Genetic and Blood Biomarkers of Alzheimer’s Disease

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Abstract: Alzheimer’s disease (AD) is the most prevalent form of dementia. AD is highly heritable, with a complex pattern. Although clinical diagnosis is based on accurate and well defined diagnostic criteria (NINCDS-ADRDA), the definite diagnosis relies on the postmortem pathological findings. The need for measures for the early detection of AD, as well as the need to distinguish between AD and other forms of dementia, has put great emphasis on the discovery of biomarkers for Alzheimer’s disease. In clinical practice, there is need for non-invasive, accurate methods for the early detection and differential diagnosis of AD. The successful identification and development of the biomarkers, depends completely on the understanding of pathology, genetic and molecular mechanisms involved in the disease. As blood is a circulating dynamic tissue and transcription reflects the ongoing changes in the system, it makes the transcriptional profiling of the blood cells, potentially, the most sensitive source for transcriptional biomarkers. A systematic comparison of the genetic and proteomic blood biomarkers in patients and healthy controls can reveal additional potential candidates for AD. In the first part of this review, we would like to discuss the most recent genetic findings and their possible involvement in the pathogenesis of AD. In the second part, we review the blood biomarkers which can be derived from peripheral blood mononuclear cells (PBMC), serum, and plasma. We discuss three main categories of biomolecules, namely DNA, RNA (miRNA and mRNA), and protein as well as their possible role in the hypothetical mechanisms involved in the pathogenesis of AD. A dynamic interaction between the genetic findings through the whole genome association studies and biomarkers discovery can advance our knowledge of AD pathogenesis beyond the scope of each field independently.

Keywords: Alzheimer's disease, genetics, blood biomarkers.

1. INTRODUCTION

Alzheimer’s disease (AD) is the most prevalent form of dementia. AD is highly heritable, with a complex pattern. Although clinical diagnosis is based on accurate and well defined diagnostic criteria (NINCDS-ADRDA) [1], the definite diagnosis relies on the postmortem pathological findings. The neuropathological lesions in Alzheimer’s brain consist of (a) intracellular neurofibrillary tangles, consisting of hyperphosphorylated tau protein, and (b) extracellular senile plaques containing β-amyloid.

There are two major types of Alzheimer’s disease 1) Early onset AD (EOAD) with the age of onset <65 and strong familial clustering, with an autosomal dominant mode of inheritance, comprising about 5% of all cases with diagnosis of Alzheimer’s disease, 2) late onset Alzheimer’s disease (LOAD), comprising 95% of AD cases with the age of onset beyond 65 and no significant familial aggregation. The rare EOAD, is mostly associated with the highly penetrant mutations in APP (Amyloid Precursor Protein) on chromosome 21 [2], PSEN1 (Presenilin-1) on chromosome 14 [3], and PSEN2 (Presenilin-2) on chromosome 1 [4] (reviewed by Hardy and Selkoe, 2002 [5]). These mutations however, have only been found in 1% of all patients with the diagnosis of Alzheimer’s disease [6], meaning the quest for the novel genetic entities involved in the pathogenesis of Alzheimer’s disease, will go on.

The genetics of late onset Alzheimer’s disease seem to be complex. Since the identification of ApoE (Apolipoprotein E) variants as a risk factor for Alzheimer’s disease by Strittmatter et al. in 1993 [7], the concept of common disease-common variant became widely acceptable for Alzheimer’s disease. Numerous association studies following the 1993 publication have consistently confirmed ApoE as a highly associated gene locus with AD.

The pathology of Alzheimer’s disease shows that it could be considered a systemic disease, as the damage to the organs is beyond the central nervous system. So far, many cardiovascular and inflammatory components have been suggested to be involved in the pathogenesis of Alzheimer’s disease. A systematic comparison of the genetic and proteomic blood biomarkers in AD and healthy controls can reveal additional potential candidates for AD. A dynamic interaction between the genetic findings through the whole genome association studies and biomarkers can advance our
knowledge of AD pathogenesis beyond the scope of each field independently.

2. RECENT APPROACHES IN THE GENETICS OF ALZHEIMER’S DISEASE

Recent advances in post human genome era have revealed astonishing information on the variations in human genome. The most common variations are single nucleotide polymorphisms (SNPs) which differ among individuals. In human genome, there are estimated ~10 million SNPs [8]. Since the establishment of dbSNP, the database for single nucleotide polymorphisms in 1998 [9, 10], and HapMap project in 2003, association studies have taken a leap towards identification of genetic factors in complex diseases. The availability of a large number of SNPs made it possible to genotype the variants in and around the genes across the genome regardless of their function and the identification of tag-SNPs through the HapMap project and characterization of common haplotype block structures in human genome. It means that the SNPs across the haplotype are in linkage disequilibrium (LD) and genotyping a number of tag-SNPs can represent the majority of the variations in the block [11]. Based on these fundamental findings, the high density genome screening has shifted the search for the new genes from the traditional candidate gene approach, to whole genome association studies, where hundreds of thousands of SNPs throughout the genome are interrogated in an unbiased manner. Genome wide association studies (GWAS) take the advantage of recent advances to test hundreds of thousands of genetic markers in a hypothesis free manner. To date, there have been numerous studies on the genetics of AD and twelve GWAS and four other large scale studies catalogued in [12], reviewed by Bertram and Tanzi, 2009 [13]. Alzgene website summarizes the results from meta-analysis. Apart from ApoE, which shows to be associated with risk of AD, the published GWA studies do not show consistency in the regions or genes being associated with the disease. The two most recent independently published studies from [14, 15], however, show the first overlap in CLU which is reported to be associated with the risk of AD in both large populations under study. The only possible way to determine which genes are truly susceptibility loci for AD would be the future attempts in replicating the current results in different populations. In the first part of this review, we would like to discuss the genes identified in previous studies and their relevance to the pathogenesis of Alzheimer’s disease. In the second part, we will discuss the blood biomarkers of AD and their possible role in pathogenesis and progression of Alzheimer’s disease.

2.1. ApoE

Since the first report of association of ApoE genotype with AD [7, 16], it has remained the single most significant risk factor since the first genetic mapping efforts [7, 17, 18] until recent GWA studies [14, 15, 19, 20]. ApoE is a member of apolipoprotein family and is known to have three common isoforms, based on the variants of two coding SNPs in the amino acids 112 and 158 of the protein. These variants create three basic alleles, ApoE 2, 3, and 4. ApoE 3 has a cysteine in position 112 and arginine in position 158. ApoE 2 contains cysteine in both positions and ApoE4 contains arginine in both positions [21]. The carriers of E2 allele have a reduced risk of AD, while E3E4 heterozygous individuals carry a two to three fold higher risk of developing AD, among European populations. The E4E4 allele on the other hand, increases the risk to twice of the E3E4 allele [22]. ApoE4 allele has been found to be a strong genetic risk factor for both AD and CAA (cerebral amyloid angiopathy) [23]. Studies suggest that ApoE4 predisposes to the earlier age of onset [13, 24], while other studies present conflicting results on the effect of ApoE4 on the rate of progression of cognitive decline [25-30]. Understanding the physical involvement of ApoE in the cascade of events leading to the pathogenesis of AD is the key to the disease mechanism, new preventive measures, and a tailored therapy for AD.

ApoE contains 299 amino acids and transports cholesterol and other lipids by binding to cell surface ApoE receptors [31]. The N-terminal domain of the protein interacts with ApoE receptors and the C-terminal binds to the lipids. Different ApoE isoforms, ApoE 2, ApoE 3, and ApoE 4, differ in their protein structure and binding properties. ApoE also binds to Aβ in the C-terminus, interfering with the lipid binding property of the protein [32]. This interaction which can interrupt the lipid transportation of ApoE, may also contribute to the pathogenesis of AD. The histopathological studies have shown a positive correlation between amyloid plaques’ density and ApoE 4 allele dose [33, 34]. ApoE 3 isoform binds to Aβ with higher affinity than ApoE 4 [35], and ApoE 2 isoforms have even higher binding affinity to Aβ. As affinity of ApoE to Aβ inversely correlates with the risk of disease, one can assume that ApoE is involved in the clearance of Aβ. The body of literature on ApoE and its role in the pathogenesis of AD suggests diverse mechanisms on how it could be a part of the cascade leading to cognitive decline and AD. These include Aβ aggregation and clearance, synaptic plasticity, neurotoxicity, tau aggregation and neurofibrillary formation, neuroinflammation, metabolism in brain, neuronal signaling, and mitochondrial dysfunction (reviewed by Bu, 2009 [36, 37]). Due to strong genetic association and functional involvement of ApoE in the central nervous system, future investigations are necessary to fully understand the role of ApoE in neurodegeneration and to identify new targets for therapy.

2.2. ACE

Angiotensin converting enzyme I is encoded by the ACE gene. ACE is a zinc metalloprotease and a potent vasopressor that modulates blood pressure and electrolyte balance. It has been suggested that ACE is involved in degradation of pathogenic Aβ-42 to Aβ-40 [38]. Variants in the ACE gene have been considered as risk factor for a number of diseases such as Alzheimer’s disease, diabetic nephropathy, coronary heart disease and stroke [39]. There are reports of a possible role for ACE gene in the risk of cognitive impairment and AD [40-42]. In a study conducted by Edwards et al. in 2008, a significant association between ACE haplotype and susceptibility for AD was shown in family-based and case-control samples [43]. The strongest association of the risk for Alzheimer’s disease with ACE gene variants has been observed in the alu repeat insertion/deletion (I/D) polymorphism rs1799752 located in intron 16. Other polymorphisms associated with the risk of AD in Caucasians include rs4343 and rs4291 [44]. Several studies have demonstrated association between different
clades of *ACE* gene [45] and *ACE* 1 serum levels. The carriers of clade A have the lowest level of ACE1, while carriers of clade B have the highest level of ACE1. The carriers of clade C have an intermediate level of ACE1 [45]. In-vitro experiments showing degradation of Aβ are relevant for understanding the pathogenesis of AD [46]. This would explain the low levels of ACE1 in carriers of I allele in AD patients as a risk factor. It must however be clearly stated that I/D polymorphism is not necessarily functional, but might be in complete linkage disequilibrium with the functional variant. Apart from the role of *ACE* gene in Aβ catabolism, ACE could also be involved in other mechanisms linked to the risk for AD, such as blood pressure regulation. Some studies have shown increased risk for AD in individuals with midlife high blood pressure [47]. These findings raise a great interest in the area of pharmacogenomics by using ACE inhibitors as preventive and therapeutic measures for AD [48, 49].

### 2.3. MAPT

The pathological hallmarks of AD are neurofibrillary tangles, consisting of hyperphosphorylated tau protein and β-amyloid plaques (mainly consisting of insoluble species of amyloid protein). The *MAPT* (*Microtubule Associated Protein Tau*) gene on chromosome 17q21, encodes Tau protein, with the primary function as a cytoskeletal protein stabilizing microtubules in the axons of neurons [50]. Tau protein has six isoforms, resulting from differential splicing of the exons 2, 3, and 10. Depending on the inclusion or exclusion of exon 10, Tau protein has three or four microtubule-binding domains, hence called 3R or 4R Tau. The function of Tau protein depends on its ability to bind to microtubules. In healthy brain, the 3R/4R ratio is ~1. Any deviation from 1 (increase or decrease), is indicative of neurodegeneration and Tau deposition. The exact pathogenetic mechanism by which Tau undergoes conformational changes and builds neurofibrillary tangles is not fully understood. Tauopathies are a group of neurodegenerative disorders with abnormally phosphorylated and aggregated Tau proteins in CNS, mainly in the neurons and glial cells [51-53]. Tauopathies include Alzheimer’s disease (AD) and frontotemporal dementia (FTD). To date, 66 different *MAPT* mutations, both coding and intronic, have been found in FTD patients (for the list of mutations see http://www.molgen.ua.ac.be/FTDMutations). In these patients with Tau mutations, a cause and effect relationship can be manifested through the Tau pathology.

*MAPT* is located on chromosome 17q21, one of the largest haplotype blocks in human genome [54]. *MAPT* haplotype spans a 900 kb region and is defined by an inversion that created the two haplotypes H1 and H2. H2 haplotype has a large number of SNPs which are in complete linkage disequilibrium and its structure does not show any variations. H2 haplotype is mainly found in Caucasian population and is almost absent in Southeast Asian populations. Studies have shown that H2 haplotype has a slight protective effect if compared to H1. This effect has been also observed in Parkinson’s (PD), progressive supranuclear palsy (PSP), and corticobasal syndrome (CBS) [55]. The importance of Tau lies in 1) the brain pathology which is manifested in a large group of diseases, 2) the established causal relationship between tau pathogenic mutations and diseases such as AD, FTD, CBS, 3) the yet to be discovered correlation between the cases with Tau pathology and no obvious Tau mutations. The causal mutation in these cases could lie upstream of Tau in the functional pathway. The exact function and structure of the Tau gene and its role in pathogenesis of AD remains to be explored.

### 2.4. Clu

Association of *Clusterin* or ApoJ with AD has been recently reported in two independently performed studies [14, 15]. These studies screened 6010 AD cases and 8625 controls [14] and 5964 AD cases and 10188 controls [15]. Both studies found significant association with the SNP rs136000, located in an intron of *CLU*, p=9.0*10^-9* reported in Lambert et al. and p=1.4*10^-9* reported in Harold et al. The association of ApoJ, an apolipoprotein, indicates involvement in the similar functional pathways as ApoE. *CLU* (*Clusterin*) or ApoJ, encodes a heterodimeric glycoprotein, consisting of two subunits, α and β [56]. Clusterin is ubiquitously expressed and its expression has been especially detected in pathological conditions such as injury and chronic inflammation of the brain [57]. It has also been shown that it plays a role in maintaining Aβ soluble in AD [58] and is overexpressed in neurodegenerative disorders such as AD and Pick’s disease [59]. Like ApoE, Clu binds to Aβ and is present in amyloid plaques. Clu-immunopositive Aβ has been isolated in AD temporal cortex and these plaques are associated with dystrophic neuritis [60]. The same study shows that 70% of Clu-positive plaques were also positive for phosphorylated Tau. In-vivo experiments have shown Clu affecting Aβ fibrillogenesis and increased neurotoxicity [61]. Both Clusterin and ApoE bind to soluble Aβ and can cross the blood-brain-barrier. It has been suggested that ApoE and Clu transport Aβ through the blood-brain-barrier. ApoJ-Aβ complex transport the plasma Aβ back to the brain [62, 63], while ApoE, transports Aβ from brain to plasma [64]. The role of ApoE and Clu in the transport, clearance and regulation of Aβ in health and disease still remains to be elucidated.

### 2.5. PICALM

In their recent publication on genome-wide association study of Alzheimer’s disease, Harold et al. reported the association of SNP rs3851179, 88.5 kb upstream of *PICALM* on chromosome 11 (P=1.9*10^-8, OR=0.849) [12]. The two previous GWA studies examining *PICALM* did not find any association between *PICALM* and Alzheimer’s disease [65, 66]. PICALM (phosphotyidylinositol-binding clathrin assembly protein) is a ubiquitously expressed protein, although highly in neurons and is involved in clathrin mediated endocytosis. As part of endocytic machinery, PICALM is involved in the formation of clathrin coated vesicles, intracellular trafficking of proteins, lipids, and neurotransmitters [67-69]. The reports show that over-expression of PICALM in the cells impairs transferrin uptake by impairing endocytosis [67]. This confirms the *in vivo* role of mouse Picalm in iron uptake. It has been known that neurons are susceptible to iron supply; low intracellular iron level impairs iron metabolism while high intracellular iron concentration impairs neuronal function. In AD brains, the regions of Aβ deposition greatly overlap with iron accumulation sites [70]. A potential
pathogenic role for iron has been recently suggested by Silvestri and Camaschella [71]. Therefore, although identified through GWAS and not through functional analysis, the role of PICALM in pathogenesis of AD could be highly important and further functional analysis and genetic screenings are recommended.

2.6. CR1

Another locus presented in Lambert et al. 2009 as a candidate region for AD is an LD block on 1q32 containing only CR1 (Complement Receptor 1) gene. The gene lies in the region of regulators of complement activation [72]. The study reveals two major haplotypes with the frequency of 97.8% and a third haplotype with the frequency of 1.2% in combined control population [14]. Lambert et al. suggest the possible role of CR1 in AD by its involvement in Aβ clearance. There are three major genetic variance classes known for CR1 [73]; 1) insertion/deletion polymorphisms causing substantial changes in the molecular weight of the protein, 2) intronic and exonic single nucleotide polymorphisms, affecting the density of the CR1 protein on the cell surface, 3) single nucleotide polymorphisms generating Knops blood group antigens.

CR1 is the receptor for C3b/C4b complement peptides [74]. CR1 is a multifunctional polymorphic glycoprotein expressed in the plasma membrane. The soluble form of CR1 (sCR1) is released in plasma as a result of cleavage of CR1 from the surface of leukocytes [75]. The CR1 protein contains four long homologous repeats (LHRs). The functional domains of the protein are distributed among these domains. The soluble form of CR1 differs from the surface form only in the cytoplasmic part of the protein at the C-terminus, removed through the proteolytic cleavage of the transmembrane protein [76]. Research has associated CR1 to a number of diseases such as autoimmune disorders [77], systemic lupus erythematosus [78-81], rheumatoid arthritis [81, 82], malaria [83], atopic dermatitis [84], HIV [77], systemic lupus erythematosus [78-81], rheumatoid arthritis [81, 82], malaria [83], atopic dermatitis [84], HIV infection [85]. Expression of CR1 has been shown to be regulated by cytokines and immune complexes in diseases (reviewed by Khera and Das, 2009 [73, 86]). Tumor necrosis factor α (TNFα), TNFβ, IFNγ, interleukin-4 (IL-4) and IL-10 have been shown to regulate expression of CR1.

Neuropathology of Alzheimer’s has been linked to inflammatory processes involving cytokines and chemokines. On one hand, it has been shown that cytokines secreted by neurons, astrocytes, and microglia, can stimulate the synthesis of Aβ [87]. On the other hand, Aβ can induce the expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-6 [88]. The inflammatory process could be the possible link between the genetic association of CR1 and its function in the pathway involved in pathogenesis of Alzheimer’s.

2.7. TNK1

Tyrosin kinase, non-receptor (TNK) 1 gene, also called thirty-eight-negative kinase-1, was shown to be associated with AD, through the silent SNP rs1554948 in exon 2 [89]. In that study, Grupe et al. found TNK1 to have the strongest signal in their GWA study. Figgins et al. however, found no association between rs1554948 and LOAD [90]. In 2007, Azoitei et al. reported that TNK1 acts as a molecular switch in TNFα pathway and blocks p65-induced NF-xB reporter gene activation [91] and induces apoptosis by blocking TNFα-induced NF-xB transactivation. It is noteworthy that according to AlzGene, TNFα gene is also one of potential candidate genes for AD.

2.8. GAB2

The GWA study [92], which found ApoE as the only gene associated with LOAD, was re-analyzed by the same group and found a significant genome-wide association (P-value=9.7*10-11) with five SNPs in GAB2 [93]. The major allele of GAB2 showed 2-4 times increase in AD risk in this cohort. The authors also pointed out to the functional data connecting the genetic association with an effect on Tau phosphorylation. Further meta-analysis, revealed that the minor allele of GAB2 SNP rs10793294 has an almost 50% reduced risk for AD, which is consistent with the original study [94]. As the meta-analysis results show, overall, GAB2 (or a locus in complete linkage disequilibrium with GAB2) represents an important susceptibility locus for AD. GAB2 is a member of GRB2-associated binding protein (GAB) gene family. GAB2 is a scaffolding protein involved in signaling pathways, which might be involved in AD pathology, affecting Tau phosphorylation and amyloid metabolism [93]. GAB2 is highly expressed in prefrontal cortex and the hypothalamus. Moreover, GRB2 (growth factor receptor-bound protein 2) which binds to GAB2, also binds to Tau, APP, presenilin 1 and presenilin 2 [95].

2.9. Other Candidate Genes

Apart from the above mentioned genes, there are a large number of genes found to have a significant association with AD in the recent GWA studies. At the AlzGene, the online encyclopedia of AD whole genome association studies, 35 genes have been listed as the top results. The genes have been ranked based on the criteria for the assessment of cumulative evidence of genetic association [96, 97].

3. BLOOD BIOMARKERS OF ALZHEIMER’S DISEASE

As defined by the Biomarkers Definitions Working Group (BDWG) at the National Institutes of Health in 2001 [98], Biomarker is “A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” Peripheral blood has the potential to monitor transcriptional changes in response to the physiological alterations due to the diseases. As blood is a circulating dynamic tissue and transcription reflects the ongoing changes in the system, it makes the transcriptional profiling of the blood cells, potentially, the most sensitive source for transcriptional biomarkers. The successful identification and development of the biomarkers, depends completely on the understanding of pathology, genetic and molecular mechanisms involved in the disease. Biomarkers are becoming more and more an integral part of medicine, particularly in the prognosis of neurological disorders, where the clinical criteria are not sensitive enough. The clinical criteria capture the signs and symptoms at the mid to advanced stages of the disease. The aim of developing the biomarkers is to manifest the pathological process prior to reaching the end point. The term “Clinical endpoint” refers
to “a characteristic or variable that reflects how a patient feels, functions, or survives” (Biomarkers Definitions Working Group (BBDWG) at the National Institutes of Health in 2001). Although clinical endpoint is a measure for the disease diagnosis and evaluation of the patient, we require identifying indicators or the so-called “Surrogate endpoint” which can be measured prior to the end point and can predict the endpoint with high precision. The “Surrogate endpoint” is defined as “A biomarker that is intended to substitute for a clinical endpoint. A surrogate endpoint is expected to predict clinical benefit (or harm or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence.”

The clinical diagnosis of early stage Alzheimer’s disease is rather uncertain. Categorically, the stage between normal old age cognition and dementia is defined as mild cognitive impairment (MCI) [99]. This group consists of cases with different neuropathological symptoms, a subgroup defined as amnestic MCI with predominantly memory impairment, which represents a group with high risk of developing AD [100]. Studies have shown that amnestic MCI shares genetic and environmental risk factors with sporadic AD [101]. The majority of studies in biomarkers of AD have focused on the analysis of cerebrospinal fluid (CSF), because it is more reflective of CNS molecular processes. The structure of Blood-Brain-Barrier (BBB) limits the flow of potential biomarkers closely associated with the CNS pathology (such as Tau) to the blood stream. Nonetheless, CSF collection is an invasive, costly and time consuming process and imposes an additional burden on the elderly population already suffering. Additionally, CSF collection is not as widely practiced as blood draw. Therefore, in the clinical practice, there is a need for a panel of biomarkers which can be derived from blood and can reflect the purpose with high accuracy. The recent studies of blood-based biomarkers have shown that the peripheral changes in pathogenesis of AD may not be only a reflection of CNS [102-104]. The blood biomarkers can be derived from peripheral blood mononuclear cells (PBMC), serum, and plasma. While serum and plasma can be used for the detection and quantification of secreted proteins including cytokines, chemokines, different species of Aβ, and a number of other markers, PBMC offers the opportunity for extraction and analysis of three main category of bio-molecules, namely DNA, RNA (miRNA and mRNA), and protein. PBMC harbors useful markers for prediction of MCI progression and diagnosis of AD and the isolation and processing of PBMC has been extensively discussed by Maes et al., 2009 [102]. Based on the pathology of Alzheimer’s disease and the possible pathways involved in the pathogenesis of AD, the blood biomarkers can be further categorized and discussed in groups of microvascular, immune, metabolic and pathological markers.

4. GENETIC MARKERS

4.1. DNA

The DNA extracted from PBMC can be screened for candidate genes (reviewed in the first part of this manuscript) by sequencing the entire coding region or genotyping SNPs with highest association. The DNA can also be tested for chromosomal changes such as deletion or multiplications as APP locus duplication reported by Rovelet-Lecrux et al. [105] by quantitative Polymerase Chain Reaction (qPCR) or using copy number variation (CNV) microarray technology. The DNA extracted from the peripheral blood can also be used for large scale genotyping platforms for identification of novel loci.

The only limitation in the testing reliability of PBMC would be the study of epigenetic alteration in Alzheimer’s cases. Epigenetics is defined as dynamic processes that modify the gene expression without changing the DNA sequence [106]. Epigenetic modifications include histone acetylation and phosphorylation, which open the chromatin structure and increase the transcription, whereas histone methylation can both activate and silence the gene transcription [107]. DNA methylation often leads to condensation of the chromatin and silencing the gene, specifically in the CpG islands. As AD is primarily a disorder of the nervous system, the epigenetic pattern needs to be investigated in the brains of the patients. A recent post mortem study in human brain identified hypermethylation of PSEN1 promoter region in comparison with normal controls [108]. This however, cannot undermine the concept that AD is a systemic disease being influenced by cardiovascular and inflammatory factors. Our knowledge of neuropathological processes involved in AD is still missing the earliest events triggering the changes leading to neurodegeneration. Probably the study of epigenetic machinery outside of CNS barriers can identify mechanisms and pathways linking AD pathology to the earlier events. The epigenetic markers of Alzheimer’s are not tested in blood and further analysis is required to test the usefulness of the detection of AD epigenetic markers in PBMC derived DNA. In their study Wang et al. showed that the methylation pattern of specific loci do not overlap with the pattern in the brain [108].

4.2. RNA

Another approach towards identification of blood biomarkers for Alzheimer’s disease is the transcriptional profiling of the peripheral blood cells. The potential of blood transcriptional profiling has been suggested previously [109-111]. Studies have shown that peripheral blood cells are sensitive to changes in the central nervous system (CNS) and share gene expression patterns [112]. Using microarray technology, expression of thousands of genes can be investigated with high precision. The key in the study of blood biomarkers is optimizing the results for the different studies and different stages of the disease, and combining and comparing the data obtained from pathology and genetic findings to obtain consistency across different fields of research [113]. The molecular mechanisms and pathways involved in the pathogenesis of AD encompass a dynamic network of gene transcription, expressional regulations, silencing and activation of genes, post transcriptional and post translational modifications and numerous other mechanisms, leading to neurodegeneration at the older age.

For the gene expression profiling, two species of RNA, messenger RNA (mRNA) and micro RNA (miRNA), are isolated from the PBMC [102]. The genetic information encoded in the DNA is copied into an intermediary molecule, called mRNA, in a process called transcription. After modifications and removal of the non-coding segments
of the molecules this intermediary molecule leaves the nucleus and enters the cytoplasm where it is translated into proteins. The microRNA (miRNA) is involved in a recently discovered gene silencing pathway. miRNA are short non-coding RNA molecules that block transcription by binding to mRNA and degrading the double stranded RNA molecule to RISC (RNA Induced Silencing Complex), to be degraded. There is commercially available microarray technology for both mRNA and miRNA. A recent study investigated the importance of miRNA expresion regulatory in CSF and brain [114].

MiRNA molecules target mostly the 3'UTR in diverse mechanisms such as mRNA degradation or protein synthesis inhibition [115]. The polymorphisms in the 3'UTR of the binding sites of the miRNAs, change the affinity of these molecules to the target [116]. The miRNA of target genes seem to be up regulated as the genes are down regulated. There is an inverse correlation between miRNA and mRNA and protein. miRNA dysregulation can be associated with the pathways involved in the mechanisms of neurodegeneration. Hebert et al. study from 2008 screened 392 miRNAs in the brains of AD patients and controls [117]. Thirteen miRNA molecules were identified to be downregulated in patients. Seven out of 13 miRNAs were complementary to the 3'UTR of BACE, APP, and PSEN1 [117]. The comparative analysis of 60 miRNAs in brain and CSF did not correlate. It was suggested that the CSF miRNA is derived from the T-lymphocytes. It is of paramount importance to translate these findings into the context of peripheral blood. Understanding the role of miRNA and miRNA in the pathogenesis of AD and their pattern of expression in peripheral blood cells, can be a challenge of the future therapeutic measures for Alzheimer's.

4.3. Proteins

The pathogenesis of neurological disorders starts decades before the clinical manifestation of the disease. The current diagnosis of MCI and AD depends on a battery of clinical and neuropsychological tests, with close to no effective method for the early diagnosis. The notion of relative separation of blood from the central nervous system (CNS) through the blood-brain barrier has dominated the field of neurodegenerative disease. The accumulation effect of the pathological lesions during time, up to the onset of the symptoms, is involved in numerous known and yet to be discovered pathways. The recent findings have revealed a relationship between the brain and the immune system which is reflected in the physiological processes in the body [118]. The study of circulating proteins in the body fluids such as blood, urine and CSF can increase the accuracy of diagnosis and prognosis of the disease. CSF is probably the most informative resource for the study of neurodegenerative diseases but the invasive procedure, through which the CSF is extracted, makes it impractical for the elderly patients. Blood however, contains cells, proteins, lipids and metabolites which reflect the physiological and pathological changes in the body. Blood and CSF have a dynamic connection making blood a good source for biomarkers of neurodegenerative disorders [119]. The process of progression from normal cognition to MCI and consequently to AD is a complicated process with a number of mechanisms, hypothesized to be involved. To identify the biomarkers which are predictive of the progression of the disease, specifically at the early stages, different mechanisms need to be considered. The biomarkers, regardless of their involvement in pathology, inflammation, oxidative stress or vascular physiology, need to 1) be highly sensitive to be used as a diagnostic test, 2) reflect the pathophysiology of organic changes in AD, 3) discriminate between AD and other types of dementia, and 4) be practical for clinical use. The biomarkers also need to be confirmed in independent studies to be able to qualify for diagnostic purposes. The current results of novel blood biomarker discoveries have been barely replicated in independent studies, probably due to the population diversity, diurnal fluctuation of physiological markers, and collection, preservation, processing, and experimental methodologies which have not been standardized. Currently, the proteomic methods involved in the biomarker identification are used in combination with the protein databases, to verify those previously identified and to discover novel pathways. The current consensus criteria proposed by the national Institute on Aging (NIA) [120] has set the standards for the reliable AD biomarkers. The following section reviews the protein biomarkers based on the pathway involved in the pathogenesis.

5. INFLAMMATORY MARKERS

Apart from the classical pathological features of AD, amyloid plaques and neurofibrillary tangles, inflammatory processes have been manifested in the brains of AD patients [121, 122]. The presence of microglia and astrocytes, the complement system and inflammatory markers such as cytokines and chemokines in AD brain, imply to the chronic inflammation in the neuropathology of the brain [123]. Concurrent conditions such as trauma [124], metabolic disorders [125], autoimmune disorders [126] and aging associated inflammation, that promote inflammatory response, such as IL-6, TNF-α, IL-1β make it difficult to interpret elevated inflammatory markers. According to the Aβ hypothesis [5], brain amyloidosis is the major cause of neurodegeneration in AD. It has been further hypothesized that extracellular insult to the neurons could trigger the production of inflammatory cytokines by astrocytes and microglia [123]. An interplay between the Aβ neurotoxicity and cytokine production in neurodegenerative conditions has been manifested through the reports that cytokines secreted by neurons, microglial cells and astrocytes, may induce production of Aβ [87], and the report showing that Aβ can induce the expression of IL-1β, TNF-α, and IL-6 in astrocytes and microglial cultures [88]. The blood-brain barrier limits the molecular exchange between the CNS and blood. A few studies compared the correlation between the levels of cytokines and chemokines in the CSF and plasma. The possible pathways involved in this molecular exchanged have been studied by Quan and Herkenham [127]. The correlation between the peripheral cytokines and chemokines in AD needs to be replicated in order to be established as routine clinical testing for AD.

Interleukin-1 (IL-1) has been shown to be associated with the expression of Aβ precursor protein and senile plaque production. Most studies that investigated the serum levels of IL-1 did not detect any difference between AD subjects and controls. One study [128] showed elevated...
levels of IL-1β in AD patients compared to controls. This correlation, however, could be manifested in a subset of the patients. These results could be due to the methodological limitations, as the serum levels of IL-1 are below the sensitivity of ELISA. Larger samples sizes and more sensitive methods are necessary for conclusive results.

**Interleukin-4 (IL-4)** is an anti-inflammatory cytokine expressed in normal brains and has been shown to be increased in response to regulate microglial response to Aβ and play a role in inflammatory response of pathology surrounding senile plaques [129]. It has been suggested that the IL-4 activity counteracts IL-1 pro-inflammatory activity. In the mononuclear cells of AD patients treated with acetycholinesterase inhibitor, expression levels of IL-4 are increased and inversely, the IL-1β levels are decreased [130].

**Interleukin-6 (IL-6)** is a pro-inflammatory cytokine which is found to be considerably increased in AD [120], though some studies found no difference in IL-6 levels of AD patients compared to the healthy controls [131]. The levels of IL-6 in serum and CSF of AD patients are correlated [132].

**Interleukin-10 (IL-10)** is an anti-inflammatory cytokine that may play a role in reducing inflammation in AD. It has been reported that level of IL-10 is elevated in the serum of patients with dementia but these levels do not discriminate between different types of dementia [133]. One of the mechanisms attributed to the role of IL-10 in reducing inflammation in AD is suppression of pro-inflammatory cytokines. The decrease of IL-10 in PBMC of patients with AD has been suggested to be correlated with the Aβ (1-40) production [134]. The IL-10 levels do not correlate with the severity of the disease.

**Interleukin-12 (IL-12)** is a heterodimeric cytokine, produced by dendritic cells, monocytes, and microglia, in the presence of immune signals [135]. IL-12 increases production of pro-inflammatory cytokines. Some studies found no changes in the levels of IL-12 in the serum of patients with AD and FTD compared to controls [136], while other studies found a correlation between the plasma levels of IL-12 and severity of AD [137].

**Interleukin-16 (IL-16)** is a growth factor that stimulates production of inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Motta et al. showed that IL-16 levels depend on the severity of the AD [137]. IL-16 was elevated in the early stages of the disease, but reduced in the severe AD.

**Interleukin-18 (IL-18)** is a pro-inflammatory cytokine produced in the brain. In a study, no difference was observed in the level of IL-18, between the AD patients and controls, although an increase in production of IL-18 was observed in stimulated mononuclear cells of AD patients [138]. This study also considers a significant correlation between IL-18 production and cognitive decline. Motta et al. found higher levels of IL-18 in mild and moderate AD and lower levels in severe AD [137].

**Tumor Necrosis Factor-α (TNF-α)** is a nonspecific factor for development of disorders such as dementias and depression. In AD, TNF-α is produced by activated microglia in response to Aβ (1-40) and Aβ (1-42) as well as oxidative stress. The results of different studies with regards to the levels of TNF-α in AD patients and controls are unequivocal. Some studies report lower levels of TNF-α in demented patients [139, 140]. Also patients with mild to moderate AD have lower TNF-α levels than severe AD. This indicates that TNF-α could possibly be used as a biomarker for the staging of the disease. In contrast, some studies report higher levels of TNF-α in patients with AD [141, 142] whereas some studies report no significant difference between TNF-α levels in patients and controls [143].

**Transforming Growth Factor-β (TGF-β)** is a cytokine involved in numerous immunological processes including cellular immune response, cell growth, and inflammation. TGF-β is a pleiotropic cytokine with an unclear role in neurodegeneration. Some studies suggest that TGF-β stimulates astrocytes to generate Aβ (1-40) and Aβ (1-42), while other studies suggest that TGF-β is involved in activation of microglia and clearance of Aβ. Similar to the results of other cytokines, Motta et al. suggest an increased level of TGF-β in plasma and serum of AD patients with the highest level in mild to moderate AD and lower levels in severe AD [137].

**Monocyte Chemotactic Protein (MCP)** is a member of CC chemokine family, small cytokines mediating chemoattraction between cells that has two adjacent cytosines near their amino terminus. Microglial cells produce MCP and stimulate astrocytes to degrade Aβ peptides. The plasma levels of MCP have been suggested to be an indicator of inflammatory processes of AD [144]. MCP has also been shown to be an indicator of age [145].

**Interleukin-8 (IL-8)** is a microglia-derived chemokine expressed in response to pro-inflammatory signals including Aβ, leading to recruiting the microglia to the pathological areas of AD brain. No significant role for IL-8 has been found, either in the diagnosis or in the classification of AD [146].

### 6. OXIDATIVE STRESS MARKERS

Growing evidence suggests that oxidative damage could be important in pathogenesis of AD. Although AD is a disease of central nervous system, peripheral manifestations have been observed in patients with MCI and AD. In the process of neurodegeneration, a variety of macromolecules seem to be damaged [147]. There are two hypotheses for peripheral oxidative stress leading to neurodegeneration. One suggests that oxidative stress initiates within the periphery resulting in the reduction of CNS antioxidants and ultimately leading to neurodegeneration [148]. The second hypothesis states that oxidative stress begins in the CNS and the byproducts of oxidative damage are formed and transferred to periphery [148]. The markers of oxidative damage of lipid peroxidation, protein carbonyl and DNA oxidation have been reported in the AD brain [149]. Increased DNA damage due to oxidative stress has been demonstrated to be associated with apoptosis in lymphocytes from AD and MCI patients [150]. Study of peripheral oxidized proteins in plasma may be useful biomarkers for AD and MCI. ELISA studies show lower protein carbonyls in serum of the AD patients but not in CSF and plasma [151]. Isoprostane is one of the products of lipid peroxidation, resulted from free-radical mediated peroxidation of poly-unsaturated fatty acids [152]. Although isoprostane is not neurotoxic, levels of F2-isoprostane (F2-
IsoP) were shown to be increased in AD patients and significantly declined after treatment with anti-oxidants [153]. Pratico et al. found elevated levels of F2-IsoPs in plasma, CSF and urine of patients with MCI and AD [154, 155]. As the results from different studies with regards to the correlation between plasma levels of isoprostane and progression of dementia are not equivocal, and the precision of laboratory methods required, are beyond capacity of clinical diagnostic procedures, isoprostane is not currently used as a diagnostic marker. Gatta et al. showed increased expression of mRNA of proapoptotic protein Bax in AD and MCI patients, whereas mRNA levels of antiapoptotic Bcl2 showed no change [156]. The mRNA of Sod1 was found to be significantly increased in the AD and MCI patients compared to normal controls and PD subjects [157]. Molecular changes in PBMCs due to oxidative stress are sensitive biomarkers which can be utilized for early detection and follow up on intervention efficiency in AD.

7. MICROVASCULAR MARKERS

Vascular changes have been observed in AD brains, but have been considered as co-pathology and an event downstream of AD pathogenesis [158]. Cerebrovascular pathology is increasingly being recognized as AD specific pathology, contributing to the amyloid pathology and cognitive decline [159]. Large population based Rotterdam study showed association of atherosclerosis as a risk factor for AD and vascular dementia [160]. Brain studies have shown Aβ (1-42) deposition within microvascular system [161]. Since vascular pathology precedes clinical onset of AD [162, 163], measuring the cerebral microvascular damage through the biomarkers, could be a sensitive tool for early detection of AD [158].

Vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) regulate transcapillary permeability and their levels are regulated by cytokines and have been linked to atherogenesis [164]. Selectins, another class of adhesion molecules, have been associated with microvascular damage [164]. Increased plasma levels of E-selectin, together with elevated levels of VCAM and ICAM, have been observed to be associated with risk for diabetes, which in turn, is a risk factor for cardiovascular disease [165]. The increased levels of VCAM-1 in AD patients were not associated with abnormal levels of E-selectin [166].

ET-1 is a member of endothelins, is a vasoconstrictor and has been implicated in the development of hypertension and myocardial infarction. ET-1 can be derived from peripheral and central endothelial and vascular smooth muscle cells, as well as astrocytes and neurons. The arterial natriuretic peptide (ANP) is a vasodilator, found in neurons and astrocytes in hypothalamus. Adreno-medullin (AND) is a potent vasodilator assuring blood supply to the organs [158]. Due to the short half-life and rapid turnover of these molecules in blood, assays have been developed to measure the inactive surrogates C-terminal endothelin-1 precursor fragment (CT-proET-1), midregional pro-adrenomedullin (MR-proADM) and midregional pro-atrial natriuretic peptide (MR-proANP) [167-169]. The study showed that the levels of vasodilators MR-proADM and MR-proANP were increased but the vasoconstrictor CT-proET-1 was decreased in subjects with AD. The vasodilator MR-proANP in blood was the strongest indicator of AD classification [170]. The concentration of MR-proANP predicted conversion of MCI to AD in a clinical follow up [171].

Sphingomyelins (SM) belong to the group of Sphingolipids and affect permeability of the cell membrane. Through the hydrolysis of SM by sphingomyelinas or de novo synthesis, are created. Ceramides are pro-apoptotic and pro-atherogenic molecules. The cascade of oxidative stress, involving ceramide, causes damage to astrocytes and endothelial cells [172] which in turn leads to leaky blood-brain barrier that may attract macrophages and neutrophiles, promoting atherogenesis [158]. Low serum levels of ceramides are associated with memory impairment, and high levels predict future impairment [173]. This might indicate involvement of ceramide in early stages of the disease.

8. PATHOLOGICAL MARKERS

Extracellular amyloid senile plaques and intracellular neurofibrillary tangles are pathological hallmarks of AD brain [174]. Since the levels of total and phosphorylated Tau in plasma are below the detection levels [175], Aβ has been the target of blood biomarkers in MCI and AD. Amyloid precursor protein (APP) is expressed in all tissues and its proteolytic cleavage by β-secretase leads to release of ectodomain. Subsequent cleavage by γ-secretase releases Aβ (38-43) [176]. As Aβ 42 is the main component of senile plaques in AD, most of studies focused on measuring the levels of Aβ 42 and other species of Aβ in CSF. The studies show that the amount of Aβ is not well correlated with the diagnosis of AD [177, 178]. Aβ (1-40) and Aβ (1-42) are the main components of plaques and in the course of plaque formation; Aβ (1-42) is first deposited. [179]. The factors affecting the metabolism of Aβ in plasma include AD-pathology, age, cerebrovascular disease and liver catabolism and renal excretion [180]. Aβ is generated in the CNS, transported into the peripheral blood through the blood-brain barrier [181], or secreted by platelets in blood [182]. In diseases such as cerebrovascular disease [183] such as ischemic stroke [184] or aging [185] levels of plasma Aβ are elevated. There are conflicting results reported in the literature with regards to plasma Aβ levels and reliability of Aβ as a marker to discriminate between AD and controls [186, 187]. Recent studies implicate slight decrease of Aβ (1-42), but Aβ (1-40) is stable [185]. Survey of the literature shows that there is no definite statement on whether plasma Aβ reflects the levels of Aβ in CNS and/or progression of the disease. Some studies have considered ratio of Aβ (1-42)/Aβ (1-40) as a risk factor and not the absolute measure [185, 188-191]. While the majority of literature hints to a negative correlation between the progression of AD and the levels of Aβ antibody [192, 193], some recent studies find elevated levels of Aβ antibody in serum of AD patients, before and after antigen/antibody dissociation [194]. Longitudinal studies with large number of patients need to be studied and screened with standardized methods to effectively assess the role of Aβ in plasma.
9. METABOLIC MARKERS

ApoE protein is a component of very low-density lipoproteins (VLDL), and is involved in cholesterol transport in CNS and in periphery. ApoE has three common isoforms, encoded by three genetic variants of the gene ApoE 2, ApoE 3, and ApoE 4 [195]. ApoE 4 allele is a risk factor for sporadic AD [196, 197] and may impair memory function [198]. ApoE is also the only established AD risk factor, increasing the risk of memory impairment in patients with AD or MCI. The negative effect of ApoE 4 is related to the cholesterol metabolism, through the increase in low density cholesterol [199]. The other metabolic risk factors for AD and dementias in general, include total cholesterol (TC), low density lipoprotein cholesterol (LDL), and lipoprotein A [122]. ApoE levels however, have not been shown to be a sensitive measure for the differentiation between the dementia or being a predictive tool for progression of MCI to AD.

Homocystein (Hcy) is a sulfur containing amino acid derived from methionine, and has been shown to be a risk factor for cardiovascular disease, cognitive impairment and AD [200]. Within the normal range, the higher levels of total Homocystein (tHcy) for the elderly AD patients are associated with higher rate of cognitive decline [201]. A cohort study showed that individuals who developed AD from cognitively normal had higher Hcy levels compared to those who developed MCI [188]. As tHcy levels are good indicators of cognitive decline, they seem to be good predictive biomarkers, regardless of possible causal effect. Interestingly, if there is a causal relationship, treatment of high Hcy with folic acid and vitamin B-12, reduces tHcy and may improve the rate of cognitive decline [202].

CONCLUSION

The key to the successful development and utilization of blood biomarkers for the prognosis and differential diagnosis of spectrum of mild cognitive impairment to Alzheimer’s disease lies in the integration of results from mRNA, miRNA and protein levels in blood cells and plasma. These results need to be combined with the DNA screening results to develop a wholesome set of diagnostic biomarkers for Alzheimer’s disease. The majority of the studies in the database have investigated the biomarkers in the brain or CSF. A novel set of studies are required to replicate the biomarkers in the blood and to identify new blood biomarkers for Alzheimer’s disease. Identification of useful biomarkers progresses hand in hand with the new genetic discoveries in AD. As the novel candidate genes or loci are being discovered for the disease, these candidates need to be tested for the expression in blood to identify the probable role in the pathogenic pathway.

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