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Evaluation of Tau Protein Fragments as Biomarkers for Alzheimer's Disease

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RESUMO

A doença de Alzheimer (DA) constitui a forma mais comum de demência a nível mundial, representando actualmente uma epidemia global associada a grandes custos financeiros e sociais. A presença de placas amiloides e tranças neurofibrilares são caracteristicas da DA, sendo compostas por péptidos Aβ e proteína tau hiperfosforilada, respectivamente.

As alterações patológicas que ocorrem ao longo da DA podem iniciar-se décadas antes da doença se encontrar completamente estabelecida. Por este motivo, torna-se essencial existir um diagnóstico precoce numa fase assintomática da DA. Além disto, actualmente o tratamento desta patologia baseia-se apenas no controlo de sintomas, o que reforça a necessidade de se desenvolverem terapias modificadoras da doença capazes de prevenir a progressão da patologia numa fase assintomática. A presença de biomarcadores é crucial para a avaliação destas estratégias terapêuticas, incluindo na identificação e monitorização dos efeitos bioquímicos das terapias. Por outro lado, o desenvolvimento de biomarcadores capazes de detectar alterações relacionadas com a DA é fundamental para permitir um diagnóstico precoce da doença e subsequentemente o início do processo terapêutico.

Neste projecto, desenvolvemos novos imunoensaios específicios para a medição de tau *Full Length* e de fragmentos no domínio C-terminal da proteína.Os ensaios foram avaliados usando a tecnologia MSD e resultaram da combinação entre distintos anticorpos monoclonais anti-humano disponíveis *in house*. Adicionalmente, testámos ensaios previamente desenvolvidos e específicos para a medição de tau fosforilada em amostras de CSF humano, de modo a determinar o seu potencial em diferenciar indivíduos controlo de indivíduos com DA.

Apesar de dados preliminares indicarem a ausência de fragmentos de tau C-terminal em amostras de CSF humano, será interessante no futuro esclarecer esta questão e testar os mesmos ensaios em amostras adicionais. Por outro lado, os resultados obtidos mostram a ocorrência de processos de fosforilação em epítopos específicos, os quais poderão estar relacionados directamente com a patologia da DA, apesar destas observações necessitarem igualmente de confirmação.

De forma geral, os nossos resultados indicam que o desenvolvimento de novos imunoensaios baseados na proteína tau poderão ser necessários para se compreender aspectos relacionados com a dinâmica da tau em sujeitos controlo e em pacientes de Alzheimer. Do mesmo modo, a optimização e avaliação de tais ensaios poderá ser importante para compreender o perfil da proteína tau total e da sua forma fosforilada em CSF humano de indivíduos controlo, DA ou noutras tauopatias. O estudo desta dinâmica poderá culminar no desenvolvimento de novos biomarcadores para a tau total e tau fosforilada nestas patologias.

Palavras-Chave: Doença de Alzheimer, Tau, Biomarcadores, Anticorpos

ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia worldwide, and is emerging as a global epidemic, being associated with high social and financial costs. The main neuropathological hallmarks of AD are amyloid plaques and neurofibrillary tangles, respectively composed of Aβ peptides and hyperphosphorylated Tau protein.

The pathological changes that take place in AD start decades before the disease onset, reinforcing the need for an early diagnosis of the disease at asymptomatic individuals. Besides this, currently only symptomatic treatments are available. This strengthens the need to find disease-modifying therapies which may halt disease progression at asymptomatic stages. Biomarkers are of critical importance to evaluate disease-modifying approaches, including the identification and monitoring of biochemical effects of treatment. Furthermore, development of sensitive biomarkers capable of detecting AD pathology related changes is crucial to allow an early diagnosis of the disease and subsequent treatment.

In this project, we developed new tau immunoassays specific for the measurement of Full Length tau and C-terminal tau fragments. Assays were evaluated with MSD technology and are based on the combination of distinct anti-human Tau monoclonal antibodies available *in house*. Furthermore, pre-existing phospho-Tau immunoassays were tested in human CSF samples, in order to evaluate its potential for discriminating Control subjects from those with AD pathology.

Although our preliminary data shows no C-terminal tau fragments detection in human CSF samples, it will be of interest to further test the developed assays in additional samples and increase the sensitivity of the assays to confirm whether these fragments are present or not in human CSF. Regarding tau phosphorylation assays, our data shows the occurrence of tau phosphorylation at the analyzed epitopes, and preliminary data suggests that this phosphorylation might be related with AD pathology. These results still need to be confirmed.

Overall, our results indicate that the development of new tau immunoassays might give new insights regarding tau dynamics in both control and AD subjects. Evaluation and optimization of these assays will be important to understand the Tau and p-Tau profile in CSF of Control, AD or other tauopathies. Eventually this could lead to the development of novel Tau and p-Tau biomarkers for these diseases.

Keywords: Alzheimer's Disease, Tau, Biomarkers, Antibodies

TABLE OF CONTENTS

ACRONYMS

Aa – amino acid

Aβ – Amyloid β protein

$A\beta_{pE3}$ - Pyroglutamate-modified amyloid-β

AD – Alzheimer's Disease

AEP – Asparagine endopeptidase

APP – amyloid precursor protein

Apo – Apolipoprotein

ApoE/ APOE – Apolipoprotein E protein/ gene

Asp – Aspartic acid residue

BACE1 - β-site APP cleaving enzyme 1

BBB – Blood brain barrier

Camk-II - Calmodulin-dependent protein kinase II

CNS – Central nervous system

CSF – Cerebrospinal fluid

CT – C-terminal

CV – Coefficient f variation

C59 – Carboxy-terminal fragment 59

C83 – carboxy-terminal fragment 83

C99 – Carboxy-terminal fragment 99

DIAN - Dominantly Inherited Alzheimer Network

DMTs – Disease modifying therapies

ECL – Electrochemiluminescent

ELISA - Enzyme-linked Immunosorbent assay

EOAD - Early-onset AD

EpoD - Epothilone D

FDG - (18F) Fludeoxyglucose

FL – full length

fMRI – functional magnetic resonance imaging

GABA - Gamma-Aminobutyric acid

Glu – Glutamic acid residue

hrTau – Human recombinant tau

IDE – Insulin degrading enzyme

ICV – Intra cerebroventricular

ISF – Interstitial fluid

IVIg – Intravenous immunoglobulin

IWG – International Working Group

LP – Lumbar puncture

LOAD – Late-onset AD

LTP – Long term potentiation

mAbs – Monoclonal antibodies

MAP – Microtubule associated protein

MCI – Mild cognitive impairment

MMSE – Mini-mental state examination

MRI – Magnetic resonance imaging

MSD – Mesoscale Discovery

MT – Microtubule

MTBD – Microtubule binding domain

NFTs – Neurofibrillary tangles

NHS – N-hydroxysuccinimide

NIA-AAA – National Institute of Aging and Alzheimer's Disease

NTs – Neuropil threads

NMDA - N-methyl-D-aspartate receptor

NPH – Normal pressure hydrocephalus

ON – Overnight

PBS – Phosphate saline buffer

PET – Positron emission tomography

PHF – Paired helical filament

PiB – Pittsburgh compound B

PRD – Proline rich domain

PS1 – presenilin 1

PS2 – presenilin 2

P-tau/Phospho-tau – Phosphorylated tau

RP-HPLC – Reverse-phase high-performance liquid chromatography

RT – Room temperature

sAPPα – Soluble amyloid precursor protein-α

sAPPβ – Soluble amyloid precursor protein-β

Ser – Serine residue

SFs – Straight filaments

T-tau – Total tau

UPS – Ubiquitin-Proteasome system

WB – Western blot

CHAPTER 1 – INTRODUCTION

INTRODUCTION

1. ALZHEIMER'S DISEASE

Alzheimer's disease is a chronic neurodegenerative brain disorder, and accounts for 60-80% of all cases of dementia. Dementia is a general term used to describe a multiplicity of disorders that develop due to a malfunction or death of neurons, leading to a decline in mental ability (e.g. memory loss) severe enough to affect a person's daily life. In AD, for instance, the damage to brain cells will eventually interfere with memory, thinking and behavior.[1] Currently, more than 20 million people around the world are affected by this neurodegenerative disease, and about 115 million are expected to develop Alzheimer's by 2050.[2,3] Given these numbers, it is not surprising that AD represents a key concern to governments of countries with an aged population, being associated with high social and financial costs.

Extracellular accumulation of amyloid-β containing plaques and intracellular formation of neurofibrillary tangles (NFTs) composed by hyperphosphorylated Tau proteins are unique neuropathological hallmarks of AD.[4] A central mechanism underlying the formation of both amyloid plaques and tau tangles is pathogenic cerebral protein aggregation. However, numerous studies suggest that these precipitated forms can be relatively biologically inert, ascribing the cytotoxic effects of the disease to soluble oligomeric forms of Aβ and Tau, which may propagate via a "prion-like mechanism".[5] Besides these two features, synapse dysfunction and neuronal loss are also major events in AD pathology. These start in the hippocampus.[6]

At a clinical level, Alzheimer's disease is defined by a gradual decline in memory and other cognitive functions. Importantly, the pathological process is thought to begin more than 10 to 15 years before cognitive impairments become clinically manifested.[7] Despite several clinical trials investigating this disease have been conducted in the last years, there is still no effective treatment. Currently, only symptomatic treatments for AD are available, and these have generally been tested in late-stage AD patients. The above mentioned facts strengthen the necessity to find disease-modifying therapies which may slow or even halt neurodegeneration at asymptomatic stages and, consequently, clinical progression.[8]

2. *Epidemiology and Risk Factors*

AD is a complex neurodegenerative disorder, leading to a progressive deterioration of memory. Based on the time of the onset, two types of the disease are considered.[9] Familial early-onset AD (EOAD) typically develops before the age of 65 and accounts <5% of the total AD cases. The more common heterogeneous and sporadic late-onset AD (LOAD) establishes only after 65 years of age. EOAD is linked with mutations in amyloid precursor protein (APP), presenilin 1 (PS1) or presenilin 2 (PS2), which are linked to APP processing and Aβ production.[10,11] LOAD affects >95% of patients with Alzheimer's and both genetic and environmental factors seem to be associated with this type of AD, with age as a major risk factor.[11–13] Although the causes of LOAD remain largely unknown, changes in Aβ clearance seem to be preponderant in development of this late-onset pathology.[14] Environmental factors known to act as risk factors include the level of physical activity, diabetes mellitus, hypertension, dietary habits, smoking, obesity, educational status and head injury.[15,16] However, it remains inconclusive whether these risk factors are truly responsible for driving the pathogenic processes, culminating in the accumulation of Aβ plaques and tangle formation, or not.[9]

Inheritance of the apolipoprotein (apo) ε4 allele is the strongest identified genetic risk factor for developing sporadic LOAD.[17] Apolipoprotein E is a major transporter of cholesterol to neurons,[18] and ApoE isoforms differentially regulate Aβ clearance and aggregation within the brain.[19] Three polymorphic alleles can be found in the human APOE gene, ε2, ε3 and ε4. The frequency of the latest may increase up to 40% in Alzheimer's patients, while in normal conditions it's of approximately 14 percent.[18] The confirmed genes for AD are summarized in **Table 1**. Besides APOE, PSEN1, PSEN2 and APP were also confirmed to be linked to AD, as previously mentioned, and associated with autosomal dominant inheritance.[20]

Table 1| Genes associated with AD. Adapted from ***Vilatela, A. (2012)*** [20]

Gene Symbol	Protein	Inheritance	Age of onset (years)
APP	Amyloid beta (A4) precursor protein	Autosomal Dominant	40-60
PSEN1	Presenilin-1	Autosomal Dominant	30-58
PSEN2	Presenilin-2	Autosomal Dominant	45-88
APOE	Alipoprotein E	Risk Factor	40-90

3. NEUROPATHOLOGICAL HALLMARKS OF AD

The two main neuropathological AD hallmarks commonly found within the brain of AD patients are extracellular amyloid plaques composed of Aβ peptides and intracellular accumulation of hyperphosphorylated Tau proteins, which generates NFTs. These are particularly found in the medial temporal lobe and cortical areas of the brain. Loss of neurons and synapses are also key features in AD.[9,21] Many hypotheses have emerged in order to explain the development of tangles and plaques, with consequent synaptic and neuronal loss, that lead to a decline in memory and cognitive functions in AD. These include the cholinergic hypothesis, amyloid cascade hypothesis, tau hypothesis and inflammation hypothesis.[22] For the last two decades, the amyloid cascade hypothesis has dominated the field, although recent studies show that it does not account for the total complexity of AD pathophysiology.[23] Nevertheless, in this introduction the focus will be on Aβ and Tau proteins, and the hypotheses related with these two cornerstones of the disease.

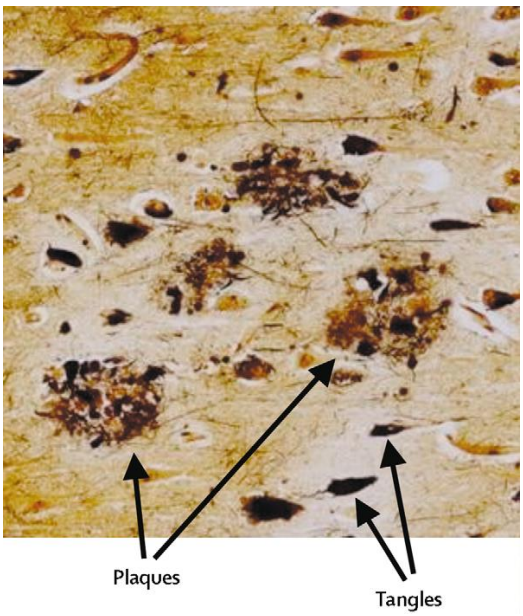

Figure 1 | Amyloid plaques and neurofibrillary tangles constitute the neuropathological Hallmarks of AD. Image shows cerebral AD cortex, with plaques of Aβ deposited extracellularly, and intracellular aggregates composed of hyprphophorylated Tau protein that form tangles. Adapted from ***Blennow, K., de Leon, M. J. & Zetterberg (2006)*** [9]

3.1. Aβ and the Amyloid Cascade Hypothesis

Amyloid plaques are composed of Aβ peptides, which arise from the proteolysis of amyloid-β precursor protein, APP.[24] APP is a transmembrane protein, encoded by a gene on chromosome 21 (in humans). Although the physiological role of APP is not entirely clear, it seems important to mediate cell-to-cell and matrix interactions.[25] APP exists in three alternatively splice isoforms, APP 695, APP 751 and APP 770. In the brain, APP 695 isoform represents the most abundant form of the amyloid precursor protein.[26]

Under normal conditions, APP is sequentially cleaved by α and γ- secretases, and this is known as the non-amyloidogenic pathway. Cleavage by α-secretase releases a soluble N-terminal fragment (sAPPα) and a C-terminal fragment (C83), which is further cleaved by γ-secretase,

generating a C-terminal fragment, p3. In this case, Aβ peptide is not generated since cleavage by α-secretase occurs within the Aβ peptide **(Figure 2a)**.[27,28] Interestingly, the p3 fragment is highly hydrophobic and its presence has been reported in diffuse amyloid plaques.[29] Alternatively, in the amyloidogenic pathway APP is cleaved by β-secretase, releasing a soluble N-terminal fragment (sAPPβ) and a longer C-terminal fragment (C99) containing the full Aβ peptide sequence. BACE1 has been identified as the major β-secretase.[29] Subsequent γ-secretase action releases the Aβ peptides, along with the C59 fragment **(Figure 2b)**.[27,28]

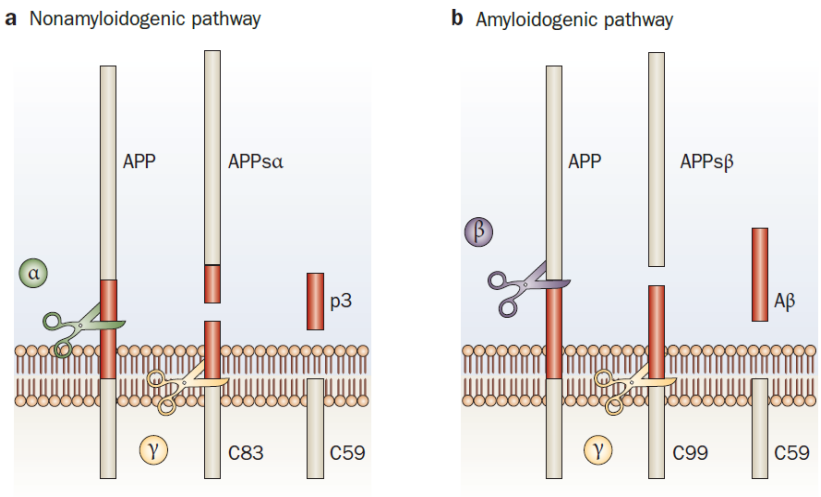

Figure 2 | The amyloid precursor protein (APP) is a transmembrane protein which is proteolysed by secretases. Under physiological conditions, APP is processed by the nonamyloidogenic pathway **(a)**, while in AD the amyloidogenic pathway is favored, leading to the formation of Aβ peptides **(b)**. Adapted from ***Strooper B., et al. (2010)*** [28]

According to the amyloid cascade hypothesis, APP is aberrantly processed by β- and γ-secretases in Alzheimer's Disease, which leads to an imbalance between production and clearance of the Aβ peptide.[30] This hypothesis proposes that Aβ peptides are able to spontaneously aggregate into soluble oligomers, which later coalesce to form insoluble fibrils containing a β-sheet conformation. These fibrils would then be deposited in diffuse senile plaques.[4] There are distinct amyloid-β species, which vary in number and sequence of amino acids. Species containing 40 ($A\beta_{1-40}$) or 42 ($A\beta_{1-42}$) amino acids are the most abundant in the brain. Despite the similarities between the different Aβ species, $A\beta_{1-42}$ is the most neurotoxic of all Aβ peptides, since it is more prone to aggregation and fibrilization, playing a pivotal role in AD pathogenesis.[31]

Aβ oligomers have been pointed out as the most toxic forms of the amyloid derivates, therefore mediating several mechanisms that contribute to the onset and development of AD.[32] For instance, it was recently shown that $A\beta_{1-42}$ are produced by neurons and its associated astrocytes,[33] and interaction of the oligomers with these cells may lead to: a) activation of pro-inflammatory cascades, b) mitochondrial dysfunction and increased oxidative stress, c)

impairment of signaling pathways, d) synaptic dysfunction, impacting synaptic plasticity processes, e) increased tau phosphorylation, f) deregulation of calcium homeostasis, and g) neuronal death.[22,32] In addition, the mentioned mechanisms might culminate in a positive feedback loop in which Aβ peptides lead to deleterious effects to neurons, and lead to further dysfunction of APP metabolism and increased production of Aβ peptides.

Besides increased Aβ production, in AD there's also a downregulation of the mechanisms promoting Aβ clearance from the brain.[32] Two proteins are important players in the clearance of Aβ, apolipoprotein E (ApoE) and insulin-degrading enzyme (IDE). The mechanisms by which these proteins promote Aβ clearance are not fully elucidated, but one hypothesis is that they can bind to the Aβ peptide, inhibiting its aggregation and promoting its clearance.[31]

An important concept in the amyloid cascade hypothesis is that NFTs formation is an event that occurs downstream from Aβ aggregation. Animal models co-expressing Aβ and tau reveal support this idea.[34,35] However, data from these models should be interpreted carefully, since overexpression of these proteins does not reflect physiological levels of expression. Thus, results might be related to the transgene expression and not necessarily reproduce the real pathological process.

3.2. Tau Protein and Structure

While plaques seem to be associated with disrupted neurite morphology, gliosis, and oxidative stress, the impact of NFTs is less known at this point.[36] Neurofibrillary tangles are composed of truncated and hyperphosphorylated tau protein.[37] Tau belongs to a group of proteins generally referred to as microtubule associated protein (MAPs). This protein is mainly expressed in neurons and largely found in axons, where it regulates microtubule (MT) polymerization and stability.[38] The MAPT human gene contains sixteen exons and yields six major isoforms of tau, which arise from alternative splicing of exons 2,3 and 10 **(Figure 3)**.[39,40] These six isoforms differ in the presence of three or four of tubulin/microtubule binding domains (MTBD) - 3R or 4R – at the C-Terminal region of the protein, and in the number of inserts containing 29 amino acids each at the N-Terminal domain of the molecule – 1N, 2N or no inserts – preceding the so called proline rich domain (PRD).[41] Amongst the several known tau isoforms, the longest isoform found so far at the human central nervous system contains 441 residues, with 85 potential phosphorylation sites **(Figure 4)**.[42,43] 71 of the putative 85 phosphorylation sites can also be phosphorylated in physiological conditions. These are mainly located in regions near the MTBD, particularly in the PRD and C-Terminal region of tau.[42]

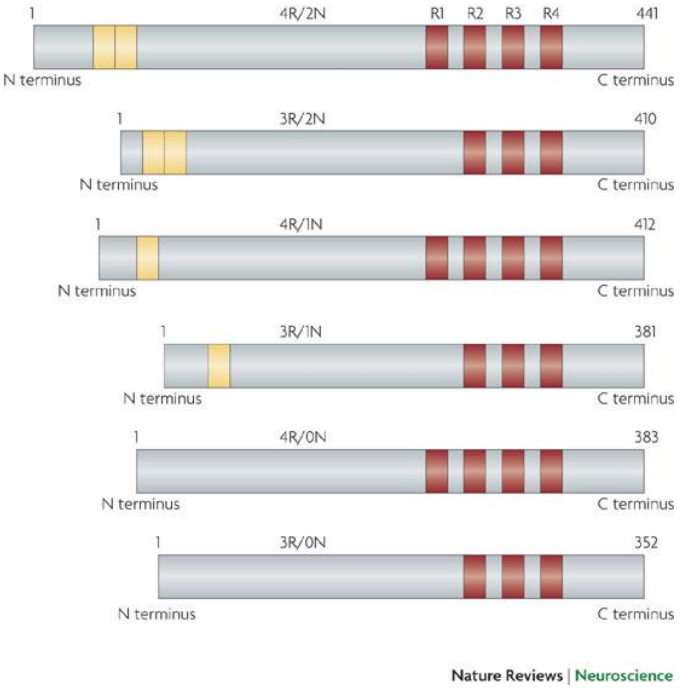

Figure 3 | Alternative splicing generates six major isoforms of Tau. Alternative splicing of tau exons 2, 3 and 10 results in six different tau isoforms. The isoforms differ in the number of repeats in microtubule-binding domains (near the C-terminal region, in red) - 3R/4R isoforms. The presence or absence of one or two highly acidic domains (in yellow) at the projection domain, (N-terminal region) also varies between different isoforms. Between the microtubule-binding domains and the projection domain, all isoforms contain a basic proline-rich region. 2N4R constitutes the longest tau isoform found in the central nervous system, containing 441 amino acids. From ***Ballatore C., et al (2007)*** [40]

Tau Pathology

While normal phosphorylation of tau regulates MT dynamics, ensuring neuronal polarity, axonal outgrowth and axonal transport,[44-46] during the course of AD tau becomes hyperphosphorylated and becomes detached from microtubules. When this happens, tau is no longer able to control MT dynamics.[47] In this pathological condition, abnormal phosphorylated tau accumulates in the somatodendritic compartment, where it displays an increased propensity to aggregate into structures called paired helical filaments (PHFs).[48] Furthermore, in these conditions Full Length (FL) tau is sequestered, as well as other MT-associated proteins, whereby hyperphosphorylation of tau seems to be a potent inducer of tau aggregation and pathology.[49] In AD, other post-translational modifications of tau may further contribute to tau aggregation and disease neuropathology. These include acetylation, truncation due to proteolytic cleavage, glycation, nitration, ubiquitination and conformational changes. Although the mechanisms by which tau becomes non-functional are still not completely understood, abnormal post-translational modifications seem to play an important role.[41]

In the next sections, we will focus on evidence concerning abnormal phosphorylation, acetylation and truncation of tau as major changes during AD pathology.

Tau hyperphosphorylation

The dominant view regarding tau pathology is that abnormal phosphorylation of this protein causes a disruption in microtubule-based cellular transport. This impairment in cellular transport interferes with trafficking of essential components towards the synapse, including mitochondria and synaptic receptors.[36,50] In 1993, there was already evidence that Tau can be phosphorylated up to fourfold more in the brain of AD patients than in matched nondemented individuals' brain.[51]

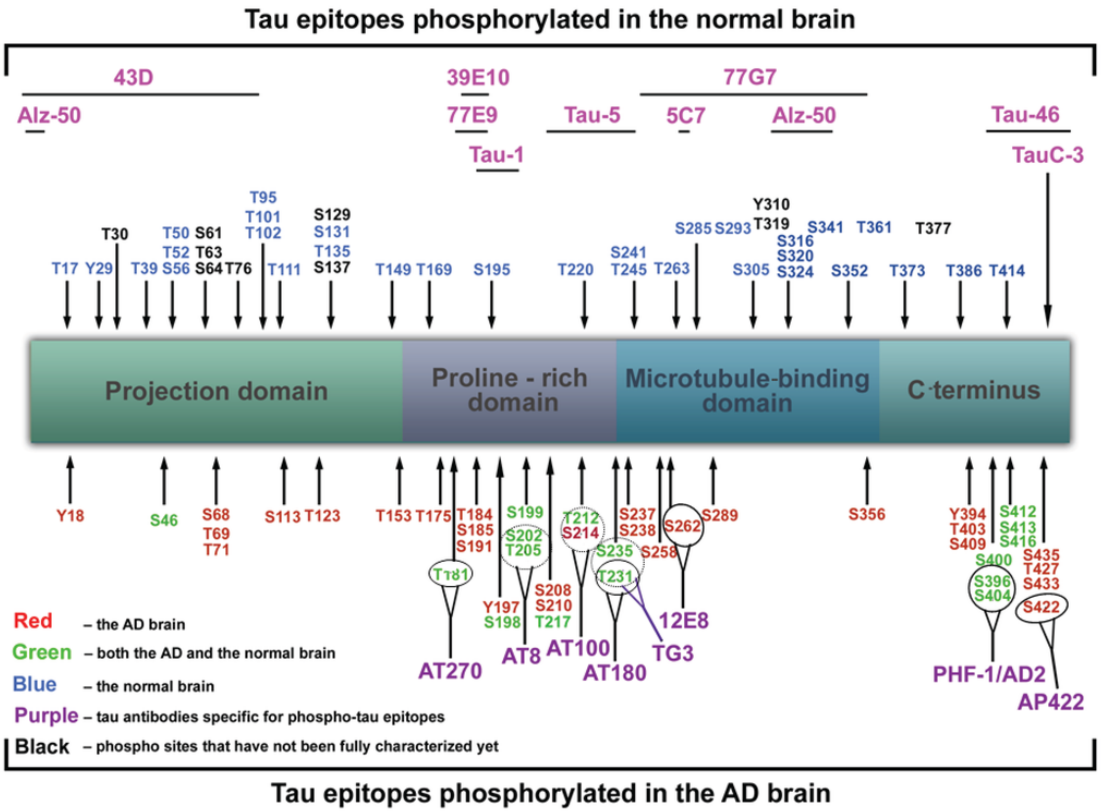

Figure 4 | **Putative phosphorylation sites on tau protein, epitopes and available tau antibodies.** Tau protein has multiple phosphorylation sites, which may or not be related with tau pathology. Recently, immunotherapy using anti-tau antibodies has raised a large interest in AD (see topic **5.**). Red color – amino acids phosphorylation in AD brain; green – phosphorylated in both normal and AD brain; blue – phosphorylated in physiological conditions; black – phosphorylated sites not fully characterized so far. Antibodies indicated in purple recognize phospho-tau epitopes; antibodies in pink color are specific for non-phosphorylated tau epitopes. Alz-50 (aa 2–10, aa 312342), 43D (aa 1–100), 77E9 (aa 185–195), 39E10 (aa 189–195), Tau-5 (aa 210–230), 5C7 (aa 267–278), Tau-1 (aa 195, 198, 199 and 202), 77G7 (aa 270–375), Tau-46 (aa 404–441), TauC-3 (tau cleaved on aa 421). From **Šimic G., *et al (2016)*** [52]

Phosphorylation-dependent anti-tau antibodies have been crucial to understand tau pathology in neurodegeneration.[53] Recently, *Hanger et al* extracted PHF-Tau from Alzheimer brain and used phospho-specific antibodies against tau and mass spectrometry to determine which sites might be phosphorylated *in vitro* by distinct kinases. The authors reported the existence of at least 39

phosphorylated sites in tau protein that can be associated with native PHF isolated from the brain of AD patients.[54] It remains unknown whether distinct mechanisms are involved in the physiological and pathological phosphorylation of tau.[48] Multiple evidences have emerged over the years pointing towards abnormally hyperphosphorylated tau as a major component of several hallmarks characteristic of AD, namely PHFs, NFTs, NTs and dystrophic neurites in the brain of AD patients.[55,56] For instance, the density of NFTs distributed in brain areas such as the hippocampus, entorhinal cortex and neocortex has been correlated with the level of dementia in AD. [57]

There is a link between tau hyperphosphorylation and tau aggregation. For instance, phosphorylation of serine and threonine residues is a major early characteristic of tau aggregation.[48] Even though tau abnormal phosphorylation seems to precede tau aggregation, whether this process is sufficient or necessary for filament assembly is unclear. In human diseases, one possibility to explain the relation between these two processes is that tau is first misfolded, rendering it a better substrate for protein kinases and a worse substrate for protein phosphatases. Thus, hyperphosphorylation of tau might result from both upregulation of tau kinase(s) and downregulation of tau phosphatase(s).[58,59] This conformational change could result in abnormal phosphorylation of tau and higher amounts of tau not bound to microtubules.

Tau aggregation

Tau is a natively unfolded protein that assembles into cross-β structure filaments through its tandem repeat domains.[60] Ultrastructurally, NFTs are mainly composed by PHFs and SFs. These structures are constituted predominantly of hyperphosphorylated tau protein.[61] In AD, these filaments might remain in the extracellular space of dead cells and give rise to ghost tangles consisted largely of the MTBD repeats of tau. Experiments have shown that tau aggregates composed of this region are able to induce neurotoxicity. [62]

Other studies suggest that oligomeric species of tau have increased toxicity over fibrillary aggregates.[63-65] A study using a specific monoclonal antibody against these oligomeric units in brains of AD patients emphasized the observation of tau dimers and oligomeric aggregates *in situ* at early stages of AD. The authors demonstrated that dimerization occurs early in the course of tau aggregation by using a light induced cross-linking technique.[66] However, this study lacks the establishment of a link between these distinct oligomeric tau forms and neurodegeneration in AD.

In another study,[67] *Tai et al* were able to isolate very small insoluble tau oligomers, corresponding to a few hundred KDa, from synaptosomes derived from AD brains, which were further associated with impairments in the ubiquitin proteasome system (UPS). Both *in vitro* and

in vivo studies support the idea that soluble tau is sufficient to mediate dysfunction and toxicity in AD. For instance, studies using mice expressing mutant tauN279K[68] have observed the existence of learning defects without observation of NFTs or insoluble tau, and also in the absence of cell death. Additional evidence supporting soluble tau mediated toxicity arises from studies reporting the rescue of tau-mediated phenotypes after tau suppression, leading to reductions in the amounts of soluble tau but not in tangle pathology.[63,69-71] Despite the growing body of evidence showing that small soluble tau oligomers represent the most toxic form of tau, it is not always clear whether the toxic soluble species of tau are composed of monomers or oligomers (or both). To address this question would be of major interest to develop strategies for treatment of AD and other tauopathies, since we do not know yet which species of tau would represent the best target for tau-based therapies.[72]

Tau truncation

Tau truncation is also a key pathological event in AD. Data from several studies suggests that tau hyperphosphorylation takes place before cleavage, and cleavage is followed by NFT formation.[73-76] This raises a possibility in which abnormal phosphorylation might be a key event that triggers the truncation and posterior pathological aggregation of tau in AD. Biochemical analysis of the PHFs structure revealed the existence of a "PHF core" composed of a fragment of tau comprising the MTBD and terminating at Glu^{391} residue.[76-78] This truncation has been demonstrated to be associated with neurofibrillary pathology in AD patients' brains.[79,80] Furthermore, several studies have shown that tau is a substrate for many proteases. A susceptible residue at Asp^{421} was reported to be cleaved by caspase 3 *in vitro*, and tau truncated at this residue was found to be a component of NFTs.[81,82] There are also studies reporting abnormal activation of both calpain 1 and calpain 2 in AD brain.[83] In 2005, *Park & Ferreira* used calpain inhibitors to comprehend the impact of proteolytic tau cleavage towards the mechanisms underlying Aβ-induced neurotoxicity. The authors observed a reduction in Aβ-induced neuronal death when calpains' action was inhibited. This study reported that activation of calpains in hippocampal neurons can be induced by pre-aggregated Aβ treatment, leading to the production of a neurotoxic 17-KDa tau fragment, tau_{45-230}.[84] In opposition, overexpression of tau_{45-230} induced neurite degeneration and cell death in neurons and non-neuronal cells, suggesting that tau truncation may be one important mediator or Aβ-induced neurotoxicity.[84] Nevertheless, it is important to take into account that in the mentioned study, tau pathology occurs as a secondary event in AD, emerging as a downstream effect of Aβ aggregation.

In a recent study by *Zhang et al*, the proteolytic cleavage of tau protein by asparagine endopeptidase (AEP) is highlighted as an important neuropathological event in AD. AEP is a lysosomal cysteine protease, and the authors showed that, during aging and in human AD brain, AEP is activated and upregulated, being able to degrade tau.[37] Interestingly, the authors have shown that tau fragmentation by AEP can be attenuated using an antibody directed against AEP, and that AEP is able to cleave tau independent of caspases or calpains. Mass spectrometry (LC-MS/MS) was also used to determine the AEP cleavage sites on tau protein, and two peptides ending at N255 and N368 were identified with this method. For instance, an anti-tau N368 antibody showed an abundancy of tau N368 immunoreactive fragments in human AD brains, conversely to what was observed in aged-matched controls. Additionally, tau N368 co-localized with thioflavin S-positive NFTs in human brains and with phosphorylated tau by immunofluorescence staining. This suggests that fragments produced by AEP cleavage may be components of NFTs *in vivo.* [37]

Tau degradation prevents its microtubule assembly function, promotes tau aggregation, and induces neurodegeneration, establishing an important link between tau proteolysis and AD neuropathology.[37] Despite these studies, many tau fragments present in AD are not well characterized, and the proteases responsible for generating these fragments are not entirely known at this point. As an example, a tau fragment of 25-35 KDa in the cerebrospinal fluid has been used as an early AD marker, although the proteases promoting its cleavage are not known at this point.[85,86]

Propagation of Tau pathology

Several studies have already demonstrated a positive correlation between NFTs burden and cognitive decline or neuronal loss in AD.[87,88] Over the years it has been established that during AD progression, NFTs spread initially along the entorhinal cortex, progressing to the hippocampal formation and association cortices. Sensory areas are only affected in late stages of the disease.[21] In recent years, cell to cell transmission was hypothesized to explain the diffusion of tau pathology.[89] According to this hypothesis, tau might spread trans-synaptically from the entorhinal cortex to the hippocampus, before severe neurodegeneration occurs.[90,91] Some studies have proposed a mechanism in which certain forms of tau may be secreted into the extracellular space, being then able to enter cells and induce further tau aggregation.[92,93] This spread of aggregated tau between neurons requires at least four steps: 1) releasing of tau seeds into the extracellular space from donor neurons, 2) tau aggregation (which might also be occurring before step 1), 3)

the uptake of seed-competent tau into receiving neurons and 4) tau seeding and putative aggregation in recipient cells and further release of seeded tau into the extracellular space.[94]

Recently, a study by *Yamada et. al* correlated neuronal activity with tau pathology, in an attempt to explain the mechanisms underlying neuronal release of extracellular tau. The hypothesis raised by the authors is that neuronal activity may regulate the release of tau from neurons, establishing a link between trans-synaptic spread of tau pathology with synaptic activity.[95] Many studies have already shown the occurrence of interneuronal tau transfer *in vitro*.[96,97] In one of these studies, Jackson *et al* observed that insoluble high-molecular weight and aggregated tau was the most efficient in seeding aggregated tau. These species were able to initiate the formation and dissemination of filamentous tau pathology *in vivo*, conversely to what was registered for monomers and oligomeric units. The authors concluded that the major seeds are composed by short tau filaments (>10 mer), and not by small oligomeric tau species (<6 mer).[96] Although this study gave new insights on tau pathology spreading, further studies are needed to understand the correlation between seed-competent tau species and neurotoxicity. Nonetheless, which tau species are seed-competent isn't completely elucidated. It will also be of interest to characterize other tau species that might play a role in tau seeding, dissemination and neurodegeneration in AD and other tauopathies.

The molecular nature of extracellular tau also remains unclear. An interesting study from *Kanmert et al* analysed three distinct neuronal models in order to characterize extracellular tau species.[98] The authors developed ELISA assays capable of distinguishing FL tau, mid-region bearing fragments, and CT fragments. They observed that although intracellular tau appears mainly as FL, the majority of extracellular tau is C-terminally truncated and lacks the MTBD, believed to play an important role in tau aggregation. Importantly, CT truncated tau was shown to be released from neurons independently of cell death. However, tau fragments containing the MTBD appear to be present in the extracellular space when cell death is observed. This study emphasizes the need to further understand whether aggregated tau species are released passively only upon cell compromise or if active processes can underlie their release from neurons. This would be of interest to target specific tau species therapeutically.[98]

4. THE ROLE OF BIOMARKERS IN AD

The pathological processes in Alzheimer's disease are thought to begin at least 10 to 20 years before cognitive impairments become clinically manifested.[99] Although several clinical trials investigating this neurodegenerative disease have been conducted in the last years, effective treatment is still lacking. Only symptomatic treatments unable to alter the course of the disease are available nowadays, and these have generally been tested in late-stage AD patients.[100] In fact, the only drugs approved by the US Food and Drug Administration (FDA) for AD treatment include four cholinesterase inhibitors and the NMDA antagonist memantine.[101] In 2007, the diagnostic criteria for AD were revised, since AD detection in asymptomatic at risk individuals remains unfeasible in clinical situations.[100,102] In December 2013, the G8 determined dementia to be a global priority, aiming to develop a cure or disease-modifying therapy by 2025.[103] The mentioned facts strengthen the necessity to find disease-modifying therapies (DMTs) and emphasize the need to develop consistent and accurate biomarkers.

By definition, a biomarker is "*a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological responses to a therapeutic intervention*".[104] There are distinct types of biomarkers, according to their purpose: diagnostic markers are helpful to guide clinical diagnosis; prognostic markers estimate the risk to develop a certain disease; and theragnostic markers are used to monitor the progression or response to therapy.[105,106] Reliable diagnostic biomarkers are also essential to facilitate an early diagnosis of AD at pre-clinical stages, and may give new insights into AD pathogenesis.[100] In addition, biomarkers play a major role in monitoring disease progression and response to therapeutical interventions. Currently, the most reliable biomarkers are measured in cerebrospinal fluid (CSF) and neuroimaging biomarkers (see topics **4.1** and **4.2**).[100] Core CSF biomarkers include amyloid-β_{1-42}, total tau and phosphorylated tau, and have shown high diagnostic accuracy but are still untrustworthy for preclinical detection. An ideal biomarker for Alzheimer's disease should have a sensitivity and specificity above 85%. Sensitivity refers to the probability of AD detection, while specificity reflects the accuracy of the biomarker in differentiating AD patients from non-diseased individuals and patients affected by other primary causes of dementia. Availability, non-invasiveness, reduced cost, and potential for repeated measurements are additional key features of a good biomarker.[100,107]

Biomarkers and AD diagnosis

The present diagnostic tests of any dementia syndrome, including AD, rely on neuropsychological evaluation and assessment of symptomatology over time. This hampers an early detection of the disease, and differentiation of AD from other types of dementia.[103,108] As a result, probable AD is only detected after the onset of the first symptoms, when neurodegeneration is already occurring.[109] A major focus in the last years has been the improvement of AD diagnosis. Consequently, diagnostic criteria have been revised in the last years. For instance, with the appearance of MRI and discovery of CSF biomarkers and amyloid PET, a new criteria was proposed by the International Working Group (IWG) in 2007, which led to a subsequent set of new criteria defined by the National Institute of Aging and Alzheimer's Association (NIA-AAA) working group.[103] An important goal of these new criteria has been to cover the full range of disease stages, acknowledging a long pre-dementia stage and culminating in most severe dementia stages.[110] This allows a refinement of AD diagnosis by defining distinct stages of the disease: at-risk state for AD (asymptomatic individuals with positive biomarkers), pre-symptomatic AD (carriers of autosomal-dominant mutations), prodromal AD or mild cognitive impairment, MCI (episodic memory loss, positive biomarkers), and AD dementia (severe episodic memory loss, positive biomarkers).[111] The most valuable application of these diagnosis criteria is the possibility to intervene at prodromal stages of the disease. In addition, they could facilitate a secondary prevention of AD at preclinical states. Likewise, biomarkers' research has helped to clarify the value of each marker in AD diagnosis.[111] For instance, the value and correlation of CSF biomarkers with pathology has largely improved. In amyloid PET, data interpretation has evolved, new ligands have been introduced, and the clinical relevance in the context of AD has been clarified. In IWG criteria, atypical presentations of the disease are also taken into account – as an example, presence of memory impairment is no longer mandatory provided that biomarker evidence is present.[103]

Although the NIA-AA diagnostic criterion shares many features with the IWG criteria, they possess conceptual differences, which will not be discussed here in depth. Essentially, both recognize the importance of biomarkers in AD diagnosis, as well as the presence of an asymptomatic biomarker-positive period. NIA-AA supports the diagnosis of AD in asymptomatic individuals, as long as Aβ accumulation is detected by biomarkers, while this is interpreted as "at risk of disease state" in IWG criteria.[103,111] Overall, the NIA-AA has the advantage of being applicable if not enough information on biomarkers is available, even though this might interfere with diagnostic accuracy and specificity. On the other hand, the major advantage of IWG criteria is consistency, which makes it more suitable for clinical trials and clinical diagnosis when biomarker

evidence is present.[111] In conclusion, validation of both diagnostic criteria is crucial in the AD field, as biomarkers play a crucial role in allowing an accurate and precise diagnosis of the disease. In the following sections, development and value of currently available biomarkers will be discussed.

4.1. CSF Biomarkers

CSF is in intimate contact with the cerebral tissue, and pathological changes within the brain are often reflected in the CSF, making this fluid an ideal source of biomarkers.[100] Core CSF biomarkers are based on the levels of Aβ and tau protein, specifically the levels of $A\beta_{1-42}$, total tau (t-tau) and phosphorylated tau (p-tau). The pattern of these three CSF core biomarkers is often referred to as the "AD signature" within the CSF.[112] However, other biomarkers of Aβ metabolism, degeneration, neuroinflammation or lipid metabolism are also emerging and may give new insights into AD pathologic mechanisms, diagnosis and treatment.

Core CSF Biomarkers (Aβ and Tau protein)

In Alzheimer's disease, APP cleavage occurs mainly through the amyloidogenic pathway, due to the action of β- and γ-secretases, and generates Aβ peptides of 17 to 42 amino acids in length, which can be detected in the CSF. The most investigated Aβ variant in the CSF of AD patients is $A\beta_{1-42}$, which is the most abundant Aβ peptide in amyloid plaques.[113] Many studies using enzyme-linked immunosorbent assay (ELISA) show that CSF $A\beta_{1-42}$ levels are reduced in AD patients, and this decrease is significant long before symptoms become clinically manifested in both familial and sporadic Alzheimer's.[114-116] The deposition of $A\beta_{1-42}$ around amyloid plaques constitutes a possible explanation for this decrease, since in these conditions the peptide would be less available to be cleared into the CSF.[100] Other Aβ truncated forms at the N- and C-terminals may be detected in the CSF. Several studies have measured the levels of Aβ oligomers, but the results are contradictory.[117]

CSF Aβ levels display high sensitivity, ranging from 78 to 100%, but the specificity in distinguishing AD patients from non-diseased controls and other pathologies is insufficient, and the values vary between 47 and 81%.[118] The fact that $A\beta_{1-42}$ levels appear reduced before the occurrence of the first clinical symptoms indicates that measurements of this Aβ peptide may facilitate the diagnosis of incipient AD in patients with MCI.[112] A more trustworthy biomarker consists of the evaluation of the $A\beta_{1-42}/A\beta_{1-40}$ ratio, even though $A\beta_{1-40}$ is marginally increased or shows no changes in AD patients CSF.[119] According to *Wiltfang et al*, one explanation for the better results obtained with this ratio is that the concentration of $A\beta_{1-42}$ may depend not only on

the physiological status of the patient, but also on the total amount of Aβ peptides in her/his CSF. The concentrations of $A\beta_{1-40}$ show high variability between distinct individuals, which may even reflect differences in the efficiency of APP processing by secretases in these individuals, or in the expression levels of APP molecules on the cell surface.[120]

The other hallmark of AD is the aggregation of tau protein, which leads to the formation of NFTs. Tau undergoes several post-translational modifications that have a key role in the neuropathology of AD.[121] Axon degradation and neuronal loss induce the release of tau proteins in the CSF. This might explain the increased levels of total tau in patients with autosomal dominant and sporadic AD, when compared with healthy controls.[105] Also, in some individuals, this increase may be detected in preclinical and prodromal stages of the disease, following the decrease in $A\beta_{1-42}$ levels.[114] T-tau levels in the CSF of AD patients have revealed a good correlation with neuronal tissue damage, whereby its measurement could reflect the intensity of neurodegeneration.[122,123] For instance, high levels of total tau have been linked with a fast progression of individuals with MCI to AD.[124] Although CSF levels of t-tau have revealed high sensitivity (around 84%) and specificity (up to 91%) in differentiating AD patients from healthy individuals,[125] it is not a reliable biomarker for discrimination between AD and other types of dementia.[126]

Though t-tau is seen as a core biomarker, CSF levels of p-tau are more specific than total tau measurements. In comparison with t-tau, levels of p-tau show a specificity of 92% and a sensitivity of 80% in differentiating AD patients from healthy controls[118] and from other primary causes of dementia.[127] In AD, levels of tau phosphorylation vary at different stages of the disease.[128] Regarding p-tau, phosphorylation at residues 181, 199 and 231 are the most studied and can be detected using ELISAs for p-tau181, p-tau199 and p-tau231, respectively. Although p-tau181 is more frequently used than p-tau231, both forms possess similar specificity and sensitivity for AD and MCI progression.[105,129] As for the p-tau199 epitope, *Itoh et al* were able to detect AD using this biomarker with a sensitivity and specificity above 85%.[130] Still, both p-tau181 and p-tau231 presented better results in early detection of AD.[131] Another study demonstrated that CSF p-tau231 is a stable biomarker of MCI conversion to AD.[127] While t-tau is an indicator of neurodegeneration and neuronal death, p-tau correlates with the phosphorylated state of tau protein and the buildup of NFTs in AD patients' brain.[132] However, the main source of p-tau in CSF remains unclear, and it is still unknown whether neurons affected by tau pathology are able to secrete p-tau in the extraneuronal space. If all three p-tau biomarkers show increased concentration in CSF, the accuracy of AD diagnosis raises up to 90%.[133]

Despite these facts, the molecular nature of tau in CSF is not completely elucidated. Although many studies have proven the presence of tau in CSF, the exact identity of tau fragment in this fluid is not well determined. Most of the data comes from two available commercial assays, INNO-BIA AlzBio3 and INNOTEST plate ELISAs, which rely on the use of anti-tau antibodies that bind to the mid-term region of tau protein and might limit the range of tau species measured in the studies. In a study by *Meredith Jr et al*, the authors developed a sensitive WB method and a series of novel overlapping tau and p-tau ELISAs in order to characterize tau fragments present in the CSF.[134] The ability of different tau species in discriminating AD from control CSF samples was also evaluated. Three distinct antibodies were used for tau detection by WB: HT7 (mid-term domain antibody), Tau12 (N-Terminal antibody) and K9JA (MTBD antibody). Bands corresponding to FL tau (~65 KDa) were not detected with either HT7 or Tau12, which indicates that FL tau is not present in the CSF or below the detection limit of this assay. Similarly, no specific bands were detected with K9JA in CSF samples, whereby CT tau fragments also seem to be absent or, again, unable to be detected. WB data analysis revealed that tau in both control and AD CSF is present primarily as N-terminal and mid-domain fragments, with an apparent size between ~20 to ~40 KDa. Levels of tau were more accurately measured by developing five tau ELISAs and three p-tau ELISAs that detect overlapping regions along the tau protein. The different tau assays were able to detect the following fragments: aa 9-163 (Tau12-HT7), aa 9-198 (Tau12-BT2), aa 159-198 (HT7-BT2), aa 159-225 (HT7-Tau5) and aa 159-335 (HT7-77G7), while the p-tau specific assays detected phospho-epitopes at aa 9-ptau181 (Tau12-AT270), aa 159-ptau181 (HT7-AT270) and aa 159-ptau231 (HT7-PHF6). The authors observed that tau assays specific for the N-terminal domain of tau displayed the most significant discrimination between AD and control samples, while fragments from aa 159 to 335 (MTBD region) were not detected in CSF. Although the lack of a quantifiable signal provided by the HT7-77G7 assay is in accordance with the inability to detect FL tau in WB, it is important to take into account that fragments containing MTBD or even more CT tau sequences can still be present, and be undetectable by this assay. P-tau assays that measured the fragments 159-ptau181 have also shown good potential in distinguishing AD from Control. On the contrary, p-tau181 did not reveal significant discrimination between AD and Control. This suggests that the value of p-tau181 as a biomarker is limited and varies according to the measured tau species. Overall, these results propose that not only CSF tau is composed of a mixture of distinct fragments, but also that the potential of tau and p-tau assays to discriminate between AD and control individuals is dependent on the tau species detected by specific assays.[134]

In summary, the combination of all CSF core biomarkers – $A\beta_{1-42}$, t-tau and p-tau – is essential to reach a high diagnostic accuracy in differentiating AD patients from control individuals, and the values of specificity and sensitivity range in this case from 75 to 95% for the prediction of dementia in patients with MCI.[119,135,136] Additional CSF measures that promote a more reliable discrimination between patients with AD, healthy controls and patients with other primary dementias comprise the use of ratios. Examples are $A\beta_{1-42}$:$A\beta_{1-42}$, $A\beta_{1-42}$:$A\beta_{x-42}$, t-tau:$A\beta_{1-42}$ or p-tau:$A\beta_{1-42}$.[113] These ratios may also be applied to predict AD progression in patients with MCI, and t-tau:$A\beta_{1-42}$ is particularly important for the prediction of cognitive decline in these patients.[137]

Currently, CSF biomarker models of AD predict a decrease in $A\beta_{1-42}$ concentrations within the CSF and an increase in t-tau and p-tau levels with disease progression. However, some studies have reported slight decreases in both p-tau and t-tau CSF concentrations,[138-140] which supports a model in which alterations regarding biomarkers may depend on where the patient falls in the AD pathological process.

Novel CSF Biomarkers

Additional biomarkers have been investigated in order to detect other pathophysiological mechanisms implicated in AD, and to detect some copathologies or drug treatment effects. For instance, APP is first cleaved by either α-secretase or β-secretase, generating sAPPα or sAPPβ, respectively. CSF levels of both of these soluble forms have been investigated in AD patients as potential biomarkers.[105,141,142] While the utility of sAPPα for AD diagnosis remains unclear, sAPPβ levels have been useful in clinical trials of β-secretase inhibitors, as this peptide reveals drug target engagement.[105,141,142] The CSF of AD patients also shows altered levels of neprilysin and cystatin C proteins.[143,144] Levels of apolipoprotein E have also been studied, and seem to positively correlate with CSF tau, where lower levels of APOE are indicative of cognitive decline and brain atrophy.[145]

An increase in CSF concentrations of neuromodulin (GAP43), neurofilament proteins, and visinin-like protein 1 (VILIP-1) has also been shown in AD patients, and these biomarkers reflect neurodegeneration within the brain.[146,147] Glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) have also been investigated as potential biomarkers for degeneration.[100] AD progression results in the alteration of several inflammatory factors in the CSF, such as interleukin-1 (IL-1), tumor necrosis factor α (TNF- α), transforming growth factor β (TGF- β) and interferon γ (INF- γ).[148,149] Lipid metabolism is also altered in AD, and some reliable biomarkers have been developed associated with this process.[150-153] Finally, several studies have

revealed alterations in neurotransmitter pathways in Alzheimer's disease, whereby the diagnostic potential of CSF neurotransmitters has been assessed in many studies.[154] However, the results have been conflicting most of the time, particularly for CSF monoamime metabolites, GABA, glutamate and neuropeptides.[155] Only the implementation of new techniques such as liquid chromatography-tandem mass spectrometry (LC-MS), which allows a more sensitive determination of different neurotransmitters and associated metabolites in the CSF, will lead to more promising results.[154,155] The main problem with CSF neurotransmitters is that they lack both specificity and sensitivity, and cannot be considered as favorable biomarkers for AD.

4.2. Neuroimaging Biomarkers

Besides CSF biomarkers, the most promising biomarkers are neuroimaging biomarkers.[110,156] Magnetic resonance imaging (MRI) is a structural imaging technique that detects abnormalities in brain structures with high resolution. The two earliest features that may be assessed by MRI are hippocampus and entorhinal cortex atrophy. However, these two changes were already seen in other types of dementia, making MRI insufficient for the diagnosis of AD.[157] Recently, functional MRI, or fMRI, has been considered as a method for AD diagnosis. It measures alterations in brain blood flow, which depend on neuronal activity. For instance, it has been reported that AD patients show decreased neuronal activity in both hippocampus and parietal lobe. In contrast, the primary cortex, which is not affected in AD, reveals higher levels of neuronal activity,.[158] The big disadvantage in using fMRI as a diagnostic method relies on its high inter and intra-individual variability.[100]

The most promising neuroimaging technique is positron emission tomography (PET), which measures alterations in brain metabolism. In brains from AD patients, hypometabolism was reported using 18F-fluorodeoxyglucose, FDG-PET, which measures glucose uptake within the brain. Many studies have shown that in this disease, FDG uptake is reduced in the parietal, temporal and posterior cingular cortices.[159] Furthermore, this method reached a specificity of 84% and a sensitivity of 93% in detecting very mild probable AD.[160] Two other PET radiotracers, Pittsburgh Compound-B (PiB) and [8F]FDDNF, have been developed to detect only senile plaques or both NFTs and senile plaques, respectively.[161,162]

When combined with MRI, amyloid imaging shows high specificity and sensitivity in detecting AD at an early stage, and the possibility to visualize the amyloid pathology in the AD brain *in vivo* has been a huge accomplishment in research. Thus, two other radiotracers have been approved for amyloid imaging: [18F]florbetapir and [18F]flutemetamol.[163] Although cerebral atrophy and

hypometabolism can be assessed using MRI and FDG-PET, respectively, only Aβ PET imaging is specific to evaluate Aβ pathology in the brain.[164] In addition to amyloid imaging, many compounds that detect tau deposits are also being developed, and both [18F]-labeled T808 and [11C]-labeled PBB3 ligands are now in phase III of clinical trials.[165] Furthermore, a recent publication revealed the retention of a tau-specific tracer ([18F]-THK5105) in AD patients' temporal lobe when compared to healthy controls.[166] The clinical application of selective tau imaging biomarkers is becoming a hot topic in AD research, since it provides new insights regarding tau pathology in AD and other tauopathies. Such biomarkers will be helpful to better correlate the tau brain load with cognitive decline, monitor disease progression and evaluate new therapeutic interventions targeting tau pathology.[153]

Although neuroimaging biomarkers have demonstrated outstanding results in early detection and differentiation of AD, their use as diagnostic tools is still limited in many clinical centers and hospitals. The high costs of the technology itself and of the radiotracers are the main reason for why their use is still restricted.[154]

4.3. Clinicobiological Correlations

In 2010, a very interesting "timeline" for alterations in Alzheimer's disease has been proposed by *Jack et al*. In this hypothetical model, the first changes of biomarkers in AD would occur in the amyloid β pathway. Thus, the decrease of CSF $A\beta_{1-42}$ levels and/ or deposition of Aβ in brain tissues by *in vivo* amyloid imaging techniques, such as PiB-PET, would be observed at an early stage of the disease.[167] The idea that Aβ pathology within the brain occurs before the appearance of clinical symptoms had been further proposed in *post-mortem* studies which showed Aβ deposition in brains of cognitively normal people.[168] On the other hand, increases in CSF tau concentrations seemed to occur later. This supports a model in which tau pathology would be established after amyloid β pathology.[40] However, when the first clinical symptoms are observed (in MCI stage), a better correlation is seen between tau pathology and loss of cognitive functions, than in the case of Aβ deposition.[169] At that time point, it was not known whether Aβ and tau pathways could arise independently in the course of AD, or if they could influence each other.[170] Still, the authors denoted that from the diagnostic point of view, the two core groups of CSF biomarkers, i.e. one group related with Aβ levels and another with t-tau/p-tau concentrations, should be evaluated separately and supplement each other.

In 2013, the same model was revised by the author, and some important modifications were implemented. For instance, the temporal ordering of some biomarkers was changed, assuming

the sequence depicted in **Figure 5**. In this revised model, CSF $A\beta_{1-42}$ is the first detectable biomarker, followed by amyloid PET and later on by CSF tau. Both FDG PET and MRI appear as the last biomarkers and precede the onset of clinical symptoms. Another major alteration was that tau and Aβ pathology might arise as independent pathophysiological events. Nevertheless, the authors consider the possibility that Aβ pathological changes may lead to an acceleration of a pre-existing subcortical tauopathy. This would facilitate the spreading of NFTs in neocortical areas.[171] Furthermore, the authors emphasize that the pattern outline in **Figure 5** is not necessarily the one occurring in all sporadic cases of the disease.

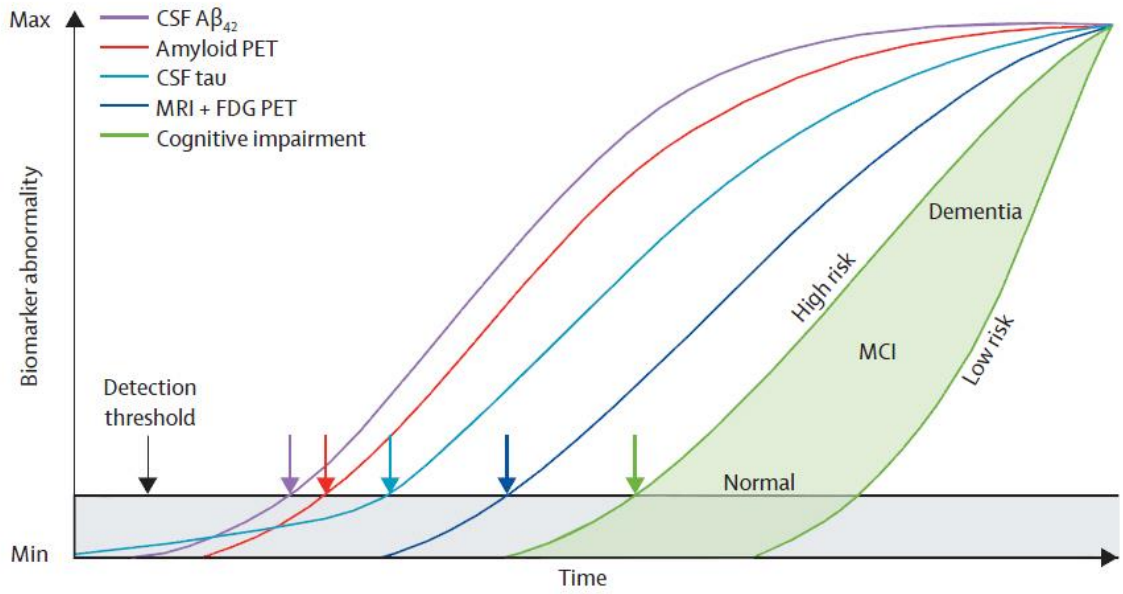

Figure 5 | Relation between Alzheimer's disease progression and Biomarkers: A hypothetical model. The black horizontal line indicates the threshold for biomarker detection of pathophysiological changes. When dementia starts, most of the biomarkers have already reached the plateau phase. The grey area corresponds to the period in which pathological events lie below the biomarkers detection threshold. In this figure, tau pathology is initiated earlier in time when compared to Aβ deposition, but remains at a subthreshold biomarker detection level. Later on, Aβ deposition occurs independently and reaches the biomarker detection threshold (purple and red arrows). At this point, changes in Aβ content can be observed in the brain using PET imaging and in the CSF by measuring $A\beta_{1-42}$ levels. The development of Aβ pathology eventually leads to acceleration in tauopathy, and allows biomarker detection of CSF tau (light-blue arrows). With disease progression, eventually FDG PET and MRI overcome the detection threshold (dark-blue arrow). At last, cognitive impairment is established, and cognitive responses can be assessed (starting at green arrow). Finding new reliable biomarkers that change significantly in preclinical AD is essential for an early diagnosis of AD, and to monitor responses to therapeutical interventions. Aβ = amyloid β; FDG = fluorodeoxyglucose; MCI = mild cognitive impairment. From **Clifford, R. J. J. *et al* (2013).**[172]

In order to reach a higher accuracy in AD diagnosis, it is essential to understand the correlations between biomarkers, particularly between neuroimaging and CSF biomarkers. Only

the combination of neuroimaging techniques with neurochemical dementia diagnostic tools may increase the sensitivity of diagnosis at early stages of the disease, i.e. at prodromal AD. [100] For instance, a very good correlation has been demonstrated between PiB binding and the deposition of fibrillar Aβ in the brains of AD patients. This confirms that PiB binds to fibrils and not to Aβ oligomers. Moreover, the regional distribution of PiB retention has been shown to correspond to the distribution of Aβ deposits observed *post-mortem*.[173] In agreement with these data, low levels of $A\beta_{1-42}$ have been linked to increased cortical amyloid burden, which can be assessed using amyloid PET.[113] The concordance between CSF $A\beta_{1-42}$ concentrations and amyloid PET is usually higher than 90%.[174] Some exceptions have been demonstrated in carriers of rare mutations in APP, which are linked to autosomal dominant forms of AD.[175] However, in the majority of the cases, there's a good correlation between soluble CSF $A\beta_{1-42}$ and amyloid pathology.

Regarding neurodegeneration, tau is considered the biomarker of neuronal injury, as previously discussed (see topic **4.1**). In AD, CSF p-tau seems to correlate with neurofibrillary tangle pathology, while CSF t-tau levels are linked to an overall cytoskeleton derangement and to neuronal damage or death.[132,176] Data from many studies involving more than 2500 AD patients and 1300 controls has demonstrated up to 3 times increased CSF t-tau levels in AD compared to age-matched controls**.**[125] One hypothesis is that increases in CSF t-tau arise from leakage of tau from damaged neurons into the CSF, and thus reflect neuronal damage and degeneration.[43] Additionally, AD patients show decreased FDG-PET uptake. This hypometabolism condition is particularly visible in temporal and parietal regions and correlates with cognitive impairment.[177,178] Volumetric measurements of cerebral atrophy provided by MRI also show a strong correlation with the levels of cognitive impairment. Furthermore, the rates of synaptic and neuronal loss seem to be linked to cognitive decline.[179] An association between the formation of neurofibrillary tangles and the topographic distribution of MRI changes has likewise been highlighted.[180]

In conclusion, a growing body of evidence in the last years emphasizes the importance of both CSF and neuroimaging biomarkers in the early diagnosis of AD. To better analyze AD, the disease should be considered as a continuum, with alterations in the CSF occurring years, or even decades, before the onset of clinical symptoms. To date, the first pathological changes that can be evaluated and used in AD diagnosis are associated with Aβ metabolism. These are reflected by a decrease in CSF $A\beta_{1-42}$ levels, as well as an increase in the brain uptake of specific tracers on Aβ PET. Despite this, it is likely that a good diagnosis of AD has to be based on a combination of different biomarkers, namely CSF, neuroimaging and genetic biomarkers.[100]

5. THERAPEUTICS IN AD

Although AD is known for about a century, the only treatment approved by the FDA is symptomatic treatment based on the use of cholinesterase inhibitors (donepezil, rivastigamine and galantamine) and memantine. Hence, current therapeutic approaches have been focused on disease modification, in order to halt neurodegeneration before it becomes irreversible. The complex and multifactorial etiology of Alzheimer's disease has hampered the production of an effective treatment. Many therapeutic strategies have been carried out till date, which target different pathophysiological mechanisms of AD. These include neurotransmission modulation, tau-based therapies, amyloid-based therapies, intracellular signaling cascades, oxidative stress reduction, mitochondria specific therapies, anti-inflammatory therapies and multi-target directed ligands, among others.[181]

In the next sections, amyloid and tau-based therapies will be discussed, with a focus on tau immunotherapy, which is currently a hot topic in AD therapeutics. Vaccination is the most common form of immunotherapy and is based on the administration of an antigen, often with adjuvant, to actively increase the production of antibodies in the body. Aβ immunotherapy started with the investigation of active immunization approaches, first in mice and then in human clinical trials. An alternative consists of the injection of purified antibodies to deliver host immunity against a specific antigen, a process known as passive immunization (see different immunotherapeutic approaches in **Figure 6**). Passive immunization has often been linked to problems such as difficulty in selecting the right targets, high costs, need for repeated injections (in chronic diseases), blood brain barrier impermeability, and triggering of an immune response against the injected antibodies. Nevertheless, several passive immunization trials are under investigation at the moment.[182]

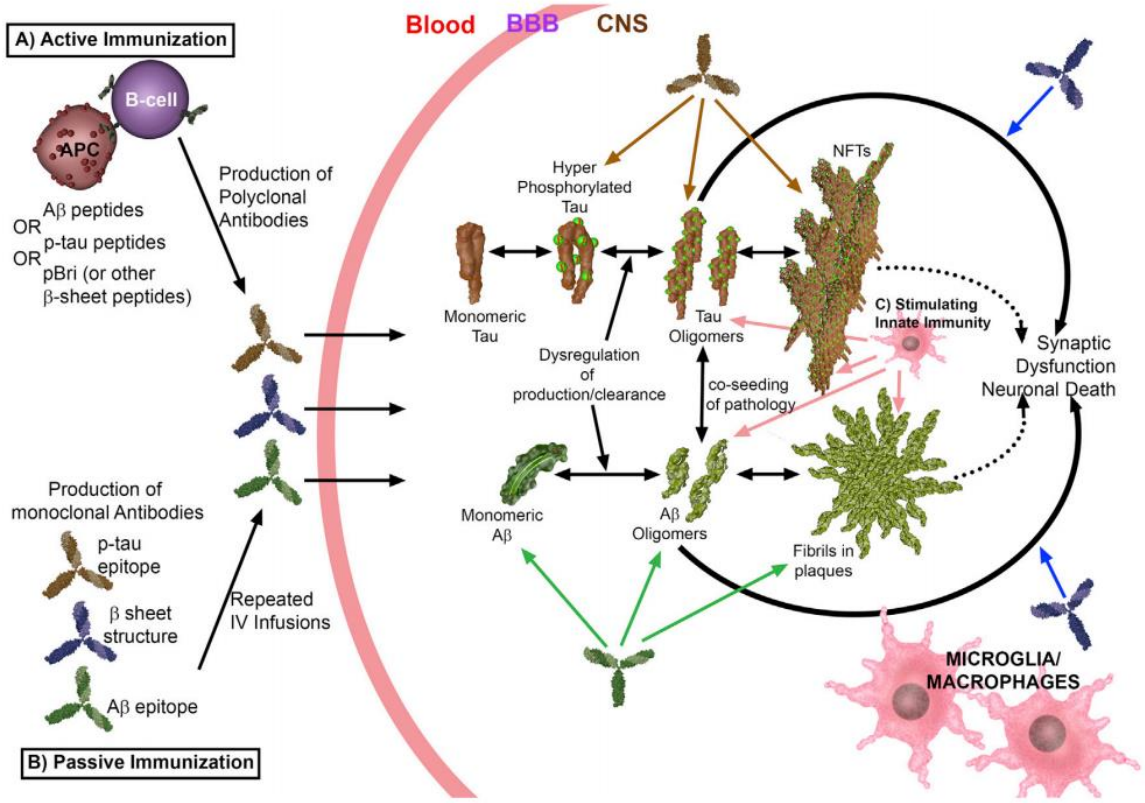

Figure 6 | Different Immunotherapeutic Approaches to Ameliorate AD.

(A) Active immunization can be performed using Aβ or phosphorylated tau (p-tau) peptides. These immunogens are presented to B cells by antigen-presenting cells (APC). Use of Aβ peptides or p-tau peptides will lead to the production of antibodies to Aβ or p-tau epitopes by B cells. **(B)** Passive immunization is based on the injection of monoclonal antibodies (mAbs) to deliver host immunity. These mAbs can bind Aβ, tau, or β-sheet pathological conformations. The infusion of these antibodies needs to be made systemically, in concentrations that allow its crossing through the BBB. In both active and passive immunization, once antibodies cross the BBB, they will promote clearance and degradation of their targets. Additional or alternative mechanisms include disaggregation or neutralization of their target (i.e., blocking of toxicity). **(C)** Potentiation of innate immunity may likewise enhance the function of microglia/macrophage via TLRs or related pathways. Microglia/macrophages are stimulated similarly by the immune complexes produced using active or passive immunization approaches. From ***Wisniewski, T. & Goñi, F. (2015)*** [182]

5.1. Amyloid-based Therapies

Despite the fact that formation of amyloid plaques represents one of the hallmarks of AD, Aβ peptides seem to have a physiological role.[176] Even though the conditions that turn Aβ into a pathological molecule are not fully understood, the concentration of this peptide may be an important aspect. One hypothesis proposes that when the production of Aβ exceeds the capacity

for its clearance, it will accumulate and concentrations of Aβ will eventually reach toxic levels. In these conditions, the equilibrium between the formation of Aβ fibrils, oligomers and monomers will also be altered, and may lead to cell damage.[177]

Several aspects of APP metabolism are often the target for amyloid-based therapies. Currently, there are three fundamental approaches targeting Aβ for treatment and prevention of AD. These comprise the inhibition of Aβ production, the prevention/promotion of its aggregation/disaggregation, and increasing its clearance from brain tissues.[178] More recently, immunotherapy approaches have gained a major importance in AD research, and the following segment will focus on results and concerns in Aβ immunotherapy.

Immunotherapy targeting amyloid-β

Currently, many of the novel therapeutic strategies for AD involve the modulation of the immune system. Due to its central role in disease progression, Aβ and downstream targets have emerged as potential targets for immunotherapies.[186-188] Though Aβ-directed immunization has revealed promising results in transgenic mice models of AD, the safety and efficacy of these therapies in humans still remains a challenge. One big question is the identification of the ideal target and timing of the therapy.[182]

Many phase III trials of anti-amyloid approaches with patients comprising mild-to-moderate AD have been published in recent years with disappointing results.[47,89,103,127,132,135,136] The first passive immunization therapy for AD treatment was the development of Bapineuzumab, a humanized anti-Aβ monoclonal antibody which targets the N-terminus of amyloid beta.[181] Although Bapineuzumab reached phase III trials, it ultimately failed to show clinical improvement and did not clearly reveal disease-modifying effects.[182] Solanezumab (Eli Lilly), a humanized version of mAb 266, was the second anti-Aβ antibody to enter AD clinical trials. The phase II trial of this compound showed no treatment related benefits, but the observation of a dose-dependent increase in Aβ plasma and CSF $Aβ_{1-42}$ levels conducted a phase III trial of Solanezumab in patients with mild Alzheimer's disease (NCT01900665).[189] Despite higher doses of Solanezumab were used in phase III trials, when compared with Bapineuzumab, amyloid related imaging abnormalities (ARIAs) were not found in treated subjects, and an increase in plasma Aβ levels was observed.[190] Therefore, Solanezumab will be used in two very early treatment trials – Dominantly Inherited Alzheimer Network (DIAN) and Anti-Amyloid Treatment for Asymptomatic Alzheimer's Disease (A4).[182,191] Another monoclonal antibody which has revealed promising results in preclinical studies is Gantenerumab (Hoffman-LaRoche). This antibody is currently in a phase III

trial.[181] Both Gantenerumab and Solanezumab are enrolled in DIAN trial, in an attempt to assess their potential in patients with dominantly inherited AD lacking clinical AD symptoms but with appreciable amyloid load on PET scan (NCT01760005).[192] Another prevention trial that will be carried out is Alzheimer's Prevention Initiative (API). This trial will examine the effect of Crenezumab (AC Immune/Genentech) only in patients carrying a presenilin-1 mutation, E280A.[182] This mutation leads to a very severe AD phenotype, in which Aβ deposition starts from approximately 25 years of age. Crenezumab is a humanized version of an anti-Aβ monoclonal antibody of an IgG4 isotype, known as MABT5102A. This antibody interacts with multiple forms of Aβ and reduces the risk of microglial overactivation.[193] Recently, analysis of a phase Ib trial of Aducanumab (Biogen/Neuroimmune), a human monoclonal antibody raised against aggregated forms of Aβ, has revealed a dose dependent reduction in amyloid uptake and a delay in cognitive impairment and global functioning in patients with early AD.[103] Currently, another approach under investigation in passive immunization trials is the role of intravenous immunoglobulin (IVIg) in AD.[182] A 6 month pilot study with 5 patients was one of the first reports on IVIg action, and a decrease in CSF Aβ levels was registered, accompanied by an increase in total levels of Aβ in the serum.[194] The basis for the use of IVIg is the fact that it contains a significant amount of naturally acting anti-Aβ antibodies.[182] Importantly, patients who receive regular IVIg infusions show a decreased risk of developing AD and related disorders.[195]

Overall, the main problem concerning passive immunization is that it lacks specificity to target the toxic Aβ species. Thus, normal soluble Aβ can also be targeted, which may interfere with its essential physiological functions, such as neuroprotection, modulation of LTP processes, and innate immunity. The risk of autoimmune effects may also be potentiated under these circumstances.[196-198] Another key aspect in these therapies is that probably they have to be started very early in the disease course to produce clinically significant benefits, preferably before cognitive impairment arises. Thus, the utility of these therapies is still considered limited in symptomatic AD.[182]

5.2. Tau-based Therapies

As previously discussed, tau is expressed by neurons, where it regulates microtubules assembly by binding to polymers of tubulin. This regulation is crucial for the establishment of a functional organization of neurons, specifically axonal morphology, and neuronal growth and polarity. Tau function is dependent on its phosphorylation status, and the protein contains several phosphorylation sites (see **Figure 4**). Therefore, tau hyperphosphorylation has been the focus of

several tau-based approaches, namely by the use of kinase inhibitors. Other therapies try to stabilize microtubules by using microtubule stabilizing agents such as paclitaxel or EpoD; prevent tau aggregation with distinct types of small molecules; enhancing metabolic processes by the action of heat shock proteins (HSP), the UPS and/or autophagy, and activation of proteolysis to degrade and clear aggregates.[199,200] While the above strategies continue to be investigated, tau immunotherapy has emerged as an important therapeutic approach.[181] This is not only due to some limitations and problems regarding Aβ immunotherapy,[201] but also because tau pathology has been shown to be more directly linked to disease symptoms, progression and severity.[202] Nevertheless, it might be that the ideal strategy for AD treatment has to target both Aβ and tau in concert.[200]

Immunotherapy targeting tau

Recently, there has been a large interest in targeting phosphorylated tau for immunomodulation in AD.[182,203,204] Although for many years it was believed that tau pathology was a consequence of the amyloid cascade effects, some studies have now suggested that tau pathology precedes formation of amyloid plaques.[205] Also, many groups have shown that cognitive decline and severity of dementia better correlate with the degree of tau pathology when compared to the amyloid plaque burden.[57,87,206] This idea is further supported by human immunization trials which showed no clinical benefits with decreased amyloid plaque load. Moreover, studies showing that tau can be actively released from cells and tau pathology may involve cell-to-cell transfer between neurons have further increased the interest in using antibodies that might bind tau and remove it extracellularly.[207] These findings have made tau a desirable target for AD treatment.[182]

In preliminary preclinical studies C57BL/6 mice were immunized with wild tau protein epitopes. This approach developed CNS infiltrates and encephalitic response.[208] Later, pathologically phosphorylated epitopes started to be used as immunogens. For instance, treatment with a phospho-tau peptide (containing the epitopes Ser 396 and Ser 404) before the onset of the pathology, prevented development of tau aggregates in Tg P301L mouse model. In this model, NFTs are established in several brain regions and the spinal cord. [209] Phosphorylation of the mentioned epitopes was shown to increase PHF formation and enhance the fibrillogenic character of tau.[209,210] Antibodies generated by this vaccination were able to cross the BBB, bind phosphorylated tau, and decrease tau pathology with no major adverse effects.[209] This provided new insights about the possibility of reducing tau-pathology using active immunization. However, these strategies comprise a risk of inducing encephalitis or neuronal apoptosis. A study by

Rosenmann et al in which female C57BL/6 mice were immunized with Full Length recombinant tau revealed the presence of neurological deficits, NFT-like changes, gliosis and inflammation after treatment.[208] The same deleterious effects were observed when immunization was carried out with phosphorylated tau as an epitope.[211]

Hence, passive immunization approaches using monoclonal antibodies against phosphorylated tau have started to emerge, which demonstrated benefits in tau transgenic mouse models.[212] Two trials have been conducted using passive immunization, that show a reduction in both tau-pathology and motor deficits when the timing of the antibody administration was prior to the onset of tau pathology.[213,214] To date, the only study showing improvement in pathology after its onset has been unable to ameliorate animal survival when compared to controls.[215] In this report, the authors administered PHF1 (specific for Ser 396/404 epitope), MC1 (conformational antibody) and the pan-tau antibody DA31 in either 4 or 7 months old P301L Tg mice. This transgenic mouse model presents disease onset at about 3 months of age. Injection of MC1 showed a decrease in tau-related pathology in the hippocampus from 7 to 10 months. However, the authors failed to identify IgG in neurons, leading to questions regarding the mechanism of action, and no change in survival was observed between mice injected with PHF1/MC1 from 6 to 14 months old and control Tg mice. Thus, although immunotherapy targeting tau revealed promising preliminary results, there is some risk of toxicity.[215]

The prevention of extracellular aggregation and "seeding" of tau pathology has been a major focus in tau immunotherapy. In 2013, *Yanamandra et al* suggested an extracellular model of tau clearance. The group studied the effects of several antibodies targeting extracellular tau on P301S mice, and proposed a mechanism in which their antibodies were able to prevent the uptake of extracellular tau aggregates by other neurons and thereby would prevent a prion-like propagation of tau between neurons. In this study, the authors tested intracerebroventricular (ICV) administration of three anti-tau antibodies (HJ8.5, HJ9.3 and HJ9.4). The antibodies were chosen based on their potential to block seeding activity present in P301S mice brain lysates and to prevent tau uptake into cells. Epitope mapping revealed that HJ9.3 recognizes a fragment in the MTBD (aa 306-320), while both HJ9.4 and HJ8.5 are specific for N-Terminal tau (aa 7-13 and aa 25-30, respectively). Importantly, the selected antibodies showed different effects in blocking seeding. HJ9.4 was the least potent antibody in blocking tau uptake and seeding activity, while HJ8.5 and HJ9.3 were the most efficient. Treatment with HJ8.5 and HJ9.3 antibodies decreased tau pathology and contextual fear conditioning deficits in P301S mice. Even though this study provided evidence that administration of anti-tau antibodies is capable of ameliorating behavior

at a point in which pathology is already established, the intraventricular route represents a major drawback.[216]

Although several tau immunization studies demonstrated some effects on AD pathology, the maximal expected efficacy of antibodies targeting tau after the onset of pathology, the ideal tau species to target and the mechanism of action of therapeutic approaches remains poorly understood.[216] For instance, while Aβ depositions are found in the extracellular space, tau lesions occur intracellularly and may be more difficult to target directly. In this context, studies that suggested a prion-like mechanism by which tau propagates to anatomically connected regions[90,91,217] had a major impact on therapeutic design, and raised the possibility that tau can also be targeted extracellularly.[216] Therefore, both intra and extracellular tau pose as potential targets for antibodies.[209,218] Addition of pan-tau antibodies to culture media has demonstrated their ability to prevent tau pathology spreading without the need to enter the cells.[216, 219] This suggest that targeting extracellular tau may be important to halt tau spreading.

Even though the development of immunotherapeutic strategies has been a hot topic in AD research, with some encouraging results in animal models, the transition of these promising strategies into clinical trials is still difficult. In human tauopathies, the nature of tau itself, e.g. the prevalence and types of epitopes, is to some extent patient dependent, and may change with disease progression. These changes have a direct implication in the efficacy of a given treatment, which will depend on the individual patient and the stage of the disease. The determination of the epitope to target is also a major concern, since targeting normal tau might have detrimental effects. Thus, it is important to ensure that therapy will be directed specifically against disease related epitopes.[220]

In summary, tau pathology seems to better correlate with disease progression in AD when compared to Aβ pathology. This makes tau an attractive target for the development of disease modifying therapies which will require specific biomarkers to monitor the effects of the therapy. In addition, certain tau phospho-epitopes may turn out to be important biomarkers that might improve the diagnostic accuracy of AD and other tauopathies. Overall, therapeutic strategies focused on tau have given important insights in recent years and are very promising to improve tau-based treatment and diagnosis in AD.

6. CONCLUSION

It is broadly accepted that abnormal post translational alterations such as hyperphosphorylation, truncation, acetylation, glycation, and others, are responsible for the altered structure of Tau in AD. Although the levels of abnormally truncated and phosphorylated tau have been correlated with disease in AD populations, there are still many obstacles to overcome in order to efficiently treat AD. For instance, although a growing body of evidence suggests that small soluble tau oligomers might represent the most toxic forms of tau, while filamentous and fibrillary tau may even constitute a neuroprotective strategy, many questions remain unanswered. There remains a knowledge gap of which tau species trigger the onset of AD pathology: is it monomers, dimers, trimers or all of them? In which conformation do they exist? What is their phosphorylation state? The sequence of events leading to tau aggregation is also not completely elucidated. Moreover, it is essential to determine which tau species could represent the best target for tau-based therapies.

Even though AD is a hot topic in research nowadays, much investigation is still needed for finding the best therapeutic intervention in AD. Our aim was to gain more insight in which form(s) tau is present in human CSF. Therefore, the work presented in this thesis will focus on the generation of several tau-detecting bioassays and evaluation of specific tau fragments and their pathological modifications in human CSF. The development of reliable and sensitive biomarkers remains essential to decrease the age limit of AD detection and enable a diagnosis in a pre-clinical stage, and consequently facilitate the evaluation of potential therapeutics before irreversible degeneration is carried out. This emphasizes the relevance of our work in the AD research field.

Chapter VI – Bibliography

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