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Characterization of antibodies recognizing pathological forms of Tau in Alzheimer's disease

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular com especialização em neurobiologia, realizada sob a orientação científica do Doutor Kristof Van Kolen (Janssen Pharmaceutica NV), do Doutor Marc Mercken (Janssen Pharmaceutica NV) e supervisão da Professora Doutora Ana Luísa Carvalho (Universidade de Coimbra)

André Filipe Domingos Marreiro

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Abstract

Alzheimer's Disease is a critical neurodegenerative disease characterized by two hallmarks, deposition of Aβ protein in plaques and incorporation of Tau proteinaceous aggregates in neurofibrillary tangles(Braak et al., 2011; Huang and Mucke, 2012). Biomarkers detecting alterations in mechanism of this disease are of utmost importance to understand disease progression (Blennow and Hampel, 2003)

Major breakthroughs have been made in the comprehension of the mechanisms of AD and potential therapies; there are however no effective disease modifying treatments of the disease(Huang and Mucke, 2012).

Progress in many fields like chemistry, radiology and systems biology are continuously providing tools giving new possibilities to develop new therapy approaches with many different strategies(Huang and Mucke, 2012).

One of AD critical alterations is Tau hyperphosphorylation and aggregation in paired helical and straight filaments, condensing in neurofibrillary tangles. The development of these aggregates is associated with the progression of neuronal loss and cognitive decline (Chai et al., 2011), therefore, hyperphosphorylated Tau targeting by immunotherapy is one of many promising approaches to treat AD.

Previous studies evidenced that Tau immunization prevents aggregation and attenuates functional impairments in mouse models (Chai et al., 2011).

To have a better insight on therapeutic effect of a pharmacological agent, it is of great importance to have efficient biomarkers of AD, that can detect if there is any alteration on the levels of a determined protein due to the effect on a specific target (Blennow and Zetterberg, 2012; Hampel et al., 2010).

One of the goals of this project was to characterize antibodies generated against different forms of Tau, both pathological and normal.

Characterization of the antibodies was performed and a better knowledge of the library of antibodies in study was obtained. Antibodies reacting with pathological form of Tau were identified, there were antibodies epitopes that could be determined, and the knowledge on the reactivity of these antibodies against normal Tau vs. phosphorylated was obtained. The reactivity of these antibodies against Tau from different species was also obtained.

Other goal of this project was to develop specific assays reacting with dog Tau, an important pre-clinical longitudinal pharmacokinetic model, that shows age related alterations in the brain and cognitive decline

(Head, 2013). With the knowledge obtained from the antibodies characterization it was possible to identify assays that could detect Tau protein in dog CSF.

The possibility to detect specific regions of Tau protein in a sensitive way can have great importance for research and therapeutics development in AD.

Keywords: Alzheimer's disease; Tau; biomarkers; immunotherapy; antibody characterization.

Resumo

A doença de Alzheimer é uma doença neurodegenerativa caracterizada por duas principais características, o deposito de placas de proteína Aβ e a incorporação da agregados proteicos de proteína Tau em tranças neurofibrilares (Braak et al., 2011; Huang and Mucke, 2012). Biomarcadores que consigam de uma forma especifica detectar alterações associadas à patologia são importantes para compreender a progressão da doença (Blennow and Hampel, 2003)

Descobertas importantes tem sido feitos na compreensão e conhecimento dos mecanismos, e possível terapia desta doença, não existindo porem actualmente nenhum fármaco que consiga modificar a progressão da doença (Huang and Mucke, 2012).

Avanços em diversas áreas, tais como a química, radiologia, e biologia de sistemas tem constantemente providenciado ferramentas que podem ser aplicadas no desenvolvimento de novas terapias com diferentes estratégias (Huang and Mucke, 2012).

Uma das alterações críticas da doença de Alzheimer e a hiperfosforilação e consequente agregação da Tau em em filamentos helicoidais emparelhados e lineares, que se condensam em tranças neurofibrilares. O desenvolvimento destes agregados está associada com a progressão da perda neuronal e declínio cognitivo, portanto, imunoterapia que tenha como alvo a proteína Tau no seu estado hiperfosforilado é uma de muitas possíveis terapias promissoras contra a doença de Alzheimer (Chai et al., 2011).

De modo a ter uma compreensão nos efeitos terapêuticos de um agente farmacológico é de suma importância ter biomarcadores que eficientemente consigam detectar alterações de níveis de uma determinada proteína associada com um efeito no alvo pretendido (Blennow and Zetterberg, 2012; Hampel et al., 2010).

O objectivo principal deste projecto foi o de caracterizar anticorpos gerados a partir de diferentes formas de Tau, tanto patológica, como normal.

A caracterização destes anticorpos foi feita e um melhor conhecimento do conjunto de anticorpos em estudo foi obtido. Anticorpos que mostram reactividade contra formas patológicas de Tau foram identificados, houve também epitopos de determinados anticorpos que foram determinados. A reactividade destes anticorpos contra Tau normal ou Tau fosforilada foi também definida, bem como a reactividade contra diferentes espécies.

Outro objectivo deste projecto foi desenvolver ensaios específicos que detectassem proteína Tau em liquido cefalorraquidiano de cão, um importante modelo longitudinal pré-clínico de farmacocinética, que

demonstra alterações no cérebro associadas com a idade e declínio cognitivo Com o conhecimento obtido através da caracterização dos anticorpos foi possível desenvolver ensaios que identificam Tau no liquido cefalorraquidiano de cão.

A possibilidade de detectar regiões especificas da proteína Tau com grande sensibilidade poderá ser extremamente relevante na investigação e desenvolvimento de terapias para a doença de Alzheimer.

Palavras-chave: Doença de Alzheimer; Tau; Biomarcadores; Imunoterapia; Caracterização de anticorpos.

Abbreviations

Ach – Acetylcholine

AchE – Acetylcholinesterase

AD – Alzheimer's Disease

ADAM – A Disintegrin And Metalloproteinase

APP – Amyloid precursor protein

Aβ – Amyloid β

BACE1 – β-site APP-cleaving enzyme 1

BACE2 – β-site APP-cleaving enzyme 2

BBB – Blood-brain-barrier

BDNF – Brain-derived neurotrophic factor

CDK5 – Cyclin-dependent kinase 5

CIP – Calf Intestinal Phosphatase

CSF – Cerebrospinal Fluid

CNS – Central nervous system

DMP – Dimethyl pimelimidate

EOAD – Early-onset Alzheimer's disease

ERK2 – Extracellular signal-regulated kinase 2

FTD – Frontotemporal Dementia

FTDP-17 – Frontotemporal Dementia with Parkinsonism linked to chromosome 17

FTLD-Tau – Frontotemporal lobar degeneration with Tau inclusions

GSK 3 – Glycogen synthase kinase 3

GWAS – Genome-wide association studies

HRP – Horse Radish Peroxidase

HSE – Heat Stable Extract

HSP – Heat shock protein

Lambda PP – Lambda Phosphatase

LOAD – Late-onset Alzheimer's disease

MAP – Microtubule Associated Protein

MARK1 – MAP/microtubule affinity-regulation kinase 1

MBD – Microtubule-binding domain

MT – Microtubule

NFT – Neurofibrillary tangle

NMDA – N-methyl-D-aspartate

NSC – Neural Stem Cell

PHF – Paired helical filament

PRD –Proline-Rich Domain

PS1 – Presenilin 1

PS2 – Presenilin 2

PTM – Post--translational modification

ROS – Reactive oxygen species

sAPPα – Soluble APPα

sAPPβ – Soluble APPβ

UPS – Ubiquitin proteasomal system

INDEX

1. Introduction

1.1 Dementia

Dementia is a syndrome, characterized by a number of disorders, that collectively and progressively affect memory, behaviour, orientation, judgment, comprehension, language, learning, and hence the ability to perform daily activities (Prince and Jackson, 2009; Wimo and Prince, 2010).

Usually dementia is associated with aging but some cases with early onset have been reported. Above the age of 65, the prevalence of people with dementia doubles every five years. Most of the cases of dementia worldwide are related to AD but dementia syndrome is also associated with other pathologies like vascular dementia, dementia with Lewy bodies and frontotemporal dementia (FTD) (Prince and Jackson, 2009).

It was estimated that in 2010, 35.6 million people above the age of 60 were living with dementia. On top of this, millions of new cases are predicted each year, nearly doubling the prevalence every 20 years to 65.7 million in 2030 and 115.7 million by 2050, shown in Figure 1 (Sosa-Ortiz et al., 2012). The worldwide cost of dementia is increasing, around US$604 billion in 2010, more than 1% of global Gross Domestic Product (Wimo and Prince, 2010).

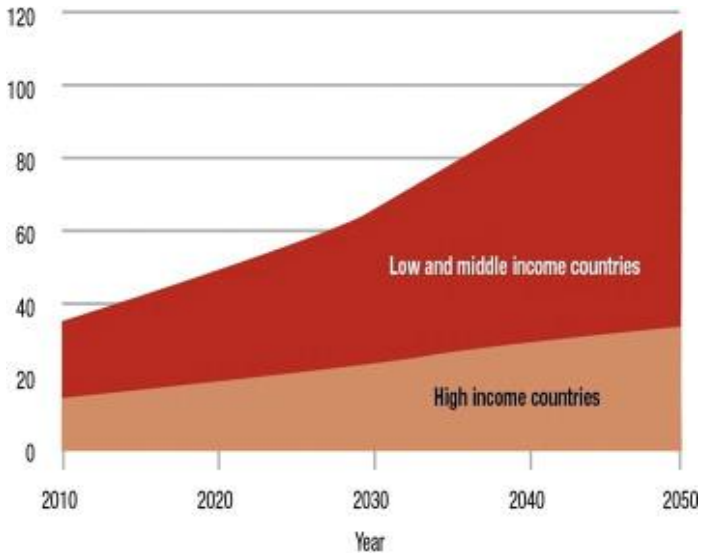

Figure 1 – The growth in numbers of people with dementia (in millions) by country income (Sosa-Ortiz et al., 2012)

Early dementia diagnosis is crucial to make advances in the knowledge and treatment of this group of diseases. For the patient it is crucial to start a potential treatment as soon as possible while it will help the family to anticipate to the circumstances (Prince et al., 2011).

1.1.1 Alzheimer's disease

In 1906, Alois Alzheimer, a German psychiatrist and neuropathologist, presented the first case of the disease which was later named Alzheimer's disease (AD) by his colleague, Kraeplin. The afterwards publication on this case in 1907 was only a relatively short communication about a woman named

Auguste Deter, who he examined for the first time in 1901. She had unusual symptoms that began at age of 51 years, having progressive changes in her personality during 8 months. Her memory slowly deteriorated, leading to psychosocial impairment, until ultimately she died in 1906. In 1907 Alzheimer treated another patient with the same clinical manifestations of Auguste D., named Johann F. This patient died after three years of hospitalization. In these three years, Alzheimer extensively described this new disease based on the observations made with the two cases (Berchtold, 1998; Möller and Graeber, 1998; Verhey, 2009). Alzheimer published a very comprehensive paper in 1911 in which he discussed the concept of the disease in detail (Alzheimer and Jahre, 1911).

AD knowledge has evolved and today it is the most common neurodegenerative disease, characterized by a progressive loss of many cognitive functions, with memory loss as the best characterized. After the initial clinical manifestations, AD pathology progresses during 10 years, evolving into a state that the patient is completely incapacitated and ultimately dies (Huang and Mucke, 2012; Prince and Jackson, 2009; Wimo and Prince, 2010).

1.2 Characterization and Mechanisms of Alzheimer's Disease Pathology

AD leads to an extensive loss in brain weight and volume, affecting some brain regions and neuronal populations more than others (Gómez-Isla et al., 1996). Even though AD promotes neuronal loss in specific brain regions, like pyramidal cells in lamina II of the entorhinal cortex and in the CA1 region of the hippocampus, most of the loss in brain volume seems to be due to a shrinkage of neurons, caused by an atrophy of axons and dendrites (Huang and Mucke, 2012).

Depending on the age of onset, it is possible to categorize AD in two types, the early-onset Alzheimer's disease (EOAD) form, and the late-onset Alzheimer's disease (LOAD) from. The EOAD is associated generally with a familial form, caused by a genetic mutation, while the LOAD, on the other hand, is usually associated with a sporadic form, which is thought to be a multifactorial disease, having influence by genetic factors (Bertram and Tanzi, 2005; Bertram et al., 2010; Kamboh et al., 2012).

1.3 Hallmarks of Alzheimer's Disease

AD is characterised by two pathological hallmarks i.e. amyloid β (Aβ) aggregation in senile plaques, and Tau aggregation in neurofibrillary tangles (NFTs) (Ballard et al., 2011; Blennow et al., 2006). Concerning the latter, progression of Tau pathology can be categorized in six distinct stages (I-VI). Stages I and II both

show alterations which are virtually confined to a single layer of the transentorhinal region (transentorhinal I-II). The key characteristic of stages III-IV is the severe involvement of the entorhinal and transentorhinal layer Pre-α (limbic III-IV). Stages V and VI are marked by isocortical destruction (isocortical V-VI) (Braak, 1991).

1.3.1 $A\beta_{42}$

The amyloid cascade hypothesis is based on the theory that Aβ aggregation will lead to neuronal dysfunction and cell death. Aβ originates from amyloid precursor protein (APP), by sequential hydrolysis (cleavage) by a group of enzymes, or enzyme complexes termed α-, β-, and γ-secretases. There are three enzymes with α-secretase activity and all of them belong to A Disintegrin And Metalloproteinase (ADAM) family of enzymes, including ADAM9, ADAM10 and ADAM17. The most studied β-secretase is β-site APP-cleaving enzyme 1 (BACE1). There is also a BACE1 homolog, BACE2, however, its expression in neurons is substantially lower than BACE1, and cellular BACE2 cleaves APP near the α-secretase site much more efficiently than at the β-secretase site (Zhang et al., 2011). γ-secretase was identified as a complex of enzymes, composed of presenilin 1 or 2 (PS1 and PS2), nicastrin, anterior pharynx defective, and presenilin enhancer 2 (LaFerla et al., 2007; Zhang et al., 2011).

The processing of APP (by cleavage) can proceed through two pathways; one called the prevalent non-amyloidogenic pathway, triggered by the cleavage of APP by α-secretase, at a position 83 amino acids from the C- terminus, producing a large N-terminal ectodomain, soluble APP α (sAPPα), that will be secreted in the extracellular medium. The membrane-localized 83-amino-acid C-terminal fragment (C83) will be then cleaved by γ-secretase resulting in a short fragment termed p3. The cleavage by α-secretase occurs within the Aβ region, thus preventing the formation of Aβ peptide (Blennow et al., 2006; LaFerla et al., 2007; Sakono and Zako, 2010; Zhang et al., 2011). The other mechanism of APP

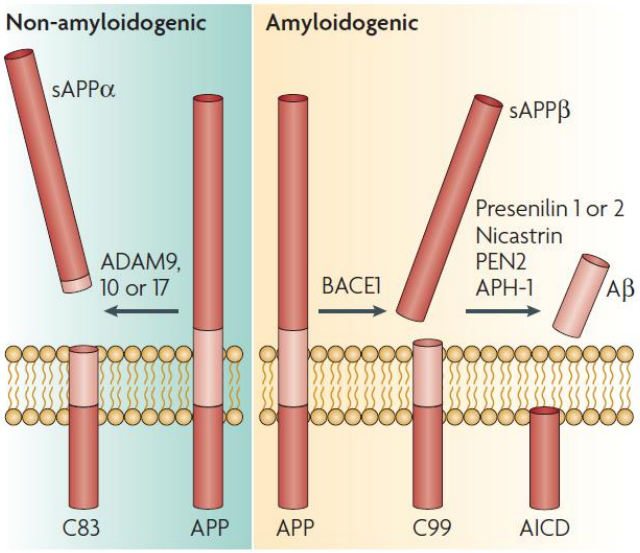

Figure 2 – Non-amyloidogenic and amyloidogenic pathways in APP processing (LaFerla et al., 2007)

processing is called the amyloidogenic pathway, which will lead to the formation Aβ. The initial cleavage is mediated by β-secretase at a position located 99 amino acids from the C terminus. This cut results in the release of soluble APP β (sAPPβ) into the extracellular space, leaving the 99-amino-acid C-terminal stub (known as C99) within the membrane. The newly generated N terminus corresponds to the first amino acid of Aβ peptide. The cleavage of this fragment by γ-secretase between residue 38 and 43 will release an intact Aβ peptide. This process is shown in Figure 2. The major part of Aβ produced by this pathway is 40-amino-acid long (Aβ40), while a small proportion of around 10% will be the 42-residue variant (Aβ42). This form of Aβ is more hydrophobic and more prone to fibril formation than Aβ40, which explains why this variant is the major Aβ species in cerebral AD plaques. Imbalances in APP metabolism or Aβ clearance through the blood-brain-barrier (BBB) or other mechanisms, like proteasomal degradation, can lead to increased levels of Aβ oligomers in the brain, which will contribute to the formation of AD. Aβ aggregates are thought to inhibit hippocampal long-term potentiation and also lead to a disruption of synaptic plasticity. Interestingly, the neurotoxic effect exerted by Aβ is believed to be mediated via Tau as this effect is abolished in Tau -/- mice. Like with Tau, Aβ plaques presence can also be categorized in different stages (0-4) (Braak et al., 2011).

1.3.2 Tau

Microtubule-associated protein (MAP)Tau is one of the major MAPs in the brain. Microtubules (MTs) are critical to cell function, especially for neurons, since neurons require assembly of MTs from tubulin for axon and dendrite growth and integrity, and also to mediate transport of cargo between the soma and distant synapses. Tau plays a major role in MT dynamics: decreased binding may destabilize MTs, and too much may lead to over-stabilization, (Wolfe, 2009). In cancer treatment with MT stabilizing drugs, like Taxol, it was observed an inhibition of dynamicity, shortening, and growing rates of MTs (Yvon et al., 1999).

Tau protein is codified by a single gene, MAPT, is located in locus 17q21.3 (Almos et al., 2008) and has 16 exons, being three of them (2, 3 and 10) target of alternative splicing (Martin et al., 2011). In the central nervous system (CNS) this splicing will lead to six different isoforms, ranging from 352 amino acids to 441(Martin et al., 2011).The isoforms of Tau protein are named by the presence of MT binding repeat sequences (named R) and N-terminal inserts (designated N). With the presence of exon 10, Tau isoform is called 4R, and without it is called 3R. In terms of N-terminal repeats, Tau isoforms can be called 0N, without the repeat, 1N with exon 2, and 2N with exons 2 and 3. Alterations in Tau gene are numbered by the location in the longest isoform, 2N4R (Martin et al., 2011; Morris et al., 2011). Tau can be subdivided

in 4 domains: 1. an N-terminal projection region, that interacts with cell membrane and regulates MT spacing (Al-Bassam et al., 2002; Morris et al., 2011); 2. a proline-rich domain (PRD), containing many phosphorylation sites, that interacts with SH3 domains of other proteins, including the tyrosine kinase Fyn (Augustinack et al., 2002; Lee et al., 1998; Morris et al., 2011; Reynolds et al., 2008); 3. a microtubule-binding domain (MBD), which can be phosphorylated decreasing its interaction with MTs (Fischer et al., 2009); and 4. a C-terminal region(Morris et al., 2011).

Recently, Tau has been found to be a key player in anterograde transport by kinesin and retrograde transport by the dynein complex. Despite the apparently essential function of Tau in MT formation, maintenance, and dynamics, Tau knock-out mice seem to display only mild phenotypes, including muscle weakness, hyperactivity, and impaired fear conditioning, but not neurodegeneration (Ikegami et al., 2000). Last year, however, a study showed neurotoxic iron accumulation in Tau KO mice (Lei et al., 2012) suggesting a loss of function phenotype. Taking in account all the information on Tau protein, it is thought that Tau pathology is not only due to Tau loss of function. However it remains possible that compensation during development exists. Therefore, a conditional knock-out of Tau in the adult mouse brain would provide more definitive answers to the question of whether loss of Tau function alone can contribute to neurodegeneration.

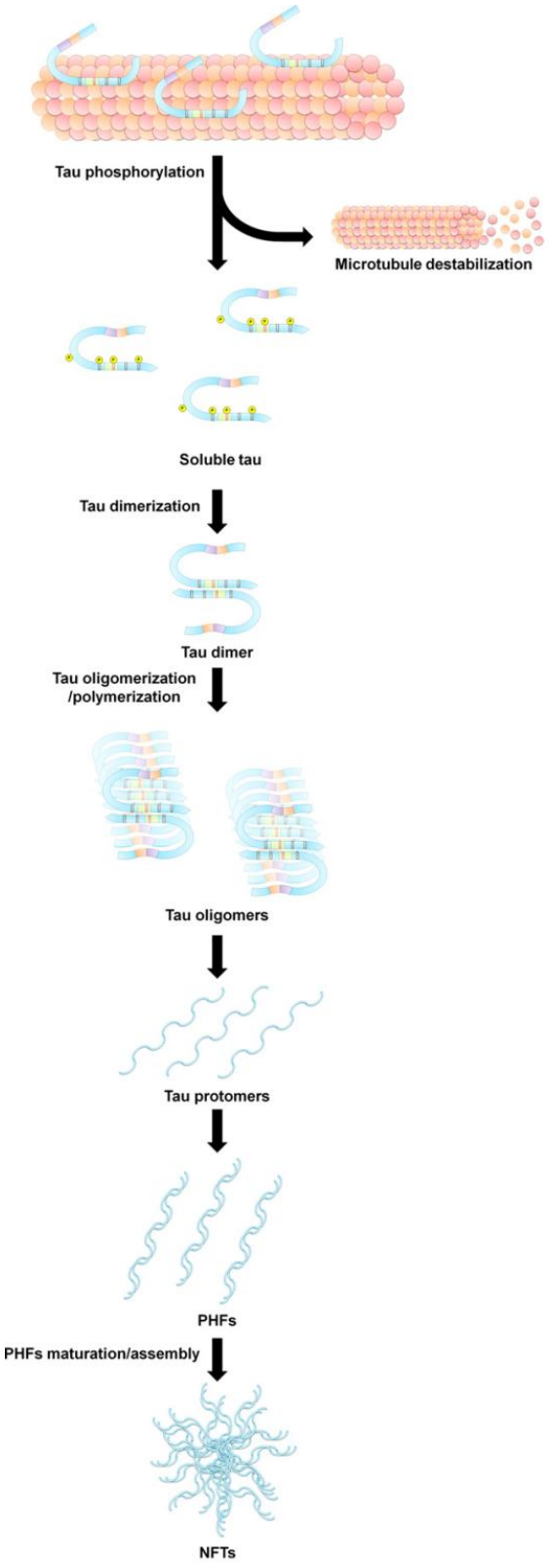

Figure 3 – Step by step representation of the process that lead from normal microtubule associated tau, to NFTs (Martin et al., 2011)

Tau protein undergoes extensive and complex phosphorylation, and the phosphorylation state can alter MT binding (Johnson and Stoothoff, 2004). Phosphorylation disrupts MT binding, whereas dephosphorylation restores binding. The kinases associated with Tau phosphorylation are GSK-3β, CDK5, DYRK1A, CDC2, MARK, MAPK, PKA, and PKC (Augustinack et al., 2002; Kimura et al., 2007). Tau is abundantly expressed in the central nervous system, especially in neurons (Binder et al., 1985), and its function and role in MT formation suggests that disruption of MTs, so critical to axonal structure and transport, may be one way by which aberrant Tau phosphorylation/modification leads to neurodegeneration (Wolfe, 2009).

Although Tau is an extremely soluble protein, its aggregation can be initiated by two biophysical triggers involving charge neutralization and β-sheet structure propensity (Mandelkow and Mandelkow, 2012). Indeed, in addition to MT destabilization, monomeric phosphorylated Tau undergoes a complex cascade leading to the formation of NFTs which is represented in Figure 3. Since many studies showed that hyperphosphorylated Tau is an important molecular hallmark of AD (Morris et al., 2011) facilitated aggregation upon phosphorylation is an attractive working hypothesis. However, the exact role of Tau phosphorylation on the aggregation process is not fully understood and remains controversial. Due to the presence of Tau inclusions, AD can be considered as a member of a group of diseases that are referred as Tauopathies, which are neurodegenerative disorders where Tau inclusions are present (Lee and Goedert, 2001). These include diseases such as frontotemporal lobar degeneration with Tau inclusions (FTLD-Tau), Pick's disease, progressive supranuclear palsy, and corticobasal degeneration; argyrophilic grain disease; and amyotrophic lateral sclerosis/parkinsonism-dementia complex (Morris et al., 2011), as outlined in Table 1. It is important to mention that with Tauopathies, some diseases do not show amyloid pathology, demonstrating that Tau dysfunction on its own can be toxic (Higuchi et al., 2002; Lee and Goedert, 2001).

The cause of this set of disorders is not established, however, the presence of NFTs in all of them supports the driving force of Tau protein in the pathological mechanism. (Lee and Goedert, 2001; Morris et al., 2011; Wolfe, 2009).

As referred earlier, Tau protein can be target of phosphorylation, but it can also be target of other post-translational modifications (PTMs) including glycosylation; glycation; prolyl-isomerization; truncation; nitration; polyamination; ubiquitination; sumoylation; and oxidation (Martin et al., 2011).Nevertheless, the most studied Tau PTM in AD is phosphorylation on serine (S), threonine (T), and tyrosine (Y) residues, since its hyperphosphorylation, is suggested to be associated with Tau aggregation (Martin et al., 2011; Morris et al., 2011).The phosphorylation sites of Tau are represented in Figure 4. Mutating Tau protein to mimic phosphorylation, changing serine residues to glutamate residues at position 262, 293, 324, and

356, resulted in an increased dissociation of Tau protein from MTs, leading also to an increased aggregation, tested by interaction with an aggregation inducer like heparin (Fischer et al., 2009).

Table 1 – Tauopathies, distinguishing absence or presence of amyloid pathology (*Diseases in which synuclein-positive lesions are the most prominent neuropathologic feature) (Adapted from: Higuchi et al., 2002)

Diseases showing coexistence of Tau and amyloid pathologies	*Diseases without distinct amyloid pathology*
	Amyotrophic lateral sclerosis/parkinsonism–dementia complex
Alzheimer's disease	Argyrophilic grain dementia
Creutzfeldt-Jakob disease	Corticobasal degeneration
Dementia pugilistica	Diffuse neurofibrillary tangles with calcification
Down's syndrome	Frontotemporal dementia with parkinsonism linked to chromosome 17
Gerstmann-Sträussler-Scheinker disease	Hallevorden–Spatz disease*
Inclusion-body myositis	Multiple system atrophy*
Prion protein cerebral amyloid angiopathy	Niemann–Pick disease, type C
	Pick's disease
	Progressive subcortical gliosis
	Progressive supranuclear palsy
	Subacute sclerosing panencephalitis
	Tangle-predominant Alzheimer's disease

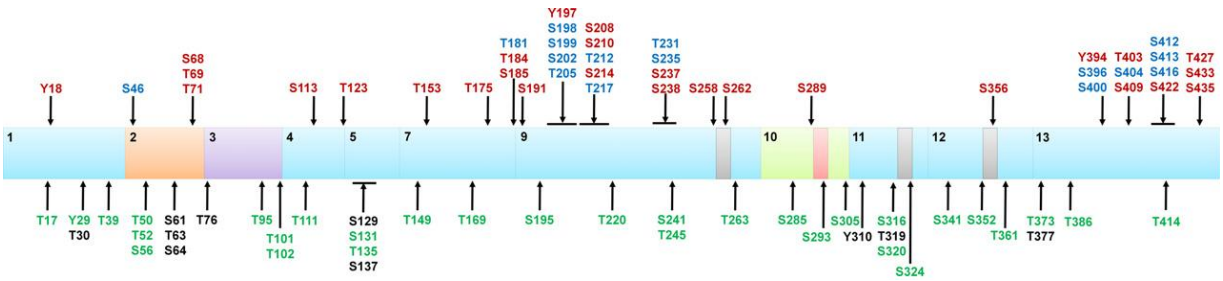

Figure 4 – Phosphorylation sites of tau. In green non-pathogenic phosphorylation sites, in red pathological phosphorylation sites, in blue phosphorylation found in both conditions, in black putative phosphorylation sites of tau (Martin et al., 2011)

The abnormal phosphorylation of Tau will start to occur several years before the onset of the disease, usually starting around the second and third decades of life, prior to Aβ aggregation (Braak and Braak, 1995; Braak et al., 2011). Braak & Braak in 1991 presented a neuropathological stageing of brain changes in AD, by the NFT tangles distribution pattern. The first two stages were an either mild or severe alteration of the transentorhinal layer Pre-α (transentorhinal stages I-II). The two forms of limbic stages (stages III-IV) were marked by a conspicuous affection of layer Pre-α in both transentorhinal region and proper entorhinal cortex. In addition, there was mild involvement of the first Ammon's horn sector. The hallmark of the two isocortical stages (stages V-VI) was the destruction of virtually all isocortical association areas (Braak, 1991; Braak and Braak, 1995; Braak et al., 2011).The results from a study on these stages, and also Aβ extracellular deposition, in 2332 brains of non-selected subjects is shown in Figure 5.

Aggregated Tau will lead to normal Tau, MAP1 and MAP2 sequestration. This sequestration will lead to a disassembly of MTs that will lead to a disturbed axonal flow and transport of essential elements from the soma to the terminal of the axon. The previously mentioned sequestration will eventually lead to a polymerization of Tau, creating paired helical filaments (PHFs), thought to be the most toxic form of Tau aggregates (Martin et al., 2011; Morris et al., 2011; Wolfe, 2009).

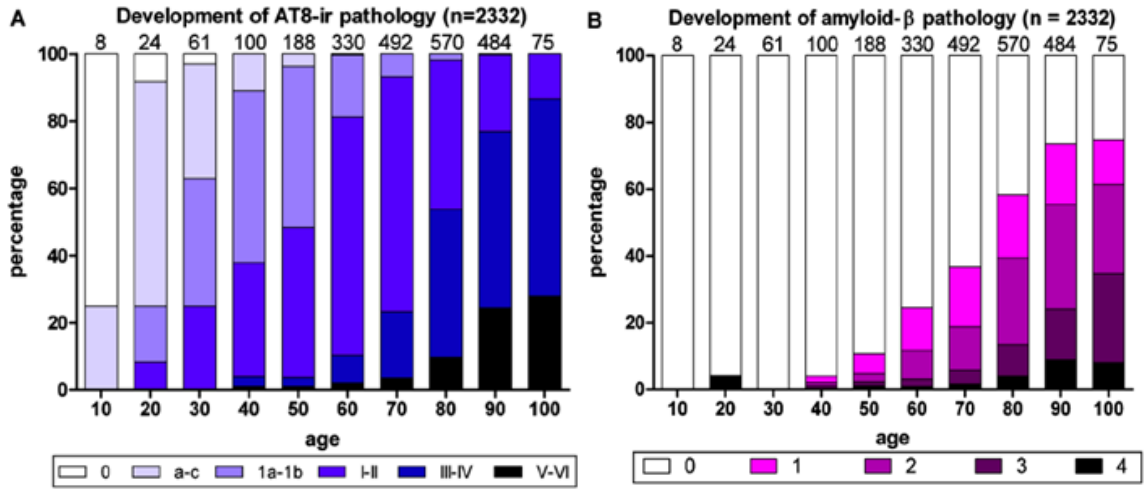

Figure 5 – Development of AT8-immunoreactivity (ir) (A) versus β-amyloid pathologic findings.(B)

1.4 Familial form

Like referred before, AD may be caused by a genetic alteration. Familial AD is associated with three genes that are related with the molecular hallmarks of the disease, being these genes APP, PSEN1 and PSEN2 (Selkoe, 2001).

1.4.1 APP

The *APP* gene encodes the APP protein that as referred above is absolutely important for AD. Some mutations in this gene have been shown to cause an increased processing of APP through the amyloidogenic pathway leading to elevated Aβ levels. On top of this, since APP gene is situated in chromosome 21, individuals with trisomy in that chromosome show an increased probability to develop AD, because this trisomy will lead to an increase in total APP levels (LaFerla et al., 2007). In addition to the mutations associated with EOAD, a recent genetic study in an Icelandic population revealed another alteration in the APP gene (A673T) that provides protection against AD. This alteration results in an approximately 40% reduction in the formation of amyloidogenic peptides *in vitro* (Jonsson et al., 2012). Alterations in PSEN1 and PSEN2 genes, which codify for PS1 and PS2 protein respectively, part of γ-secretase, are associated with familial form of AD (LaFerla et al., 2007).

1.5 Sporadic form

While the genetic causes of the rare familial inherited forms of AD are well known, the causes of the sporadic forms of the disease are not. Molecularly, these two forms cannot be distinguished (Götz et al., 2011). There are, however, factors, both genetic and non-genetic, that are known to increase the susceptibility to develop AD. Recent genome-wide association studies (GWAS) have been made to better establish the genetic factors associated with sporadic AD (Bertram et al., 2010; Huang and Mucke, 2012).

1.5.1 ApoE

The first established genetic risk factor associated with AD is the presence of ApoEε4 allele. This alteration in ApoE gene will lead to an increased susceptibility to develop AD (Huang and Mucke, 2012). GWASs on LOAD in different populations around the world identified ApoEε4 as the top LOAD gene with extremely high confidence (with p values down to ≈ $1x10^{-160}$ (Bertram et al., 2010).

1.5.2 Other Genes

Other genes have been discovered to possibly have an influence in AD emergence. These genes have also been discovered by GWAS, being ATXN1, BIN1, CD33, CLU, CR1, GAB2, PDCH11X, PICALM, among others (Bertram et al., 2010).

1.5.3 Non-genetic risk factors

The most important non-genetic risk factor for LOAD is aging. There are other potential environmental risk factors for LOAD, like head injury, low educational levels, hyperlipidemia, hypertension, homocysteinemia, diabetes mellitus, and obesity. But several of these associations remain controversial. On the other hand, combinations of apoE4 with one or more of these environmental risk factors may further increase the risks for late-onset AD and age-related cognitive decline (Huang and Mucke, 2012).

1.6 Models of Alzheimer's disease

To study AD, animal models of the disease are needed. Although, at this point in time, no real AD model, explaining biochemical and behavioural changes associated with the disease is available, a number of transgenic mice have been reported to recapitulate biochemical hallmarks of AD. In this respect, many mutations in APP, PSEN1 and PSEN2 genes have been identified to cause AD. Therefore, these mutations associated with Aβ plaque formation have largely been used to mimic the disease in mice and study it (Wisniewski, 2010). No mutations in MAPT have been found in patients with AD (Götz and Ittner, 2008), however, mutations in this gene have been found in patients with Frontotemporal Dementia with Parkinsonism linked to chromosome 17 (FTDP-17). Interestingly, these patients do not develop amyloid deposits (Hutton et al., 1998; Poorkaj et al., 1998; Spillantini et al., 1998). Because of the similarities in Tau aggregation between AD and FTDP-17, these mutations are useful for Tau aggregation models (Götz and Ittner, 2008). There are 42 known mutations in MAPT (Cruts and Van Broeckhoven, 2008), and several of them have been used in transgenic mice models (Cruts and Van Broeckhoven, 2008; Götz and Ittner, 2008).

The first Tau transgenic mouse model expressed the longest human wild-type (WT) Tau isoform in neurons (Götz et al., 1995). Pre-tangle formation and hyperphosphorylation of Tau was observed. However, it was another 5 years before the expression of human FTD mutant P301L Tau reproduced aggregation and NFT-formation in mice (Götz et al., 2001; Lewis et al., 2000). These mice have become a

widely used tool to study disease-related pathogenic mechanisms (Götz et al., 2004, 2007) and recent models have built on their success (Götz and Ittner, 2008). Another mouse model showing expression of a human FTD mutant Tau protein is P301S, which exhibits neurodegeneration and extensive Tau aggregation (Allen et al., 2002). There are also models that are based both in Aβ processing and Tau protein mutations, being the 3xTg-AD mice an example of this. These animals harbour three mutations, βAPP_{Swe}, $PS1_{M146V}$, and Tau_{P301L} (Oddo et al., 2003). Tau protein can also be aggregated in vitro for many uses, and for that, an inducer, like heparin is used (Ramachandran and Udgaonkar, 2011).

1.7 Therapies of Alzheimer's disease

Up to date, only symptomatic treatments are available to treat AD. Since this kind of treatment does not change the progress of the disease, there is an urgent need to develop disease-modifying therapies (Blennow et al., 2006; Tayeb et al., 2012).

1.7.1 Targeting neurotransmitter dysfunctions

With neuronal degeneration associated with AD, degeneration of cholinergic neurons in basal forebrain nuclei will cause disturbances in presynaptic cholinergic terminal situated in the hippocampus and neocortex, resulting in memory disturbances and other cognitive symptoms (Terry and Buccafusco, 2003). The principal strategy to enhance cholinergic neurotransmission is to increase the availability of acetylcholine (Ach) by inhibiting acetylcholinesterase (AchE) (enzyme responsible to degrade acetylcholine in the synaptic cleft) (Blennow et al., 2006). Taking into account the mechanism of action of AchE inhibitors, it is not expected that these change the natural course of AD, only mitigating some of the symptoms (Blennow et al., 2006). Some studies have shown, however, that these treatments can be effective for up to 2 years (Bullock et al., 2005; Courtney et al., 2004), having even some studies suggest benefits of this treatments for up to 5 years (Bullock and Dengiz, 2005).

More recently, a new approach in neurotransmitter targeting emerged, i.e. Memantine acting on glutamatergic signalling (Tayeb et al., 2012). In normal conditions, glutamate and N-methyl-D-aspartate (NMDA) receptors have an important role in learning and memory processes (Blennow et al., 2006). It is suggested that during AD progression an increased glutamatergic activity, which can lead to sustained low-level activation of NMDA receptors, leads to neuronal toxicity and dysfunction (Areosa et al., 2009). In that respect, Memantine, a non-competitive NMDA-receptor antagonist, is believed to provide

neuronal protection against glutamate-mediated excitotoxicity, without changing the physiological activation of NMDA-receptor, needed for proper cognitive function (Wilcock, 2003).

1.7.2 Aβ-directed therapies

In order to treat AD, investigation in disease-modifying strategies is ongoing (Tayeb et al., 2012). In the last years this investigation has focused mainly on reduction of Aβ toxicity (Blennow et al., 2006). In a Aβ-directed therapies, three classes of medications were developed: secretase modulators (decrease of Aβ production); anti-aggregants (which prevent aggregation); and immunotherapy (focusing Aβ clearance) (Tayeb et al., 2012).

1.7.2.1 Secretase modulators

β-secretase inhibitors have been demonstrated to reduce brain Aβ concentrations in AD transgenic mice (Chang et al., 2004), however these inhibitors had many problems in development, since β-secretase has other substrates other than APP (Tayeb et al., 2012). Another drawback of this approach is the reported behavioural profile of BACE1-knockout mice varying from, similar to wild-type phenotypes (Cai et al., 2001; Luo et al., 2001), behavioural and memory dysfunction (Harrison et al., 2003; Ohno et al., 2004), to even deadly phenotype with early mortality (Dominguez et al., 2005). Also, β-Secretase inhibitors have been a challenge to develop, because the structure of BACE1 is a member of the class of aspartyl proteases, and so, inhibitors would have to be large and hydrophilic molecules because of BACE1 catalytic site is unusually large (Dislich and Lichtenthaler, 2012). These properties pose problems for the pharmacokinetics required for blood–brain-barrier penetration, and subsequent therapeutic efficacy (Tayeb et al., 2012). Many compounds have been in investigated in order to overcome these obstacles, however limited candidates are suitable to start a clinical trial. Even the ones that reached Phase II/III clinical trials, have not shown to hold significant disease-modifying effects (Karran et al., 2011).

Inhibitors and modulators of γ-secretase activity has been an attractive and promising target for disease modification (Tayeb et al., 2012). It was first shown to decrease *in-vivo* Aβ production in 2001 (Dovey et al., 2001). This was a real promising development, however a valid concern about γ-secretase inhibitors existed and was related to the fact that γ-secretase is a protease for a number of essential substrates in addition to APP (Tayeb et al., 2012). One of them is the “Notch” receptor protein, a transmembrane protein that must be cleaved by γ-secretase in order to release its intracellular domain to perform its essential functions related to intracellular signalling and modulation of differentiation and proliferation of

various types of dividing cells (Pollack and Lewis, 2005). Non-selective inhibition of γ-secretase was shown to inhibit Notch signalling in animals, producing pathologies such as gastrointestinal fucoid enteropathy, and abnormal lymphocyte differentiation(Wong et al., 2004). Moreover, γ- secretase knockout mice were shown to have a fatal phenotype similar to the fatal phenotype of Notch knockouts (De Strooper et al., 1999). These side effects posed serious limitations to clinical trials.

Recently, the interest in γ-secretase research focused in so-called selective inhibitors or modulators. These compounds do not completely block the function of γ-secretase, but rather selectively alter the enzyme's function of APP cleavage without altering cleavage of essential substrates such as Notch. Several mechanisms were proposed to explain how this group of agents accomplishes this desirable outcome. These include: 1. non-competitive binding to the γ-secretase enzyme, at a site different from the catalytic one, inducing a disruptive conformational change; 2. binding to the APP itself and rendering it unavailable for cleavage; and 3. binding to the cell membrane inducing allosteric modifications in the enzyme-substrate complex. In any case, these medications have the potential to modulate production of amyloid species, shifting production away from amyloidogenic to non-amyloidogenic ones (Oehlrich et al., 2011).

Another approach in APP associated secretases is α-Secretase pathway stimulation that will lead to a reduction of the sAPPα substrate available for the amyloidogenic pathway, leading to the formation of a soluble segment (sAPPα), which was shown to be neuroprotective (Furukawa et al., 1996), and a stimulant for synaptogenesis (Small et al., 1994). Stimulation of this pathway, therefore, was another attractive way for the potential development of disease-modifying drugs. So far, there have been no major compounds modulating these pathways that have emerged from animal studies to reach clinical trials (Tayeb et al., 2012). However, activators of PKC were shown to clear Aβ load in transgenic mice with a mutation in APP gene, with the activator in this case being bryostatin (Etcheberrigaray et al., 2004), and also shown to clear Aβ in cell lines with different activators, AA-CP4, EPA-CP5, and DHA-CP6 (Nelson et al., 2009). One of the thought mechanisms that lead to Aβ clearance, is PKC-mediated activation of α-secretase (Etcheberrigaray et al., 2004; Nelson et al., 2009). Another proposed mechanism of PKC action is by inhibition of BACE1 activity (Wang et al., 2008).

1.7.2.2 Anti-aggregants

Another attractive point of intervention in AD is the creation of a way to prevent aggregation of amyloid species. Amyloid species are normally present in healthy brains, and their mere presence does not lead to neurodegeneration. This neurodegeneration is thought to require aggregation of Aβ species to form

oligomers, fibrils and protofibrils, and then deposition in the form of amyloid plaques (Geula et al., 1998; Pike et al., 1991, 1993, 1995). Recent literature, however, provides evidence that the earlier soluble oligomers are also neurotoxic (Walsh and Selkoe, 2007). Many anti-Aβ aggregation agents have been tested in clinical trials (Tayeb et al., 2012).

1.7.2.3 Amyloid removal

Another approach to modify the amyloid cascade is removal of amyloid from the brain. Theoretically, removal of amyloid can be achieved through activation of its degrading enzymes, enhancing of its transport mechanisms from the brain to the peripheral circulation, and direct removal of amyloid species through an immunological response (Citron, 2010). The most important known Aβ-degrading enzymes include neprilysin, insulin-degrading enzyme, and plasmin (Eckman and Eckman, 2005). Tissue plasminogen activator inhibitor inhibition was achieved in transgenic mice, leading to a reduction of plasma and brain Aβ levels (Tayeb et al., 2012). Regarding Aβ transport, the receptor for advanced glycation end product (RAGE) mediates transport of Aβ into the brain, whereas low-density lipoprotein receptor-related protein 1 (LRP-1), mediates its transport from the brain to the peripheral circulation. A RAGE inhibitor developed by Pfizer, PF-04494700, was tested in a 10-week placebo-controlled clinical trial in 55 mild to moderate AD patients, showing tolerability but an inconsistent effect on plasma Aβ levels and cognitive performance (Sabbagh et al., 2011).

As an alternative to small molecule approaches, Aβ immunotherapy principle was first reported in a paper showing that active immunisation of AD transgenic mice with fibrillar Aβ attenuated Aβ deposition (Schenk et al., 1999). Similar results were obtained by use of passive immunisation with antibodies against Aβ (Bard et al., 2000). The effect might be mediated by anti-Aβ antibodies that bind to Aβ plaques and induce Aβ clearance by microglia (Bard et al., 2000; Schenk et al., 2004) or alternatively, bind soluble Aβ in the periphery, thereby driving an Aβ efflux from the brain. These results were the basis for initiating clinical trials with active immunisation with the vaccine AN1792, composed of preaggregated Aβ42(Schenk et al., 2004). However, the phase IIa AN1792 trial had to be interrupted because 6% of cases developed encephalitis (Orgogozo et al., 2003). This side-effect has been suggested to be due to a T-cell response against the mid-terminal and C-terminal part of the peptide (Schenk et al., 2004). The second generation of immunotherapy, Aβ immunoconjugates composed of the N-terminal part of Aβ conjugated to a carrier protein (Schenk et al., 2004), or virus-like particles, could allow for active immunisation with reduced risk of Th-1 mediated side-effects. Both active immunisation with N-terminal Aβ fragments (AN1792, Phase II clinical trial) and passive immunisation with humanised anti-Aβ monoclonal antibodies (Bapineuzumab and Solanezumab, both in Phase III clinical trials) have been tested and showed limited or

no clinical efficacy (Delrieu et al., 2012). Despite the strong scientific base of the amyloid hypothesis, clinical trials targeting Aβ with small molecules or immunotherapy were unsuccessful or showed limited improvement of mild cognitive impairment (Communications Eli Lilly at the AD/PD conference 2013). As initial neuropathological changes occur 19 years before the clinical approaches become apparent, it is plausible to attribute these failures to the fact that disease modifying approaches targeting Aβ should be initiated much earlier. The ultimate proof of this assumption will be provided by different prevention trials on clinical cohorts having individuals that are genetic at-risk (DIAN, API) or biomarker positive (A4) (Sperling and Johnson, 2013). In this latter group, cerebrospinal fluid (CSF) Tau (total and phosphorylated) is one of the biomarkers.

1.7.3 Anti-inflammatory and neuroprotective approaches

Several treatment approaches have been based in epidemiological studies. Observational studies have suggested a protective effect of different types of drugs or supplements, but when tested in randomised controlled clinical trials designed to avoid the many potential biases and inherent methodological problems in epidemiological studies, beneficial effects have been difficult to establish. These drugs and supplements are anti-inflammatory drugs, cholesterol-lowering drugs, oestrogens, and antioxidants (Blennow et al., 2006; Tayeb et al., 2012).

1.7.4 Tau-targeted treatment strategies

Many therapeutic approaches to target Tau pathology have been pursued in recent years in animal models (Brunden et al., 2009). Transplantation of cells with the potential to differentiate in situ either into neuronal or glial cell types can be an interesting field of research (Ferrari et al., 2000). This approach was successfully applied to mice with a combined Tau and Aβ pathology in which neural stem cell (NSC) transplantation improved cognition via brain-derived neurotrophic factor (BDNF) (Blurton-Jones et al., 2009). With this method, spatial learning and memory deficits were rescued without altering the Aβ or Tau pathology. Antioxidant strategies may also be possible as both Tau and Aβ cause mitochondrial dysfunction and increased levels of reactive oxidative species (Eckert et al., 2008). The more studied approaches are focusing Tau aggregation, MT stabilization, target of Tau phosphorylation, and also Tau-based immunization approaches. Tau-targeted treatments are resumed in Figure 6.

1.7.4.1 Anti-Tau aggregation therapy

It is possible to aim inhibition of Tau oligomer and eventually, fibril formation. Blocking Tau/Tau aggregation with small-molecule drugs is generally thought to be difficult because of the large surface areas that are involved in such interactions. There is however growing evidence that Tau multimerization can be disrupted with low-molecular-mass compounds (Brunden et al., 2009). Methylen blue dye, e.g., has been reported to inhibit Tau aggregation successfully. However, while phase II data presented at the International Conference on Alzheimer's Disease (ICAD) in 2008 suggested that this compound had a positive therapeutic effect (Wischik and Staff, 2009), more results of this compound in a phase III clinical

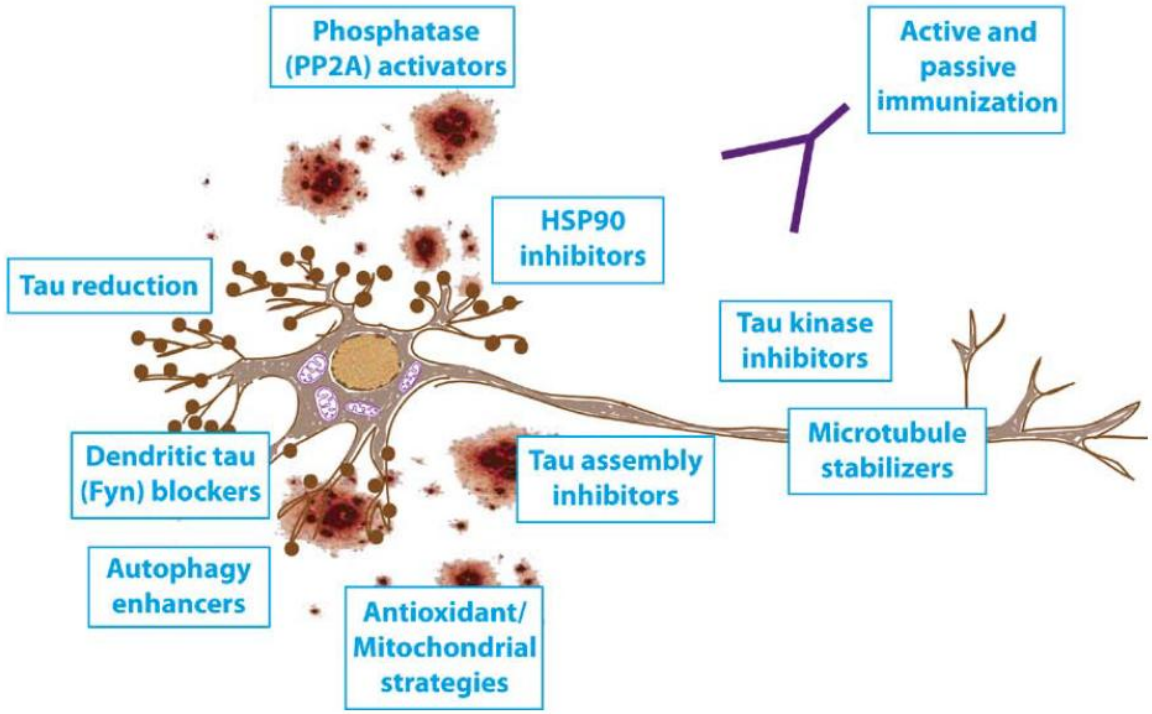

Figure 6 – Tau-related strategies and their site of action in the cell (Adapted from Jürgen Götz et al., 2012)

trial are needed. Many research teams have identified several chemical entities that may inhibit fibrillization (Brunden et al., 2009). The most useful compounds to target Tau aggregation are those that prevent the initial stages of Tau/Tau interaction, so that they lead to an increase of Tau monomers and not an accumulation of intermediate multimeric structures, which could have detrimental biological effects (Brunden et al., 2008).

1.7.4.2 Targeting of microtubule stabilization

A damaged axonal transport and MT function are a central pathomechanism in Tauopathies (Götz et al., 2006). In Tau transgenic mice with axonopathy, amyotrophy and consequently, a motor phenotype, a

reduction in MT density and fast axonal transport was found (Ishihara et al., 1999, 2001). After treatment with MT-stabilizing drug paclitaxel, these mice showed a significant improvement of fast axonal transport and MT density compared with vehicle-treated mice. Furthermore, their motor function markedly improved (Zhang et al., 2005). Epothilone D, a brain-penetrant MT-stabilizing agent, showing reduced axonal dystrophy and increased axonal MT density to improve fast axonal transport and cognitive performance in an aged PS19 mouse model. These mice also had less forebrain Tau pathology and increased hippocampal neuronal integrity. These data reveal that MT-stabilizing drugs hold promise for the treatment of AD and related Tauopathies, and that this drug could be a candidate for clinical testing (Zhang et al., 2012). To develop successful treatments for humans for AD using this approach, it is important to keep peripheral levels of MT-stabilizing drugs as low as possible (Götz et al., 2012).

1.7.4.3 Targeting Tau folding

There are cellular mechanisms described to clear unfolded or misfolded proteins. These require refolding by molecular chaperones, such as the heat shock proteins (HSPs), or eliminated by the ubiquitin proteasomal system (UPS) (Petrucelli et al., 2004). In AD, protein aggregation can also trigger cellular stress that may initiate autophagy, a cellular degradation pathway, which involves the lysosomal machinery (Higgins et al., 2010). There is evidence that mutant Tau transgenic mice have alterations in UPS, to change turnover of Tau, and an enhanced stress response (David et al., 2006; Dickey et al., 2009). In AD, there is increasing evidence that both systems, autophagy and UPS, are affected (Keck et al., 2003; Nixon et al., 2005). Targeting of these systems can be a possible way to treat AD (Götz et al., 2012).

1.7.4.4 Inhibition of Tau phosphorylation

The correct identification of Tau phosphorylation sites that lead to Tau dissociation and aggregation is believed to help to identify a biological role for the kinases and phosphatases involved in its hyperphosphorylation and dephosphorylation, respectively (Ferrari et al., 2003; Hoerndli et al., 2004; Pennanen and Götz, 2005; Steinhilb et al., 2007). These enzymes are therefore excellent targets for a therapeutic intervention in AD and related dementias (Iqbal and Grundke-Iqbal, 2008). There are several kinases that have been shown to phosphorylate Tau in vitro. MAP/MT affinity-regulating kinase 1 (MARK1), that phosphorylates two serine residues that are located within the (Ser262/Ser356) is one of those enzymes. Other enzymes that are known to be involved with Tau phosphorylation are cyclin-dependent kinase 5 (CDK5), glycogen synthase kinase 3 (GSK3), extracellular signal-regulated kinase 2

(ERK2) and p38, among others. All of these phosphorylate various epitopes outside of the MBD region (Dolan and Johnson, 2010). Within the AT8 antibody epitope Ser202 and thr205 phosphorylation is mediated by CDK5, GSK3β, MAPK, and PKA kinases, (Goedert et al., 1995). To test Tau phosphorylation, a number of antibodies were developed to recognize pathological forms of Tau protein. Some of these antibodies are: pT153, pS262, TG3, pT175/T181, 12E8, pS422, pS46, pS214, AT100, AT8 and PHF-1 (Augustinack et al., 2002). Some of these antibodies, e.g. AT8, show strong staining of NFT.

As mentioned above, different kinases have been reported to phosphorylate Tau and accordingly it is hypothesized that inhibitors of these kinases would decrease Tau hyperphosphorylation. Indeed there are some examples that support this hypothesis. One of the most studied molecule is the GSK3 inhibitor lithium chloride (however targeting other molecules like inositol monophosphatase) that reduced levels of insoluble Tau, hyperphosphorylated Tau and behavioural impairment in various Tau transgenic mouse models (Caccamo et al., 2007; Engel et al., 2006; Noble et al., 2005; Pérez et al., 2003; Reynolds et al., 2008). Another inhibitor valuable for the validation of therapies for AD is the non-specific kinase inhibitor K252a (for CDK5, GSK3 and ERK1), having also shown to reduce levels of hyperphosphorylated Tau. In this transgenic mouse model, soluble aggregated hyperphosphorylated Tau was markedly reduced, and motor deficits typical of the model were prevented, however NFTs were not reduced (Le Corre et al., 2006).

Recently it was shown that a small orally delivered compound, sodium selenate, induced dephosphorylation of Tau by a protein phosphatase 2A (PP2A)-dependent way in two Tau transgenic mouse lines, pR5 and K3 (Ittner et al., 2008; Pennanen et al., 2004). This resulted in a reduction in Tau phosphorylation and aggregation and also reduced behavioural impairment in memory and motor functions as well as preventing neuronal loss (Van Eersel et al., 2010). Until now, only three Tau-directed drugs have progressed into human clinical trials, but results on their efficacy are not yet available (Brunden et al., 2009; Hampel et al., 2009). Given this fact and the major role of Tau in disease, there is a great need for new therapeutic approaches targeting Tau pathology (Götz et al., 2012).

1.7.4.5 Tau-based immunotherapy

Immunotherapy focusing Tau protein is a recent field of research in AD (Götz et al., 2012). Immunotherapy can be either active (with the immunization with a immunogen in order to develop immunity), or passive (with the injection of antibodies recognizing specific epitopes) (Chai et al., 2011). The first Tau-based immunization approach used full-length recombinant human Tau to immunise C57BL/6 wild-type mice. With this approach, anti-Tau antibodies in the serum were detected in mice that

developed neurological symptoms including tail and hind limb paralysis. Tau-related abnormalities were visualized by Gallyas silver impregnation and were detected in both neurons and glial cells in brain stem and spinal cord. In order to confirm the presence of Tau aggregates, the phosphospecific Tau antibodies AT8 (Ser202/Thr205) and AT100 (Thr212/Ser214) were used. Testing with these antibodies confirmed the results observed with Gallyas silver staining. Axonal damage and inflammation, due to the immunization, was revealed without associated demyelination. Because the axonal damage in the Tau-immunized mice occurred in close contact with cellular infiltrates, it was presumed that a local disruption of the blood-brain barrier would facilitate the passage of serum anti–Tau antibodies. It was concluded that with all these results taken in account, a link between Tau autoimmunity and Tauopathy-like abnormalities was established, indicating potential risks of using Tau for active immunotherapy (Götz et al., 2012; Rosenmann et al., 2006).

While the first attempt caused encephalitis (Rosenmann et al., 2006) subsequent active immunization methodologies using a Tau phospho-peptide, showed efficacy by preventing a pathology in Tau transgenic models, with absence of obvious side-effects (Asuni et al., 2007; Boimel et al., 2010; Boutajangout et al., 2010).

In 2007, Asuni and colleagues used a 30 amino acid peptide that included the PHF1 phospho-epitope of Tau (Ser396/Ser404) with aluminium adjuvant to immunize 2 months-old P301L Tau transgenic JNPL3 mice. This approach strongly reduced Tau phosphorylation and led to an increase in Tau solubility. The Tau antibodies generated in the animals recognized pathological Tau on brain sections, and levels correlated inversely with the pathology. The fact that immunotherapy was performed from 2 to 8 months of age (with 2 months being prior to when JNPL3 mice develop NFTs) leaves the question if an immunization at a later age would also remove existing NFTs and the associated Tau pathology, in other words, if this approach leads to treatment instead of delaying pathology (Asuni et al., 2007).

In another experiment, similar results were obtained by immunizing mice that express all six human Tau isoforms on an MAPT-/- background, the hTau model (Andorfer et al., 2005), together with M146L mutant PS1 (Boutajangout et al., 2010). When the mice were 3–4 months old, they received peptide that comprised the PHF1 phospho-epitope of Tau (Ser396/Ser404) intraperitoneally in aluminium adjuvant, like in the previous study, with the first three injections every 2 weeks, until 7-8 months of age. After that time, administration was performed at monthly intervals. This approach strongly reduced Tau pathology throughout the brain. The solubility of Tau was not altered at statistically significant values but there was a trend towards a reduction in the PHF1-immunized group. In mice (PHF1-immunized and control-immunized) microgliosis and astrogliosis shown similar levels, suggesting that the gradual removal of Tau aggregates is not due to gliosis. Besides the biochemical reversal of pathology, it was revealed that the

cognitive impairment, characteristic for the model system could be prevented by vaccination. The improvement in the cognitive impairment was tested by learning and memory tests, including radial maze learning and retention, closed-field symmetrical maze, and object recognition. As in the study conducted by Asuni and colleagues, autoantibodies were found in the controls, being likely to be present in the immunized mice (Boutajangout et al., 2010). In AD patients, autoantibodies are reduced, but their role in pathogenesis remains unclear, although naturally occurring auto-antibodies have been suggested as a treatment approach in AD (Dodel et al., 2011; Götz et al., 2012).

In a different study (Boimel et al., 2010), 3 month-old K257T/P301S double mutant Tau-expressing mice were immunized with a mix of three short peptides comprising the phosphorylation sites Ser202/Thr205 (PHF1), Thr212/Ser214 (AT100) and Thr231 (AT180), respectively. Tau phosphorylation and NFT formation, seen by immunohistochemistry and Gallyas silver staining, was significantly reduced. Like in the previous studies, treatment was started before the onset of NFT formation and efficacy of the immunization approach after NFTs have formed is awaited with great expectation. Like in the study of Boutajangout and colleagues in 2010, immunization of these mice did not result in astrocyte activation, existing however a slight increase in the number of lectin-positive, but inactive, microglia observed (Boimel et al., 2010). Infiltration with peripheral monocytes was not shown in this study. Contribution of glial cells in this mechanism remains still to be completely understood. In a study with a similar approach, P301L mice at 4, 8 and 18 months were treated with small phosphorylated Tau fragments, and it was seen that there is reduction of Tau aggregation and phosphorylation even in aged mice, well after onset of NFT pathology, which starts at 6 months. This means that Tau-targeted immunization can have an effect in aggregation after pathology is settled (Bi et al., 2011).

Like referred before, passive immunization is also possible to target Tau pathology (Götz et al., 2012), and a passive immunization approach has been tested by two groups. The first study used immunization of two to three-month old JNPL3 mice weekly with either PHF1 (250µg/125µl) or pooled mouse IgG for a total of thirteen injections (Boutajangout et al., 2011). Three behavioural tests were made, the traverse beam, rotarod and locomotor activity. The treated mice performed better in one of these tests, the traverse beam test. Insoluble Tau levels were reduced (particularly of CP13-Tau) while those of soluble Tau stayed unaffected. PHF1 immunoreactivity in the dentate gyrus was reduced twofold in the immunized group compared to the control. With this testing there was no evidence of an increased astrogliosis. The second study employed antibodies for the PHF1 epitope Ser396/Ser404 as above, and in addition for the early conformational epitope MC1, and a control mouse IgG (Chai et al., 2011). Two mouse models were tested, JNPL3 and P301S. In JNPL3 study, antibodies were administered at 15 mg/kg three times a week for 2 months and then at 10 mg/kg twice a week for the remaining two months, while in the P301S study, antibodies were administered at 15 mg/kg twice weekly. The vaccination with the two

Tau-specific antibodies caused reduced levels in hyperphosphorylated Tau (such as of the 64 kDa species), however, total transgenic Tau levels (HT7) were not affected. The treatment delayed the onset of motor function decline (as determined on the RotaRod) and also weight loss (in both strains). This was accompanied by a concomitant reduction in neurospheroids (undifferentiated neural stem cells) in the spinal cord. Interestingly, both therapeutic Tau antibodies, despite recognizing different pathological epitopes, produced very similar levels of phenotypic improvement (Chai et al., 2011). This experiment showed however different effects on different phospho-epitopes in the two treated groups. Moreover, therapeutic long-term effects on (motor) neuron degeneration still need to be established.

The mechanisms, by which Tau-directed antibodies improve the Tau-associated pathology are far from being understood, and further investigations beyond these studies are essential (Sigurdsson, 2009). While one study has revealed the presence intraneuronal antibodies upon Tau-targeted immunization (Asuni et al., 2007), another study showed antibodies in brain vessels, but not neurons or brain parenchyma (Boimel et al., 2010). It was found that in an active immunization trial of P301L Tau mutant pR5 mice using the Aß peptide, anti-Aß antibodies bound to the intracerebrally injected Aß aggregates (Kulic et al., 2006). There is not a consent as to whether and to which extent antibodies enter the brain and in particular, the cytoplasm of neuronal and glial cells (Winton et al., 2011).

Concerning Tau-based immunotherapy, a new and important aspect has emerged very recently, the concept that Tau is secreted and is spreading. Tau pathology in AD starts in the medial temporal lobe, but with the progression of the disease, Tau pathology shows throughout the brain, in a particular known sequence of affected brain areas (Braak and Braak, 1995).

The essential molecular mechanisms of this spreading are not fully understood, but there is data suggesting that non-NFT forming Tau has been converted to NFT-forming Tau (Allen et al., 2002; Clavaguera et al., 2009; Probst et al., 2000). A recent study conducted by Liu and colleagues has shown that there exists a trans-synaptic spread of pathological forms of Tau in the brain (Liu et al., 2012). Studies have shown that Tau can be released from and taken up by cultured cells and in vivo (Frost et al., 2009; Kim et al., 2010). This suggests that the sink hypothesis could also relate to Tau, with Tau being sucked away from the cytoplasm into the interstitial space (Götz et al., 2012). As has been suggested, it is probable that Tau antibodies can target pathological Tau both extra- and intracellularly (Sigurdsson, 2009). Extracellular Tau clearance can be predicted to occur similar to what is thought to take place with antibodies targeting Aβ. Antibody binding may directly promote disassembly and as well as signaling microglia to clear the antibody-protein complexes. Intracellular clearance may possibly involve direct antibody uptake. The place where antibody-Tau interaction occurs within the cell is likely to be in the endosomal/autophagy-lysosomal system (Sigurdsson, 2009). It may however well be that there is not one

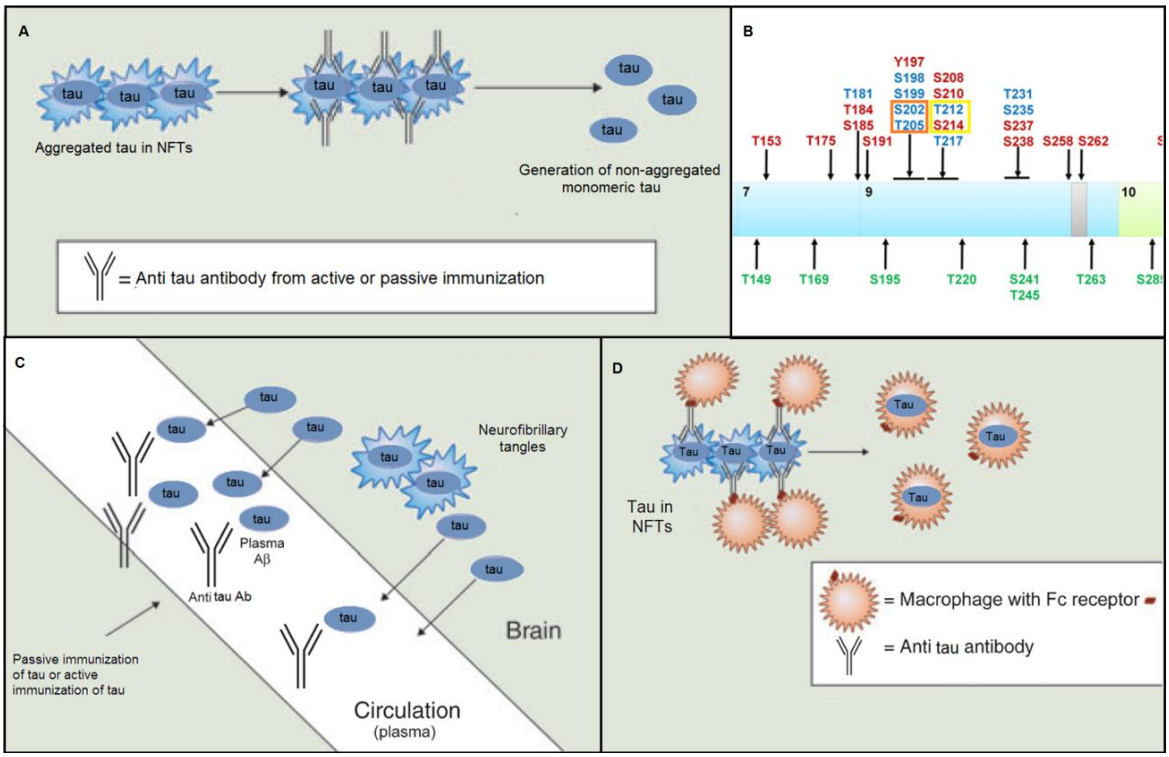

Figure 7 – Possible mechanisms of action of Tau targeted passive immunotherapy

A – Degradation of tau aggregates mediated by antibodies (Antibodies bind tau and modifies the secondary structure to one which minimizes the formation of aggregates);

B – Phosphorylation epitopes recognized by anti tau antibodies (in orange epitope for AT8, in yellow epitope for AT100);

C – Sink hypothesis of tau clearance with anti tau antibodies;

D – Macrophage mediated degradation of NFTs by anti tau antibodies.

(Martin et al., 2011; Morgan, 2011)

mechanism, but that different mechanisms are combined in action and that their relative role is different depending on the mouse strain and the immunogen used (Götz et al., 2012). Possible mechanism of Tau-directed antibody action is represented in Figure 7. Recent research has shown that Tau can be extracellularly secreted, by mechanisms that can be involved with phosphorylation or truncation in aspartic acid 421 (Garringer et al., 2013; Plouffe et al., 2012), and with exosome-associated secretion which was detected in CSF of patients with mild AD (Saman et al., 2012). This secretion of Tau can be related to mechanisms of spreading and could be a potential target for immunotherapy, by blocking the entrance of these forms in other cells. Since there is evidence that this forms of Tau can be present in the CSF of patients, a reduction on the levels of specific fragments on the CSF can be a way to determine if a potential therapy being tested is having its expected effect, and for that is essential to have specific biomarkers for the disease.

1.8 Biomarkers

A fluid biomarker is an entity (metabolite, protein, or modified protein) of which the quantity correlates with disease progression on clinical diagnosis with therapeutic efficacy. For neurological diseases, the most evident source is the CSF although for some markers, plasma can be used as well (Blennow et al., 2012).

The CSF is in direct contact with brain tissue, fact that makes it is a relevant source of biological material to identify biomarkers for neurodegenerative disorders. It is a transparent fluid mainly produced in the choroid plexus but 20% of the CSF consists of interstitial fluid (ISF) containing proteins secreted from neurons and glial cells. Therefore, changes in neuronal cell homeostasis have the potential to be reflected in CSF.

For AD, several biomarkers including Aβ and Tau are currently used to define stages in AD. The main functions of CSF are to protect the brain and spinal cord, and transport waste products from the central nervous system into the blood. Although it is a painful and uncomfortable fluid to collect, CSF is probably the most informative obtainable fluid for neurodegenerative disease prognosis. The amount of material per collection is however limited.

As indicated before, Aβ is a product from APP processing and is released into the extracellular environment after it has been produced. Accordingly, CSF levels of Aβ reflect the level of APP processing. In a dog model, modulation of γ-secretase complex by inhibitors/modulators has a profound effect on different amyloid species, Aβ(37), Aβ(38), Aβ(40), and Aβ(42) in CSF (Borghys et al., 2012). From these C-terminal proteolytic fragments Aβ(40) is the most abundant product found in CSF but on its own, this form does not correlate with plaque load. On the other hand, a decreased Aβ(42)/Aβ(40) ratio is observed in AD and MCI (Mehta et al., 2007). In addition Aβ(38)/Aβ(42) ratio's correlate with pre-symptomatic plaque load (Fagan et al., 2009a) suggesting that looking at combination of biomarkers increases clinical accuracy.

Collectively, these observations position CSF Aβ(42) as an early pre-clinical biomarker in the absence of cognitive symptoms. Nevertheless, as mentioned above, a combination of biomarkers will be required to reach the required sensitivity and accuracy (lower Aβ(42) has been seen in patients with Lewy body dementia and FTD). In addition, it does not correlate very well with disease progression (transition from MCI to AD). The combination of lower Aβ(42) with elevated CSF Tau (total and phospho Thr181) has as 90% sensitivity for AD and is considered as the current standard for AD biomarker based diagnostic (Andreasen et al., 2001; Hansson et al., 2006).

Tau is the main component of NFT and the current assumption is that neuronal loss results in passive release of this protein in the extracellular space resulting in an increase in both total and phosphorylated Tau in different neurodegenerative diseases. In AD, CSF levels of total Tau are increased 2-3 fold in comparison to non-demented elderly (Blennow and Hampel, 2003). This increase can be clearly observed at early clinical stages (MCI) but also in pre-clinical AD (in conjunction with decreased Aβ(42) (Fagan et al., 2009a, 2009b). Interestingly, therapeutic intervention using anti-amyloid-based immunotherapy was able to attenuate this increased levels of CSF Tau (Blennow and Zetterberg, 2012).

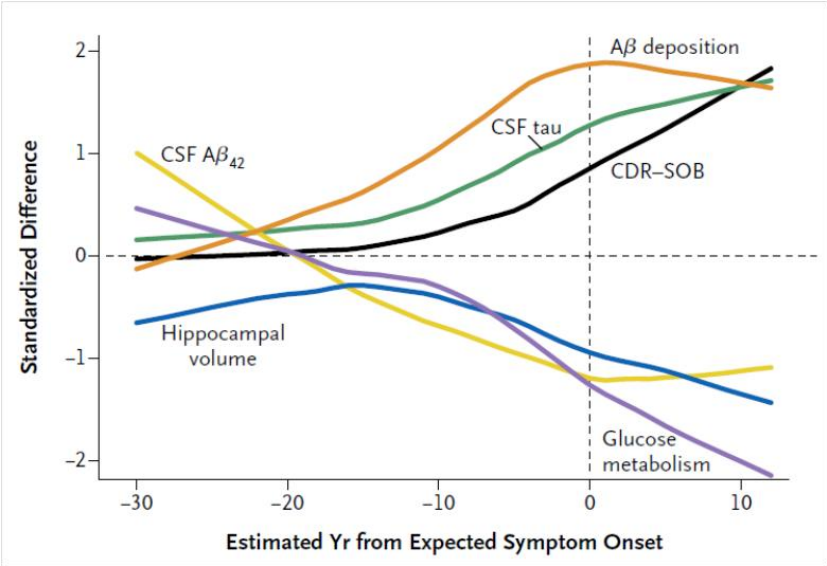

Figure 8 – Comparison of clinical, cognitive, structural, metabolic, and biochemical changes as a function of estimated years from expected symptom onset. (CDR – Clinical Dementia Rating) (Bateman et al., 2012)

Similar to Aβ(42), using Tau as a single biomarker with the current available assays has its limitations. First of all, AD is not the only neurodegenerative disease showing elevated CSF Tau. FTD, CJ but also acute brain trauma has been shown to be accompanied by elevated CSF Tau levels (Andreasen et al., 2001; Hesse et al., 2001). In addition, increased levels of Tau are observed in a later timeframe than alterations in Aβ(42) suggesting that changes in Tau correlate with disease progression but lack the predictive value of identifying the earliest biochemical events underlying AD (Figure 8).

These limitations are the result of the poor understanding of the presence of Tau and phospho Tau in CSF. In light of the recent Braak study positioning the first signals of Tau pathology (AT8 staining) two decades earlier than initial stages of amyloid β pathology, (Figure 4; Heiko Braak et al., 2011) the relatively late changes of Tau in the CSF (Figure 8) are somewhat counterintuitive. One could speculate that increases in extracellular Tau are a result of neuronal cell death, which is occurring after amyloid deposition and correlates quite well with increases in CSF Tau (at least in FAD; Figure 8). On the other hand other Tauopathies like FTLD have clear neuronal cell death in the absence of amyloid deposition. This, together with the fact that Tau levels in CSF are extremely low, could point to the need of higher sensitivity of the assays. In addition to these, quantitative aspects, some qualitative aspects are playing as well. Which of the six isoforms is/are present in the CSF, and why these actually exist in this type of

biofluid, is not known. Also PTMs of CSF Tau are poorly characterized and giving the observation that NFT Tau is hyperphosphorylated, comprehensive analysis of these PTMs is desired and could reveal novel epitopes for development of better assays with full understanding of what the readout stands for.

The identification and characterization of Tau species in CSF, has been hampered by different challenges, being the fact that Tau protein exists as different splice variants and is also subjected to a plethora of PTMs including phosphorylation, glycosylation, proteolytic cleavage etc. one of them. In healthy individuals, levels of Tau are very low (300 ng/L). Despite these challenges, LC-MALDI MS analysis of a tryptic digest on immunopurified Tau revealed fragments encompassing the entire protein with exception of the last 50 amino acids from the C-terminus (Portelius et al., 2008). As these peptides are derived by a tryptic digest, it is not known whether fragments are derived from is an intact or proteolized protein.

In order to properly test pharmacological agents, it is needed to have animal models to test efficacy and kinetics of these agents. One animal model used for this is the dog, that was used in the study of modulation of γ-secretase complex by inhibitors/modulators referred before (Borghys et al., 2012). In this study dogs were treated with these γ-secretase inhibitors or modulators and an effect on Aβ levels on the CSF was observed. The dog model is an ideal one to use due to the possibility of doing longitudinal studies where effects of treatment agents are assessed over time. The dog show also many alterations related with aging, from Aβ deposition, to cognitive impairments, in similar way of AD patients (Borghys et al., 2012; Head, 2013). It is observed also some extent of Tau hyperphosphorylation (Pugliese et al., 2006). This Tau hyperphosphorylation is not, however, associated with aggregation, like in AD patients and many transgenic mouse models. The explanation why there is presence of Aβ pathology but no neurofibrilary tangles on dog brain remains to be explained, and could be a way to understand what are the human specific mechanisms that lead to the development of AD. The exact sequence of Tau protein on dog, unlike that of Aβ, is not know, being currently only predicted from the genome sequencing (Lindblad-Toh et al., 2005; Supplemental Data 1). When analyzing an alignment of the predicted dog Tau protein, and human Tau protein isoform 2N4R (Supplemental Data 2), it is clear that the most conserved region is the C-terminal region, while the N-terminal region show less identity between species.

1.9 Conclusions and objectives

Tau pathology has been widely accepted as an important hallmark of AD. Tau-focused immunotherapy appears as an emerging field of research in the urgent need of development of an AD disease modifying therapy, showing great results in fundamental studies in animal models. This project aims to provide data to answer some questions in Tau immunization and biomarker studies mechanism. What is the epitope of interest of an antibody against human pathological forms of Tau? Has Tau aggregation an impact on extracellular Tau levels and which antibodies are suitable for detection of these forms of Tau? Further investigation on this theme should be conducted in order to provide further knowledge and a possible treatment for AD.

By characterizing antibodies developed against different forms of Tau, both pathological and normal, we hope to answer some of these questions, and apply the knowledge obtained with testing of these antibodies to develop specific assays detecting Tau, both in human, as in other species, such as dog.

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