treatment and a matched diseased tissue as control. The samples are collected mostly using non invasive techniques. The primary objective of this stage is to estimate the true positive rate (sensitivity) and false positive rate (specificity) of the developed assay for biomarker detection.

3.1.3 Retrospective Screening Studies

The retrospective training studies are longitudinal studies based on the evaluation of the efficiency and capability of the developed assay to detect disease in its preclinical stage. Also, the effect of covariates such as demographic and geographic characteristics on the efficiency of biomarker before their validation in Phase IV is studied during this phase [76].

3.1.4 Prospective Studies

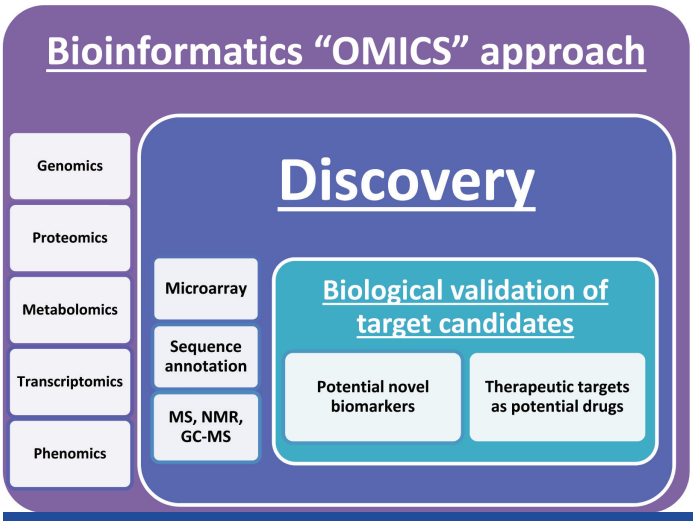
This phase focuses on evaluating the efficiency of the developed biomarker assay by screening in a specific demographic population for determining the false referral rate and also the disease detection rate of the assay. This stage basically contributes in describing the stage-specific characteristics of the disease, assessment of screening on cost and mortalities due to the disease.

3.1.5 Validation

Once the efficiency of biomarkers is assessed after retrospective and prospective screening, the results are published in peer reviewed journals so that they can be repeated and verified in other laboratories around the globe to produce similar results to check the authenticity and competency of the developed biomarkers in disease detection.

3.1.6 Randomised Clinical Trials

In the final phase, randomised clinical trials are conducted to detect whether the biomarker based screening is able to reduce the disease burden on the target population.



[Table/Fig-4]: Application of 'omics' data in Biomarker discovery.

3.2 Techniques for Biomarker Discovery [Table/Fig-4]

Bioinformatics Tools for Biomarker Discovery

Various 'omics' tools and databases available online based on different types of data mining techniques such as Decision Trees, Clustering, Regression, Association Rules, Artificial Intelligence, Neural Networks, Genetic Algorithm, Nearest Neighbour method, Classification and other pattern based searches are being used as discovery tools for easier understanding of biological systems by incorporation of systems biology in the process of biomarker discovery [Table/Fig-4,5] [77,78]. Some of the major databases used by researchers have been described in Supplementary [Table/Fig-1].

DNA and RNA microarray chips allow studying differentially expressed genes on a single chip-plate, however, similar development for providing conclusive multiple gene signatures to serve as biomarkers appearing in AD patients is still under progress [79,80].

Various techniques being used nowadays for biomarker discovery include, mass spectrometry imaging and profiling which basically explores the idea that miRNA might not provide complete information of the diseased state and the altered proteins could be assessed by mass spectrometry and used for diagnostic purposes by combining with mathematical algorithms. One of the most widely used technologies include SELDI-TOF-MS (Surface Enhanced Laser Desorption Ionization Time Of Flight Mass Spectrometry) which involves analysing small amount of unfractioned serum samples added on a protein chip. These patterns reflect the blood proteome without actual identity of proteins [81,82].

[Table/Fig-5]: Various techniques for biomarker discovery.

Another approach to biomarker discovery being explored is biomarker family approach, where in an assumption that a miRNA is already a member of biomarker family or have a potent target gene, then other miRNAs belonging to the same family or having same gene target might also be potential biomarkers for diagnosis is considered [83,84].

Serum proteome or low molecular weight plasma is another domain in which biomarkers are being investigated. Wijte et al., (2012) conducted a study on peptidome analysis of CSF from AD post-mortem brains and respective controls, and concluded with results of difference in profiles of endogenous peptides and protein bound peptide fractions [85]. The discriminating factors included VGF nerve growth factor inducible precursor, and complement C4 precursor, whereas the discriminating peptides in the protein-bound fraction were identified as VGF nerve growth factor inducible precursor, and alpha-2-HS-glycoprotein [86].

The role of auto-antibodies in pathology of neurodegenerative disorders by evaluating the changes in the spectrum of auto-antibodies in human sera is being carried out using High Throughput Protein Microarray Technology in most laboratories [87-89]. Researches show that in case of AD, early loss of pyramidal neurons may lead to breakdown of antigenic cellular

products which then enter CSF and then enter into blood and lymph. This leads to production of auto-antibodies in the blood, which can then be analysed for their potential role as biomarkers for AD. This also can lead to identification of antigen targets as well as disease relevant pathways for further investigation [90].

Although various strategies are being discussed for biomarker discovery, however a major future challenge is defining a routine procedure [91,92]. These procedures should provide clear guidelines on

- Collecting, transport, processing and storage of samples
- Analysis of the samples
- Interpretation and cut off values.

CONCLUSION

Till date, researchers vouch on amyloid beta, tau protein and phosphor tau as confirmed biomarkers for AD. However, with increase in knowledge of genomics, proteomics and systems biology a number of novel blood based biomarkers- circulatory miRNA and inflammatory biomarkers are being developed for better diagnosis by the research community for AD.

In the current scenario, research for biomarkers is not limited to diagnosis for neurodegenerative disorders. With new advances in technologies for testing and implementation of emerging therapeutic approaches, recognition of "at-risks" individuals also becomes crucial for clinical trials. AD patients have been known to show neuropathology in their brains for almost 10-20 years before the actual onset of disease. The new biomarkers should hence act as an asset for preclinical and early diagnosis of onset of AD should be sensitive, specific and reproducible biomarkers for detection of AD related neuropathological disorders. Thus, efforts are needed to be made in validating reliable, and inexpensive blood based methods for proper diagnosis, detection and monitoring of AD progression and estimation of therapeutic relevance.

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