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Current Alzheimer Research, 2019, *16*, 518-528

REVIEW ARTICLE

Biomarkers for Alzheimer's Disease

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ARTICLE HISTORY

Received: December 04, 2018
Revised: April 08, 2019
Accepted: April 25, 2019

DOI:
10.2174/1567205016666190517121140

Abstract: Alzheimer´s disease (AD) and related forms of dementia are increasingly affecting the aging population throughout the world, at an alarming rate. The World Alzheimer´s Report indicates a prevalence of 46.8 million people affected by AD worldwide. As population ages, this number is projected to triple by 2050 unless effective interventions are developed and implemented. Urgent efforts are required for an early detection of this disease. The ultimate goal is the identification of viable targets for the development of molecular markers and validation of their use for early diagnosis of AD that may improve treatment and the disease outcome in patients. The diagnosis of AD has been difficult to resolve since approaches for early and accurate detection and follow-up of AD patients at the clinical level have been reported only recently. Some proposed AD biomarkers include the detection of pathophysiological processes in the brain *in vivo* with new imaging techniques and novel PET ligands, and the determination of pathogenic proteins in cerebrospinal fluid showing anomalous levels of hyperphosphorylated tau and low Aβ peptide. These biomarkers have been increasingly accepted by AD diagnostic criteria and are important tools for the design of clinical trials, but difficulties in accessibility to costly and invasive procedures have not been completely addressed in clinical settings. New biomarkers are currently being developed to allow determinations of multiple pathological processes including neuroinflammation, synaptic dysfunction, metabolic impairment, protein aggregation and neurodegeneration. Highly specific and sensitive blood biomarkers, using less-invasive procedures to detect AD, are derived from the discoveries of peripheric tau oligomers and amyloid variants in human plasma and platelets. We have also developed a blood tau biomarker that correlates with a cognitive decline and also with neuroimaging determinations of brain atrophy.

Keywords: Alzheimer's disease, molecular biomarkers, blood markers, CSF markers, neuroimaging, amyloid β and tau protein targets.

1. INTRODUCTION

1.1. Alzheimer's Disease

Alzheimer's disease (AD) like other neurodegenerative disorders is a major puzzle for modern medicine. In this context, it is critical to detect the presence of the disease based on reliable and quantitative diagnosis approaches. The discovery by Alois Alzheimer of Neurofibrillary Tangles (NFTs) in the brains of patients with a neurodegenerative disorder named after him AD, provided a pivotal impetus for the study of molecular substrates [1]. Only in the decade of 80s, we began to understand the biochemical processes responsible for neuropathological changes. The major components of senile plaques were observed to be aggregated forms of Amyloid-β peptide (Aβ), while NFTs were composed of paired helical filaments (PHFs), mainly formed by the self-assembly of hyperphosphorylated forms of tau [2]. AD is a multifactorial disorder in which protein alterations, oxidative stress, neuroinflammation, innate immune deregulation, impairment of neuronal-glial communication, and neurotoxic agents appear as major factors triggering neuronal degeneration. Although diverse, these factors induce deleterious signaling through different sets of neuronal receptors that finally lead to amyloidogenic processes of amyloid-β precursor protein (AβPP) to generate Aβ peptide, and to the hyperphosphorylation of tau protein [3]. This raises the question as to precisely what triggers the pathologic protein post-translational processing and aggregation. Currently, it is not clear what is the role of Aβ peptide under physiological conditions, but AβPP processing and Aβ production appear as a neuroinflammatory response [4]. On the other hand, structural studies together with the signaling cascades in neurodegeneration, suggest that tau hyperphosphorylation constitutes a final common pathway in the pathogenesis of AD. This means, that different signaling cascades lead to final phosphorylation of tau [5]. Tau is the major component of Micro-

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1875-5828/19 $58.00+.00 © 2019 Bentham Science Publishers

Current Alzheimer Research

tubule-Associated Proteins (MAPs) in axons, and plays critical roles in stabilizing microtubules and inducing its own assembly [6].

The determination of peripheral markers of AD pathology can help us understand the pathophysiology of neurodegeneration in AD. Besides their relevance for patients, the use of biomarkers to identify individuals with AD before clinical symptoms is critical for the development of potential drugs for early intervention. Projections for year 2020 indicate that over 50 million people will have AD if no cure or an effective treatment is found within the next few years. Around 6 million people suffer from AD in the U.S.A. (World Alzheimer´s Report, 2018). In fact, the prevalence of AD is 3% of people age 65-74, 17% of people age 75-84 and 32% of people 85 or older [7] a matter of great concern, considering the growth rate in the elderly population. Innovative approaches that rely on clinically relevant quantitative molecular markers of what is occurring in the brain are necessary. It is not surprising that many proposed markers have not helped to tackle the key matter of early diagnosis and treatment and resulted in much confusion instead. Promising results have been obtained in the search for markers based on the amyloidogenic mechanisms - *i.e*., Aβ and AβPP - and markers of neurodegeneration like tau protein. After an in depth study on the Proteomic Pattern of Aβ and tau, in the cerebrospinal fluid (CSF) of patients with mild cognitive impairment (MCI) and with AD, they have been validated as core biomarkers of AD pathophysiology [8-11].

Worldwide efforts to establish a bridge between basic research discoveries and the clinical applications are critical at the present stage of research on neurodegenerative disorders. In this context, efforts have been directed to find links between immunological, biochemical and genetic alterations in human populations with neurodegenerative disorders including AD, tauopathies, frontotemporal disease of chromosome 17 Frontotemporal dementia with parkinsonism-17 (FTDP-17), Parkinson's disease and amyotrophic lateral sclerosis [12, 13]. Several hypotheses have been postulated on the pathogenesis of AD, studies that provide useful ideas in order to establish reliable markers for AD. The amyloid hypothesis [14], accepted for many years that Aβ was solely responsible for AD, now has been revised considering the facts that senile plaques of the amyloid are only one of the pathological changes in the brains of AD patients, and may not be involved in the initial phases of the pathogenesis of the disease. The role of neuroimmunological and neurovascular alterations, the importance of synaptic dysfunction and the hypothesis of prions mechanisms in Aβ and tau misfolding [15], constituted solid basis for experimental evidence [16]. The pathological role of tau has been supported by the observations that all the alterations in the signaling cascades of AD have downstream hyperphosphorylations as a common feature and that tau is a possible target for treatment of AD [17]. In this context, we also demonstrated that Aβ can trigger independent mechanisms that lead to protein kinase activation and phosphorylation of tau [18]. The idea that alterations in the immunomodulation are critical for AD pathogenesis provides an integrative view on this brain disorder, considering that converging research lines revealed the involvement of inflammatory processes in AD. The "Damage Signal Hypothesis" as a unifying scheme in the release of endogenous damage/alarm signals, in response to accumulated cell distress (Aβ, dyslipidemia, vascular insults, head injury, oxidative stress, iron overload, folate deficiency), is the earliest triggering event in AD, leading to activation of innate immunity and the inflammatory cascade [12, 19]. Thus, the delicate equilibrium between neuroprotection and neuronal degeneration is shifted toward the neurodegenerative phenotype upon the action of several risk factors that trigger innate damage that activates microglia and the release of TNF-α, IL-6 [20], and some trophic factors [21]. In the neuroimmunomodulation theory, we integrate different risk factors with microglial activation, the resulting neuronal alterations and tau hyperphosphorylation [22]. Understanding of this conceptual framework appears to be essential to analyze the rationale and clinical significance of the different molecular markers, the focus of this review article.

2. THE SEARCH FOREARLY DIAGNOSIS TOOLS FOR AD

For decades, the neuropathologists indicated that the final diagnosis of AD depends on demonstration of histological lesions in *postmortem* brains. However, the development of neuroimaging and molecular markers, together with clinical evaluation, provide reliable information for an accurate diagnosis of AD. In this context, it is critical to search for valuable biomarkers that improve not only the specificity and sensitivity of a diagnosis based on clinical grounds, but that permits the presently unrealized goal of early diagnosing AD well before the clinical evidence. Over the past 40 years, there has been an increase on research for biomarkers in order to detect AD. All of them have contributed to our knowledge on AD, but unfortunately, only a few of them have shown potential clinical applications. Detection of Aβ, the most important component of senile plaques has long been considered as a reliable biomarker of AD pathophysiological processes. The same is true for tau protein, the protein that aggregates to form NFTs [2]. Tau has been considered as a main marker of neurodegeneration, since this intracellular protein is liberated to the extracellular space after neuronal death. Detection of senile plaques and NTFs in brain can only be achieved *in vivo* with specific radiotracers as will be analyzed later; on the other hand, tau and Aβ can be detected in CSF easily with relatively simple methods like ELISA with specific antibodies [23]. The most reliable biochemical method being used for accurate detection of AD is based on an analysis of the CSF for measurements of Aβ and tau, but this method is highly invasive for ambulatory clinical attention, because of the rigorous requirement for lumbar puncture [9, 10, 24]. The searches for peripheral biomarkers in amyloid protein in blood, saliva and urine have not achieved the level of acceptance of CSF analyses. The existence of a reliable and highly efficient biomarker such as Alz-Tau® is relevant as a diagnosis tool for early detection of AD, but also for the search of new drugs against the disease for early intervention, when the disease may be controlled [3]. The treatments currently available only temporarily alleviate some symptoms but do not cure the pathology.

3. THE AD MARKERS IN THE CSF

CSF is in proximity to the Central Nervous System (CNS) and so, it has been considered as a valid representa-

tive of the CNS. CSF is produced mostly at the choroid plexus, it circulates trough the ventricular system of the brain and is resorbed at arachnoid granulations. CSF is in close contact with the brain and any exchange with blood contents is closely regulated by the blood-brain barrier (BBB), so changes in any substance in CSF may be considered representative of CNS processes [25].

Senile plaques deposition is an early process in AD pathophysiology and precedes clinical symptoms. Detection of Aβ, the most important component of senile plaques has been postulated as a reliable biomarker of disease physiopathological processes. The same is true for tau protein, the protein that aggregates to form NFTs [2]. Tau has also been considered as a marker of neurodegeneration, considering that tau pathology correlates with the clinical observations in AD. Detection of senile plaques and NTFs in the brain can only be achieved *in vivo* with specific radiotracers as will be analyzed later; on the other hand tau and Aβ can be detected in CSF easily with relatively simple methods like ELISA by using specific antibodies [23]. Tau is post-translationally modified in order to regulate its function. Phosphorylation in serine and threonine residues plays an important role in AD since hyperphosphorylated forms of tau dissociate from microtubules and are prone to aggregation to form Paired Helical Filaments (PHFs) that later polymerize to generate the NFTs.

Tau can be detected in the CSF of control individuals, but the concentration of tau and particularly hyperphosphorylated forms of tau (p-tau) are increased in AD [26, 27]. Measurements of tau and phosphorylated forms of tau in CSF are increased in MCI and AD [10].

Aβ can also be found in CSF in normal individuals, but interestingly its concentration is lowered in AD, it is postulated that the cause of this reduction is the consumption of Aβ due to its aggregation process in the brain in order to form senile plaques. Aβ is produced by the proteolytic cleavage of APP by beta and gamma secretases into 38 to 43 amino acids peptides, and particular attention is being paid to Aβ with 42 amino acids residues Aβ(1-42) that is considered particularly toxic and prone to aggregate.

Aβ has a half-life of approximately 9 hours in CSF and 30 to 50% of blood Aβ is generated in the CNS, accounting for Aβ transportation throughout the BBB. Aβ turnover rates slow down with the age increase in, but Aβ 1-42 kinetics are particularly altered related to Aβ(1-38) and Aβ(1-40) in the brain amyloidosis states and this may be related to the role of Aβ(1-42) in plaque deposition [28]. In healthy individuals, Aβ(1-42) modifications in the CSF appear around the fifth decade of life, and this can be related to a large preclinical stage of the disease. This gives rise to the notion that the presence of Aβ and p-tau biomarkers in the CSF of asymptomatic subjects signals to a window of opportunity for new treatments in presymptomatic subjects [29].

In the CSF measurement, a better correlation with p-tau than Aβ(1-42) was found [30]. Since oligomerization process of Aβ and tau is also closely related to disease process, the detection of Aβ oligomers has also been postulated as a good marker of disease and methodologies for such determinations in CSF have been developed [31].

There are other N- and C- terminally truncated species of Aβ, these species are derived from primary enzymatic cleavage of APP by secretases or secondary processing of Aβ by exopeptidases. These truncated Aβ variants have increased capacity to induce full-length Aβ seeding and show increased aggregation and toxicity [32]. Considering that these truncated Aβ variants may serve as biomarkers for AD, specific assays have been developed to detect them [33]. Dunys *et al*. (2018) showed that Aβ(11-x) and Aβ(17-x) variants in the CSF may discriminate MCI subjects at very early stage -*i.e*. before other "core" biomarkers- [32], while Aβ(2-42), decreased in the CSF as an isolated marker, allowed discrimination of AD from controls, and -contrary to Aβ(1-42)- also from Frontotemporal dementia [34].

Even though, there is a clear role of Aβ measurements in the CSF for AD diagnosis, the utility of this biomarker is less clear for the disease follow up and for monitoring treatments response -that is crucial for future drugs trials [35]. Tau and p-tau determinations appear as a more reliable tool for disease monitoring since this marker predicts conversion from MCI to dementia and correlates with the cognitive status and with the burden of neocortical NFTs [10, 35, 36]. Nowadays, there is no consensus on the ideal biomarker for AD follow-up [37].

Neurofilament Light Protein (NFL) is another marker of neurodegeneration that has been evaluated in CSF as a suitable biomarker. A recent meta-analysis considered that NFL as well as the core markers of AD -*i.e*. total tau, p-tau and Aβ(1-42) - was strongly associated with AD, while other new emerging biomarkers in the CSF were moderately associated with AD [38]. The latter include neuron-specific enolase, visinin-like protein 1 and heart fatty acid binding protein, considered as markers of neuronal injury and the inflammatory glycoprotein YKL-40.

Ubiquitin has been considered a good marker of dysfunctional proteostasis since aggregates of pathological proteins and ubiquitin are detected in many neurodegenerative diseases including AD and Parkinson's disease (PD). Kandimalla *et al*. (2014) reported an increase in ubiquitin in the CSF of AD subjects with diagnostic performance comparable to core biomarkers [39]. Mass spectrometric analysis in the CSF showed a 1.2 - 1.5 fold increase in ubiquitin in AD, without demonstrating changes in PD and progressive supranuclear palsy [40].

Inflammatory mediators have been measured in the CSF as markers of neuroimmunomodulatory changes that are related to AD pathophysiology. Increased levels of proinflammatory cytokines have been described in the CSF of AD, but results are not consistent enough as a reliable biomarker. Decreased levels of interleukin (IL)-1β, tumor necrosis factor-α but not IL-6 have been reported in amnestic MCI (aMCI) subjects [41]. Neurotrophic factors have also been studied in AD. Levels of brain derived neurotrophic factor (BDNF) are decreased in the brain of AD subjects and also decreased in the CSF and blood of those subjects [42-44].

There is increasingly more acceptance of the role of CSF biomarkers in improving the diagnostic accuracy in AD, or even in estimating the elevated risk of conversion to demen-

tia in asymptomatic subjects [45]. However, nowadays, there is not a single universally accepted method for AD biomarkers assay in the CSF and there is a wide variety of markers, methodologies and cut off points that have been used to estimate pathophysiological signatures of AD [46].

A recent Cochrane database reviewed data on the value of total tau, phospho-tau and the ratio of phospho-tau to Aβ in the CSF for the diagnosis of AD, and concluded that those biomarkers have better sensitivity than specificity, but their clinical usefulness is still unclear [47].

4. PERIPHERAL MARKERS FOR ALZHEIMER´S DISEASE

In regard with AD, peripheral markers currently investigated are: i) apolipoproteína E (ApoE) polymorphism, ii) inflammatory markers, iii) microRNAs, iv) alterations of the p53 protein, v) amyloid precursor platelet peptide, vi) lipid metabolism and vii) tau protein [11, 48, 49]. The latter marker has been investigated in platelets since they carry 95% of the circulating AβPP. The relationship between AβPP variants of 130 and 110 kDa is modified in AD, and it has been postulated that there is a close correlation between this and the presence of AD. However, it is important to consider that changes in Aβ(1-40) plasma concentration are not specific for AD, even though they are closely related to age [50]. Moreover, changes in the Aβ levels in plasma samples have failed in providing a reliable tool for diagnostic biomarker [51, 52]. Despite their academic interest, these studies did not lead to a diagnostic avenue since fluctuations in the AβPP ratios varied too much among patients, and due to the lack of reliability of the method. Alterations in the ratios of AβPP isoforms in platelets in AD have been reported [23, 53]. Similar abnormalities have been found in MCI subjects [54]. The ratio of truncated forms of higher molecular weight (120-130kDa) to the lower molecular weight form is reduced in AD subjects [23, 53, 54] but not in other dementia [23]. Sensitivities and specificities for AD diagnosis were in the 80% range, based on post hoc cutoff scores [23, 54]. Reduction in the AβPP isoform ratio correlated with disease progression [23, 55]. Cholesterol reduction, simvastatin, and cholinesterase inhibitors corrected the abnormally low AβPP isoform ratios in AD cases [56-58], but statistical analyses were not stimulant for a biomarker. In summary, determinations of Aβ(1-42) levels either in CSF or in plasma have not allowed an AD certainty marker *in vivo* [10].

Mass spectrometry has been also used in the search for biomarkers, but its low throughput and the inability to measure intact large proteins are a downside for this approach [59]. New platforms for ultrasensitive immunodetection of biomarker proteins have been developed in later years. These include Single-Molecule Array (SIMOA) and the MagQu platform based on Immuno-Magnetic Reduction (IMR) [60]. These platforms have been increasingly used to detect AD biomarker proteins in blood, including Aβ(1-40) and Aβ(1-42); tau and phosphorylated forms of tau (p-tau), especially tau phosphorylated at Threonine 181 (p-tau181) [61-63]. A different approach is to detect biomarker proteins in specific blood compartments where they may be concentrated, like in the case of neuronally-derived exosomes [64]. Janelidze *et al*., (2016), in a cohort of 719 individuals divided into 3 groups (AD, subjective memory complaint and MCI) plus the control group (without cognitive impairment), analyzed the plasma levels of Aβ(1-40) and Aβ(1-42) by using the ultrasensitive immunoassay (Simoa platform). The ultrasensitivity of the Simoa assays allows the samples to be diluted, either plasma and/or serum (1:4 dilution), thus allowing to minimize these interference effects of the matrix (which affects both traditional ELISA assays), greatly improving the sensitivity and precision of the assay. In these trials, the authors determined that in AD, the plasma levels of Aβ(1-42) and Aβ(1-40) were reduced, when compared with the other study groups, because they observed a weak positive correlation between plasma and CSF levels for both Aβ(1-42) and Aβ(1-40). Additionally, the authors observed a negative correlation between plasma Aβ(1-42) and neocortical amyloid deposition (measured with PET). However, although the low plasma of Aβ(1-42) and Aβ(1-42)/Aβ(1-40) ratio was associated with the deposition of amyloid in the brain, unfortunately, these biomarkers did not show a diagnostic value in AD, although more studies are required about the usefulness of this technique [61].

On the other hand, the group of Patrick McGeer evaluated by ELISA, the salivary levels of Aβ(1-42) in both control subjects and AD, without detecting major differences. However, patients with AD showed significant differences in the secretion of Aβ42, when compared with the control group [65].

5. miRNAs AS BIOMARKERS OF AD

miRNAs are small regulatory RNAs -*i.e*. around 22 bp- that can be detected in the brain, but also in plasma, serum, blood cells, saliva and urine. These small non-coding RNAs bind to regulatory sites in mRNAs. miRNAs are dysregulated at multiple neurodegenerative diseases like PD, amyotrophic lateral sclerosis and AD.

There are many methodological disparities in studies design and data analyses related to miRNAs as AD and MCI biomarkers, and analyses of pairs of miRNAs have shown better performances in diagnostic discrimination [66]. Studies have shown that certain miRNAs related to the modulation of ceramide levels (miR-137, -181c, -9, -29a / b) in patients with AD were downregulated, which led to an increase in the expression of two subunits of Serine Palmitoyltransferase (SPT). The levels of mRNA did not differ in relation to the control subjects, which shows that these are regulated post-transcriptionally, SPT1 by miR-137/-181c and SPT2 by -9, -29a/b, postulating these miRNAs as possible markers. A higher level of ceramides in the brain has been associated with sporadic AD [67]. In addition, members of the downregulated miR-9 and miR-29 families were shown to control BACE 1 in sporadic AD patients, which induces the accumulation of Aβ [68]. Studies by Leidinger *et al*. in 2013, identified, a total of 140 unique mature miRNAs with significantly modified expression levels from blood samples of 48 patients with AD and 22 control subjects, of which, 12 miRNAs together allowed to differentiate the patients with AD of control with an accuracy of 93%, a specificity of 95% and a sensitivity of 92%. In turn, the authors indicate that these miRNAs can be used to differentiate AD from other neurodegenerative diseases [69]. Nagaraj *et al.* (2017), proposed

a panel of 6 plasma miRNAs -3 previously not reported as associated with AD- that allowed discrimination with high sensitivity -0.75 to 1- and specificity -0.78 to 1- [70].

Recently the roles of miRNAs as epigenetic regulators in the development AD have been studied in further detail [71] and their function as regulators of AD-related genes is being recognized [72] as well as their association with changes in the brain cortical metabolism [73].

Numerous investigations have provided significant evidence that the metabolism of lipids is affected in AD. These dysfunctions lead to abnormal levels of certain lipids (cholesterol and oxysterols, fatty acids, phospholipids) in the brain, CSF and plasma. Among these lipids, 24S-hydroxycholesterol has opened new therapeutic perspectives, particularly in gene therapy. The results of very long chain fatty acids suggest their potential for peroxisomal dysfunction in AD. As for the phospholipids, they could be interesting biomarkers to detect the disease in the prodromal stage [74]. With respect to ApoE, it is accepted that the presence of the ε4 allele of the gene encoding ApoE is the strongest genetic risk factor for the development of sporadic AD. It is presumed that melatonin, cortisol, homocysteine and prolactin are risk factors and may lead to biomarkers for disorders related to stress and age. Studies conducted by Zverova *et al*., (2018) analyzed the ApoE genotype and the plasma concentrations of melatonin, cortisol, homocysteine and prolactin in 85 patients with AD and 44 elderly controls. They confirmed a significant association between AD and frequencies of the allele (ε4) or genotype (ε3/ε4 or ε4/ε4) of ApoE. The levels of homocysteine in plasma and cortisol increased significantly in patients with AD compared to the control subjects, independently of the presence of comorbid depressive symptoms or the degree of dementia of the patient. A significantly lower plasma concentration of melatonin was found in patients with AD but not in controls, who were not carriers of the ApoE ε4 allele, independently of the presence of depression or severity of dementia in AD [75].

6. TAU PLATELET AS A BIOMARKER FOR AD

It is essential to know the presence of the disease based on a reliable quantitative diagnosis [3, 76]. Among the proposed peripheral blood markers for AD, plasma tau was the only one associated with AD in the metanalysis by Olsson *et al.,* (2016) [38], but there was a significant dispersion in the results of the studies. On the other hand, neither Aβ(1-40) nor Aβ(1-42) were correlated with the presence of AD. We developed an innovative detection method for AD, based on molecular biomarkers. In this context, the platelets of patients with AD were analyzed, evaluating the presence of tau in this cell type. Initial studies with antibodies that recognize total tau protein (tau5) showed the presence of this protein in immunoblots of platelet extracts obtained from peripheral blood [76]. In these studies, the presence of tau-immunoreactive bands migrating at higher molecular weights than expected under denaturing conditions is striking. These high molecular weight forms (HMWtau) appear to be oligomeric forms of tau protein, which are higher in patients with AD as compared to healthy elderly subjects (Fig. **1**). The low molecular weight species of tau (LMWtau) are considered those whose molecular weight is ≤55kDa. On

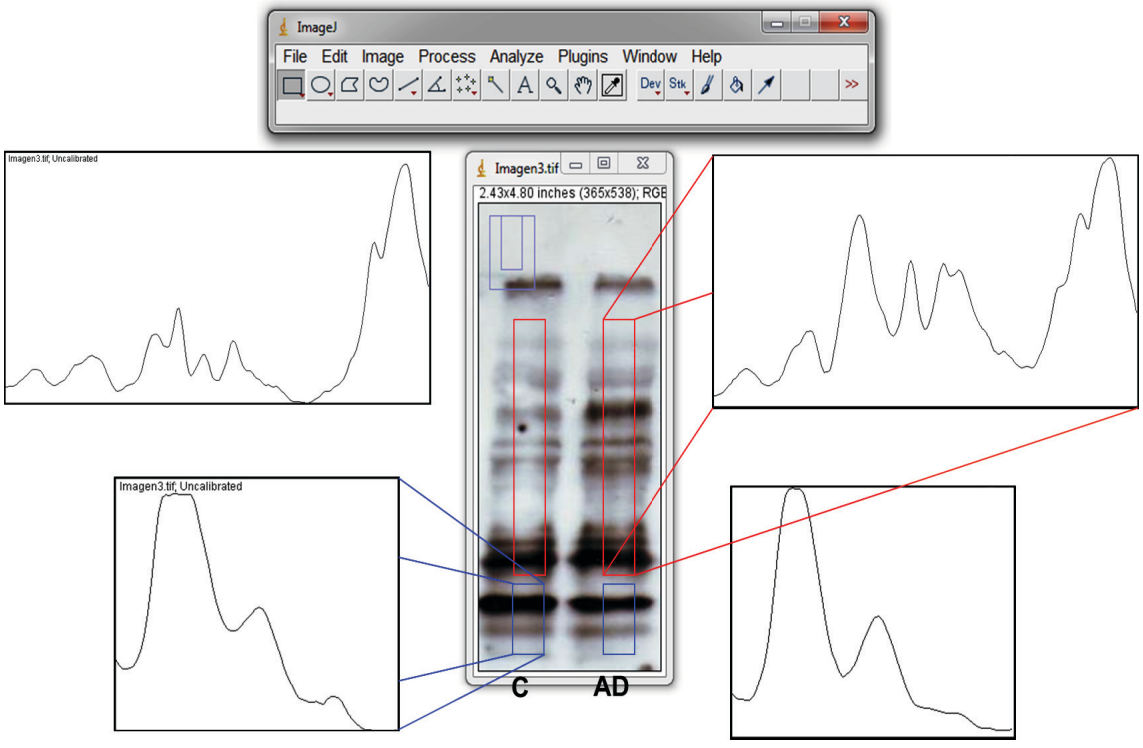

Fig. (1). Representative Immunoblots of platelet tau with tau-5 antibody. High molecular weight tau bands (about 80kDa) can be appreciated, with significantly greater immunoreactivity in patients with AD as compared with healthy subjects. Densitometric analysis of the computer program ImageJ (Wayne Rasband, National Institutes of Health, USA) for quantifying the density of bands, thus enabling the estimations of the relationship between HMWtau versus LMWtau.

the other hand, HMWtau species are considered tau oligomers between 75 and 240kDa. These findings indicate that HMW/LMW patterns of platelet's tau could be used as a biomarker of AD, in addition to being able to track the progression of the disease [3]. In turn, it has implications for the potential development of biomarkers for other neurodegenerative diseases based on tau [3, 76, 77]. Farias and collaborators (2012) [3], established a cut-off point of 1.11 for the HMWtau/HMLtau ratio using a Rho curve analysis, showing a sensitivity of 75.7% and a specificity of 73.7% to discriminate AD and control subjects. No correlation was found between the tau ratio of platelets and age in these analyses [3].

In an exploratory study using neuropsychology, neuroimaging and the relationship with HMWtau/LMWtau, Slachevsky *and coworkers* [78] confirmed that the ratio of HMWtau/LMWtau is significantly higher in patients with AD as compared to healthy subjects, and is associated with specific atrophy of brain regions such as: decreased brain volume in middle and right anterior cingulate gyri, right cerebellum, right thalamus, left frontal cortex and right parahippocampal in AD patients. A correlation was demonstrated with overall measures of cognitive performance in all the subjects [78]. In turn, authors found an association between the HMWtau/LMWtau ratio and the brain volume in the region of the mesial temporal lobe, the cingulate cortex, the pulvinar nucleus, the frontal cortex, and the cerebellum. The peripheral changes in platelet proteins (HMWtau/LMWtau) are associated with the distribution of NFT in the cerebral cortex [78]. In summary, this information indicates that the tau ratio of HMW/LMW is a valuable biomarker for AD because of the correlation between the tau ratio with brain atrophy in AD [78].

7. INFLAMMATORY MARKERS FOR AD

Neuroinflammation is involved in the onset of several neurodegenerative diseases, including AD (for further information, please refer to Morales *et al*., 2010 and 2014) [79, 80]. This immune process includes the glial cells, cytokines and the complement system. Proinflammatory mediators are produced locally within the CNS or are recruited from the peripheral circulatory system after discontinuation of the BBB. This, in turn, leads to the activation of glial cells, such as microglia and astrocytes [79-82].

The identification and validation of molecules involved in this process could be a good strategy to find new biomarkers. Inflammation is involved in the neurodegenerative process, because a constant inflammatory process leads to the chronic activation of astrocytes and microglial cells, contributing to the progression of the disease through a mechanism involving proinflammatory cytokines, activation of protein kinases, tau phosphorylation and PHF formation [82].

Changes in intracellular calcium can be observed in blood samples from patients with AD [83]. There is a decrease in IL-1β and IL-6 release in response to lipopolysaccharide (LPS) stimulation. Studies by Peskind [84] and coworkers measured levels of the s100B protein secreted by astrocytes in the CSF of patients with AD at various stages of the disease and in healthy subjects, finding significantly elevated levels of s100B in patients with mild or moderate disease, but decreased to normal levels in patients at advanced stages of the disease [84]. S100B induces the release of IL-6, which is one of the components involved in the neuroinflammatory process [85]. However, conflicting results have been obtained regarding IL-6 levels in the CSF. Some have observed no changes in IL-6 levels [86, 87], others have observed an increase in the concentration of this cytokine [88]. One study found a correlation between CSF levels of IL-6 and tau in patients with AD [85], but serum levels of IL-6 have reported either to be increased [89] or to remain normal [90].

The pro-inflammatory cytokine TNFα is an important mediator of systemic inflammation, which activates the central innate immune response [91]. Studies on the CSF of patients with AD are contradictory, since one group found elevated levels of TNFα in the CSF [92], but a second group did not [93]. On the other hand, most studies report an increase in serum levels of TNFα in patients with neurodegenerative diseases [94, 95]. In turn, several studies have examined the correlation between TNFα levels and age. It has been observed that TNFα production is significantly higher in the elderly than in younger healthy volunteers. The results showed a significant positive correlation with age [89]. Holmes and coworkers [94] demonstrated that elevated serum levels of TNFα and IL-6 were associated with an approximately 2-fold increase in the Neuropsychiatric Inventory (NPI) scores and an increase in the frequency of adverse neuropsychiatric symptoms, irrespective of delirium [94]. These studies suggested that acute and chronic systemic inflammation, associated with increased serum TNFα, results in an increased cognitive decline in AD [95].

Currently, there is no profile of inflammatory markers in CSF or plasma that can be used in the diagnosis of AD, however, none of these cytokines have been validated at the clinical level. In this context, Ray and coworkers [96] suggested a combined multivariate analysis of plasma signaling and inflammatory proteins and found 18 plasma proteins that can detect patients with AD, and predict AD in patients with MCI [96]. Another research group, Martins and coworkers [97] found that a set of 18 markers in blood had sensitivity and specificity of more than 80% to distinguish patients with AD from healthy controls [97]. This is relevant because all these results could suggest a potential tool for clinical diagnosis [97]. Thus, it will be important for future clinical applications to find markers capable of differentiating AD from other dementias[98]. Wei *et al*. (2018) proposed a panel of serum biomarkers to detect sensitivity to memantine in treatment of moderate AD. This panel includes BDNF, VEGF, IL-6 and IL-1β [99].

Decreased levels of IL-1β, TNF-α but not of IL-6 have been reported in amnestic MCI subjects. Although there is increasingly more acceptance of the role of CSF biomarkers to improve diagnostic accuracy in AD or even to estimate an elevated risk of conversion to dementia in asymptomatic subjects [45], there is a wide variety of markers, methodologies and cut off points that have been used to estimate pathophysiological signature of AD [46].

Microglial activation is an early process that also can be evaluated with specific PET ligands like 11C-PK11195. In this context, Feminella *et al*. (2016) [100] showed that mi-

croglia activation evaluated with PET correlates with brain atrophy and low glucose metabolic rate.

8. PRECLINICAL DIAGNOSIS OF ALZHEIMER'S DISEASE USING NEUROIMAGING

It is established that while Aβ peptides are formed in the course of normal brain metabolism, their overproduction and inadequate clearance in people with AD cause them to accumulate and self-aggregate in the extraneuronal neuritic plaques [101]. The abnormal accumulation of intracerebral Aβ may begin even decades before the symptoms of cognitive impairment begin to appear [102].

We know that the definitive diagnosis of AD requires both clinical features and histopathological confirmation at autopsy, and that from the first neuropathological and molecular alterations until the appearance of the symptoms, there is a continuum that can be closely monitored by certain biomarkers, highlighted for their sensitivity and precocity. Methods of structural and functional neuroimaging, which today constitute the so-called multimodal neuroimaging for AD are important. As an example, neuroimaging of amyloid with PET could allow to know the status of cerebral amyloid *in vivo* and open the possibility to confirm or invalidate the presence of AD, even though clinical applications are still limited. In addition to its diagnostic and prognostic value, there is great research and development for neuroimaging biomarker that actually indicate that a particular drug is producing a visible effect that is associated with an objective cognitive and functional improvement.

Biomarkers through neuroimaging are often used as endpoints in clinical trials for which they have been designed as methods for detecting Aβ aggregates in the brain in people with AD; however, the specific impact of amyloid aggregation on biomarker abnormalities remains elusive. Cuello *et al*. in 2017, used the transgenic rat McGill-RThy1-AβPP as a model for the selective pathology of Aβ, where it was possible to characterize longitudinal anomalies in the biomarkers commonly used in AD investigations. They proposed that abnormalities based on Aβ pathology are more evident at the level of large-scale brain network connectivity and regional measurements of cerebral metabolism than in measurements of brain atrophy or memory impairment [103].

9. STRUCTURAL NEUROIMAGING BIOMARKERS

Structural MRI. The concept of the preclinical stage of AD, with three levels of severity, has suggested the amyloid positivity plus the evidence of synaptic dysfunction and/or early neurodegeneration for AD diagnosis. Cortical thinning with loss of gray matter in a specific anatomical distribution including lateral temporal cortex, posterior cingulate and medial and lateral parietal cortex and/or hippocampal atrophy in hippocampal volume are features of AD that can be visualized with MRI [45]. In addition, the atrophy of basal forebrain appears to be a potential imaging biomarker of early detection of AD.

Both medial temporal atrophy and hippocampal atrophy have been the most common and reliable structural markers in MRI progression to AD. The three-dimensional patterns of progression of cerebral atrophy in serial MRIs were revealed to be consistent with the stages of neurofibrillary pathology, evidencing that the earliest changes in the medial temporal lobe and fusiform gyrus occur at least three years before conversion into the clinical AD [104]. Differentiating the progression of MRI atrophy from normal to AD status has progressed from manual volumetry to specific voxel-based regional analysis using more accurate volumetric software [105]. Other topographic MRI markers indicating morphological changes such as white matter hyperintensities, global ventricular or cerebral volume, and entorhinal cortex thickness would have a lower predictive value for cognitive decline, especially in the early stages of disease [106].

10. FUNCTIONAL NEUROIMAGING BIOMARKERS

PET imaging for metabolism. PET-FDG has long been used to measure rates of cerebral glucose metabolism as an indicator of neuronal activity, and metabolic reductions have been found to occur decades before the onset of AD symptoms. Specific regional patterns of hypometabolism have been found in the parietal-temporal cortex and posterior cingulate with a clinical-pathological correlation greater than 85% [107].

Longitudinal changes in the spatial pattern of cerebral glucose metabolism with PET-FDG show correlations with the cognitive decline of MCI and AD, although the method shows high sensitivity and only moderate specificity [108].

PET imaging for the amyloid. The discovery and subsequent validation of amyloid PET imaging have been able to change the clinical approach to the diagnosis of AD. The presence of amyloid burden is consistent with a pathological diagnosis of AD, and as this type of imaging technique allows the detection of moderate to severe amyloid deposition in the brain. Its sensitivity and specificity are well documented, but its accessibility has not been able to enter the routine of clinical practice and its main use has been limited to the field of research.

However, the use of diagnostic markers is essential to ensure the enrollment of subjects who effectively have the AD pathology, since diagnosis based only on the clinical evaluation has an important margin of error. Approximately 25% of clinically diagnosed individuals with mild AD turn out to be negative amyloid and this proportion increases more in MCI [109]. Also, substantial deposits of cerebral amyloid in PET are common among 10 to 30% of cognitively healthy older people, depending on age and even more significant in individuals bearing the ApoE-4 allele [110].

Nonetheless, amyloid PET has advantages, despite being a slightly invasive procedure, it provides a direct measure of amyloid status related to specific molecular mechanisms, allowing the early inclusion or exclusion of AD cases and eventually becoming a marker of therapeutic efficacy. As a counterpart, the questionable cost-benefit relation for the diagnostic purpose has been pointed out as a disadvantage. Detecting a latent state of AD in the form of asymptomatic cerebral amyloidosis could provoke a significant emotional overload in the affected subject, given the absence of an effective therapy to delay the onset of the disease or modify its course.

In response to these concerns, the appropriate criteria for amyloid PET imaging [111] recommend this imaging only in three specific clinical settings: i) patients with mild amnestic cognitive impairment or minor neurocognitive impairment, ii) patients with a suspicion of atypical AD or mixed etiology, and iii) patients with early onset of AD, *i.e.*, those younger than 65 years of age.

At present, the true impact of the amyloid imaging technique with PET for clinical practice and for possible health benefits is still unknown. As there are no disease-modifying therapies yet, the rationale that justifies the use of amyloid PET imaging in clinical practice includes improving diagnostic accuracy and medical reliability, providing counseling to patients and families about the possible clinical course and prognosis with decision-making [112].

Among the drawbacks for PET-CT uses as a diagnostic tool are: a) It exposes patients to a considerable amount of ionizing radiation, b) adverse reactions are possible, c) it is expensive, d) it requires rigorous training for clinicians who report, and e) the usefulness in predicting the development of AD in patients with MCI has not been established [113].

It is also relevant to point out clinical uses of the PET amyloid imaging, including as unacceptable screening in those who have not demonstrated an objective cognitive decline, or indicate it only based on family history or ApoE-4 status and to determine the severity of dementia. It has been estimated that this examination would be appropriate only if the expected results could alter clinical management, so that responsibility for determining patient eligibility should lie in the domains of specialist physicians experienced in evaluating and treating patients with dementia.

In the neuroimaging amyloid by PET, the radiotracer is only linked to amyloid senile plaques in the brain, *i.e*. it is specific for beta-amyloid v/s tau or other proteins and produces the signal that will be detected by the PET scanner. Images are acquired in 30 to 120 min post-injection of a radiopharmaceutical, whose history began with the Pittsburgh Compound-B

PET imaging for tau. As a diagnostic marker of AD, the image with PET and radiotracers for amyloid provides information on the extent of the loading of amyloid plaques and, in turn, with radiotracers for tau information on the loading of NTFs in the brain [114]. Unlike amyloid, tauopathies are part of a broad set of neurodegenerative diseases and not only of AD, but since tau measurements are known to be more closely related to cognitive impairment of patients, hence the relevance of its accurate and early detection and the natural interest in rapid development arise.

A growing number of radiotracers have been developed in recent years, such as AV-1451, THK5351, PI 2620 and other compounds that seem very promising candidates to reliably detect tau deposits using PET, such as benzimidazoles, lansoprazole and astemizole [115] and other second generation benzimidazoles. These derivatives have shown to bind pathological tau aggregates and cross the BBB [116]. Among them, the compound [18F] T808 is under clinical trials labeling pathological tau *in vivo* [93]. The variety and complexity of different types of tau deposits in different brain diseases have become the major challenge for the implementation of this method of examination in clinical practice. Thus, the objective is to achieve good cortical retention of the substance in specific areas, to allow differentiation of tau deposition patterns in normal subjects and in different dementia, especially in the preclinical stage, associated with this proteinopathy [117].

In summary, a valuable approach is the search of neuroimaging tools to evaluate certainty of AD based on PET imaging, but Pittsburgh compound has not yet turned into a clinical application, despite its enormous interest in the medical community. The papers by Klunk and coworkers [118] are the most cited in the medical field, however this compound has not found reliable clinical applications as an accurate diagnostic tool for AD. The reasons for that failure reside in that amyloid detection does not constitute any pathognomonic sign for AD. Improved detection approaches have reached with compounds such as [18F] Florbetapir and Florbetaben. Beyond that methodology, a novel approach has been developed on PET technology based on the detection of finely distributed tau aggregates by using benzimidazoles that have shown to interact with tau oligomers and that cross the blood-brain barrier [115].

CONCLUSION

In summary, there is a need to establish validated and reliable molecular markers and neuroimaging radiopharmaceuticals, in the clinical diagnosis of Alzheimer´s disease. This is relevant for patients in order to have quantitative tools for an improved diagnosis of AD and other tauopathies, but also for the pharmaceutical companies in the search for novel drugs to control the progress of these diseases. Fortunately, several markers have progressed from the academic level of knowledge to the clinical uses, thus facilitating the evaluation of patients with these diseases. More interesting, some of these markers provide the information that opens the possibility for early interventions in treating the diseases, considering that they can provide valuable data on their preclinical detection. Besides the early biomarkers in the CSF, that provided data on the increase in the levels of p-tau and decrease in amyloid, new peripheral markers have emerged such as the detection of aggregated Aβ levels in platelets, and our recent platelet tau biomarker that showed a correlation with neuropsychology and changes in brain atrophy. A similar situation is with with PET tracers, in which new radiopharmaceuticals that tag amyloid have been developed, with the second generation of tau tracers that have shown to be of utility for early detection of AD and tauopathies.

CONSENT FOR PUBLICATION

Not applicable.

FUNDING

Research was financed by INNOVA CORFO projects and by the ICC.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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