DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Development of plasma Aβ assays for disease modifying approaches in Alzheimer's disease

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Ana Sofia Soares Nogueira

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Resumo

A doença de Alzheimer (DA) é uma doença neuro degenerativa progressiva sendo a maior causa de demência no mundo. As caracteristicas neuropatológicos desta doença são a acumulação extracelular de péptidos de beta-amilóide (Aβ) e a agregação intracelular de tau hiperfosforilada. Os péptidos Aβ são formados na via amiloidogénica, através do processamento proteolítico da proteína precursora do péptido Aβ (APP) pelas enzimas β- e γ-secretase. Tem sido sugerido que a formação destes péptidos é o evento que desencadeia o desenvolvimento de AD. Hoje em dia, apenas tratamentos sintomáticos se encontram ao dispor destes pacientes. A procura de fármacos com potencial de alterar a progressão da doença é uma área activa de investigação na indústria farmacêutica, encontrando-se alguns compostos em avaliação, em ensaios clínicos. Vários alvos envolvidos na produção e eliminação do péptido Aβ têm sido estudados como potenciais alvos terapêuticos. Inibidores de β-secretase diminuem a produção das formas do péptido Aβ mais longas e com maior potencial de auto-agregação, tais como o péptido Aβ1-42. Biomarcadores permitem não só prever e observar a progressão da DA, mas também monotorizar a eficácia de compostos que permitam alterar a progressão da doença. Biomarcadores actualmente disponíveis para o diagnóstico e avaliação da eficácia de tratamentos incluem marcadores bioquímicos no líquido cefalorraquidiano (LCR) e imagiologia cerebral. No entanto, ambos apresentam limitações: a recolha de LCR é um procedimento invasivo e com possíveis efeitos secundários para os pacientes e imagiologia cerebral é uma técnica com custos elevados. Recentemente, tem sido sugerido que a medição do péptido Aβ em plasma é uma ferramenta de baixo custo e não invasiva para o diagnóstico de DA e para monotorizar a eficácia de terapias que visam as alterações no péptido Aβ. Plasma é barato e fácil de colher, permitindo a recolha rotineira de amostras. No entanto, oferece vários desafios devido ao seu alto teor proteico e devido à presença de anticorpos de interferência. Estes anticorpos influenciam manifestamente a immunodetecção dos péptidos Aβ, impedindo a sua correcta quantificação.

O principal objectivo deste estudo foi então quantificar de forma precisa e correcta os níveis de péptidos Aβ (Aβx-37, Aβx-38, Aβx-40 and Aβx-42) presentes no plasma de caninos e correlacionar o efeito de inibidores de β-secretase nos níveis de péptido Aβ no plasma com o efeito dos mesmos compostos nos níveis de Aβ no LCR. Uma quantificação precisa do péptido Aβ1-40 em plasma foi alcançado com o pré-tratamento das amostras de plasma canino com agentes de bloqueamento de interferências. Os inibidores de β-secretase mostraram diminuir os níveis de Aβ40 no plasma e no LCR. Foi encontrada correlação entre o efeito destes compostos nos dois fluidos.

Palavras chave: Doença de Alzheimer, beta-amiloide, β-secretase, plasma

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the world's major cause of dementia. The neuropathological hallmarks of this disorder are the extracellular accumulation of amyloid beta (Aβ) peptides and the intracellular aggregation of hyperphosphorylated tau. Aβ peptides are formed through the cleavage of APP by β- and γ-secretase in the amyloidogenic pathway and accumulation of Aβ in brain is suggested to be the primary event in AD. Nowadays, only symptomatic treatments are available for AD patients. The search for disease-modifying drugs is an active area in the pharmaceutical industry, and some compounds are being tested in clinical trials. Several targets involved in the production and clearance of Aβ peptides are being studied as therapeutic targets. β-secretase inhibitor (BACEi) compounds decrease the generation of longer and more prone to self-aggregation Aβ peptides, such as Aβ1-42. Biomarkers allow not only to predict and observe the progression of AD but also to monitor the efficacy of disease-modifying drugs. Currently available biomarkers for diagnosis and treatment efficacy evaluation include biochemical markers in CSF and brain imaging. However, both techniques have limitation: the collection of CSF is an invasive procedure with possible side effects to patients and brain imaging is an expensive technique. The measurement of Aβ peptides in plasma has been suggested as an inexpensive, non-invasive tool to diagnose AD and to monitor Aβ-modifying therapies. Plasma is easy and cheap to collect allowing routine sampling over time. Nevertheless, this fluid offers several challenges of its own due its high protein content and the presence of interfering antibodies. These antibodies can interfere with the Aβ immunoassays, leading to an inaccurate measurement of Aβ levels.

The main purpose of this study was to accurately measure Aβ peptide (Aβx-37, Aβx-38, Aβx-40 and Aβx-42) level in plasma samples from canines, and correlate the effect of different Aβ-modifying compounds (BACEi) in plasma with the effect of the same compounds in CSF. An accurate measurement of Aβ1-40 peptide in plasma was achieved with the pre-treatment of dog plasma samples with interference blocking agents. BACE inhibitors were shown to decrease Aβ40 levels in both plasma and CSF. A correlation between the effects of these compounds in the two fluids was found.

Key words: Alzheimer´s disease, amyloid-beta, β-secretase, plasma.

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Abbreviations

Ach - Acetylcoline
ACH - Amyloid cascade hypothesis
AchEIs - Aceylcolinesterase inhibitors
AD – Alzheimer's disease
ADAM - A disintegrin and metalloprotease
AICD – β-amyloid precursor protein intracellular domain
Akt - serine/threonine kinase
Aph-1 - Anterior pharynx-defective 1
ApoE – Apolipoprotein E
APP - β-amyloid precursor protein
APPs-α – Soluble β-amyloid precursor protein derivates (cleaved by α-secretase)
APPs-β - Soluble β-amyloid precursor protein derivates (cleaved by β-secretase)
ATP – Adenosine-5'-triphosphate
Aβ - Amyloid β peptide
BACE - β-site APP cleaving enzyme
BBB - Blood–brain barrier
Ca^{2+} - Calcium
CDK5 - Cyclin-dependent kinase 5
CJD - Creutzfeldt–Jacob disease
CNS - (central nervous system)
COX1 – Ciclo-oxigenase 1
CRP - C reactive protein
CSF - cerebrospinal fluid
Cu - Cupper
DMSO - Dimethylsulphoxide
DR6 – Death receptor 6
ELISA - Enzyme-linked immunosorbent assays
EOAD - early-onset forms of Alzheimer's disease
FDA – Food and Drug Administration
FTLD - Frontotemporal lobar degeneration
Glu - Glutamate
GSIs - γ-secretase inhibitors
GSK-3 - Glycogen synthase kinase 3
GSMs - γ-secretase modulators
HA - Heterophilic antibodies
HAAA - Human anti-animal antibodies
HAMA - Human anti-mouse antibody
HBR - Heterophilic Blocking Reagent

HRPO – Horseradish peroxidase
HSA - human serum albumin
IAPP - Islet amyloid polypeptide
ICAM-1 - endothelial intercellular adhesion molecule 1
IDE - Insulin degrading enzyme
Ig - Immunoglobulin
IRR - Immunoglobulin Inhibiting Reagent
ISF - Interstitial fluid
Iso-Asp - isomerization of Asp
LBD - Lewy body dementia
LOAD - Late onset forms of Alzheimer's disease
LRP - Receptor-related protein
LTD - Long term depression
LTP - Long term potentiation
Lys - Lisine
MAPs - Microtubule-Associated Proteins
MCI - Mild Cognitive Impairment
mg – Milligrams
ml - Milliliter
mM - Millimolar
MRI - Magnetic resonance imaging
MSD – Meso scale Discovery
Nct - Nicastrin
NFTs - Neurofibrillary tangles
ng – Nanograms
NHP - Nonhuman primates
NICD - intracellular domains
nM - Nanomolar
NMDA - N-metil D-Aspartato
NRG1 - Neuregulin-1
NSAIDs - Nonsteriodal anti-inflammatory drugs
N-terminal – Amino-terminal
P3 – 3kDa products of γ-secretase cleavage
p75TNFR - p75 tumor necrosis factor receptor
PBMCs - Peripheral blood mononuclear cells
Pen-2- Presenilin enhancer protein 2
PET - Positron emission tomography imaging
pg - Picograms
pGlu - pyroglutamate
PHFs - Paired helical filaments

PIB - Pittsburgh compound B

PKA - Protein kinase A

PKC - Protein kinase C

PLPH - Post-lumbar puncture headache

PP2A - Protein phosphatase 2

PS - Presenilin protein

PSEN - Presenilin gene

p-tau - Phosphorylated tau

pyro-Glu - cyclized Glu

QuantaBlu – QuantaBlu Flourogenic Peroxidase Substrate

RAGE - Receptor for advanced glycation end products

RIP - Regulated intrammembrane proteolysis

SAP - Serum amyloid P

SNPs - Single-nucleotide polymorphisms

SPs – Senile plaques

STAT3 - Signal transducer and activator of transcription 3

TGF-α - transforming growth factor-α

TNF-α - Tumor necrosis factor-α

t-tau - total tau

VaD - Vascular dementia

VCAM-1 - vascular cell adhesion molecule 1

VEGFR1 – Vascular endothelial growth factor receptor 1

VLP-1 - Visilin-like protein 1

Zn - Zinc

α2M - α2-macroglobulin

αAPP-CTF - Carboxyl terminal fragment products of α-secretase cleavage

βAPP-CTF - Carboxyl terminal fragment products of β-secretase cleavage

µl -Microliter

µM – Micromolar

Chapter 1

Introduction

1.1 Alzheimer's disease

1.1.1 - Definition, History and Epidemiology

Alzheimer disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of tau and β-amyloid (Aβ) aggregates in the brain, progressive neuronal loss, inflammation, and progressive decline of memory and cognition (De Strooper 2010). Degeneration of limbic and association cortices and related subcortical nuclei (Figure 1) slowly robs its victims of their most human qualities: memory, reasoning, abstraction, and language (Selkoe 2011). With disease progression, non-cognitive symptoms such as delusions, agitation, changes in personality, and mood disturbances may also occur (Papassotiropoulos et al. 2008).

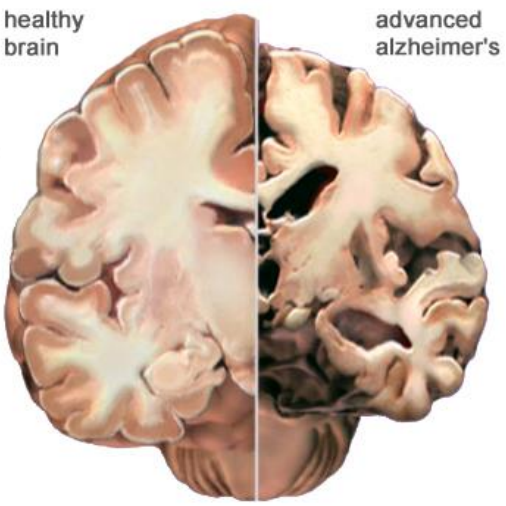

Figure 1 - On the left side a healthy brain; on the right side an advanced AD brain with shrinkage of cortex and hippocampus and enlarged ventricles (http:// www.alz.org).

AD has existed for millennia but was often confused with other syndromes. Just in 1906, Alois Alzheimer described this incurable degenerative disease, establishing a neuropathological phenotype that has enabled considerable diagnostic specificity (Alzheimer 1907), although even today this dementia can only be definitively diagnosed post mortem (Ballard et al. 2011). In his presentation, he described the "miliary bodies" (senile plaques) and "dense bundles of fibrils" (neurofibrillary tangles) that are now recognized as the neuropathological hallmarks of AD (Blennow et al. 2010).

AD is the most common type of dementia (Chopra et al. 2011), representing one of the major health and socioeconomic problems in the world (Kolarova et al. 2012). It is estimated that there are currently about 18 million people worldwide with AD. This disease affects 10% of individuals older than 65 and nearly 50% of those older than 85 years (Chopra et al. 2011). It is predicted that in 2050, 80 million people will suffer from AD in the entire world (Humpel 2011), which is in part caused by the growing elderly population.

1.1.2 – Diagnosis of AD

New criteria and guidelines for the diagnosis of AD have been published recently (Sperlinga et al. 2011), where the different stages of the disease are acknowledged and biomarkers of the underlying disease state are integrated. These are the main differences from the last set of guidelines published by NINCDS–ADRDA, which only recognized the dementia phase of AD.

The recent guidelines recognize and propose 3 different stages for AD (Figure 2):

Preclinical AD is defined as a long asymptomatic period during which the pathophysiological process is progressing and there are measurable changes in biomarkers, before symptoms are visible. The preclinical stages of AD represent a continuum where 3 phases are distinguished: asymptomatic cerebral amyloidosis, asymptomatic amyloidosis + downstream neurodegeneration, amyloidosis + neuronal injury + subtle cognitive/behavioral decline. While the first two may never progress beyond the stage of Aβ accumulation and may never manifest clinical symptoms in their lifetime, the last one is more likely to progress to MCI due to AD and AD dementia. This long preclinical phase of AD provide a critical opportunity for potential intervention with disease-modifying therapy.

Mild Cognitive Impairment (MCI) due to AD refers to the symptomatic predementia phase of AD and it is identified when there is some cognitive impairment unusual for the patient's age and educational background, but not prevents the patient to complete activities of daily livE.

Dementia due to AD refers to the phase of AD where the symptoms such as memory impairment, thinking and behavioral symptoms impair a person's ability to perform daily basis activities. The new terminology for classifying individuals with dementia caused by AD recognizes 3 stages: probable AD dementia, possible AD dementia, and probable or possible AD dementia with evidence of the AD pathophysiological process. (Dubois et al. 2007).

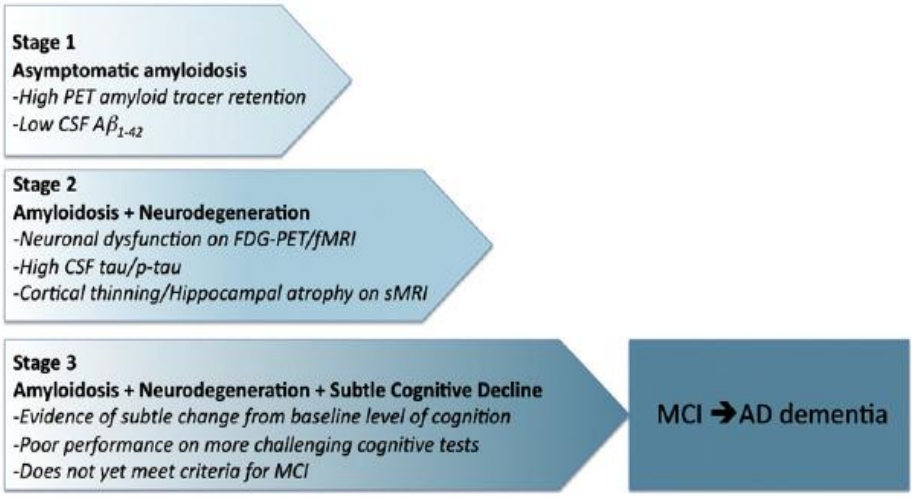

Figure 2: Graphic representation of the proposed staging framework for preclinical AD. Aβ: Amyloid-β; PET: position emission tomography; FDG: fluorodeoxyglucose; fMRI: functional magnetic resonance imaging; sMRI: structural magnetic resonance imaging. (Sperlinga et al. 2011).

Distinguishing AD from other dementias is very difficult because there is an overlap of symptoms and even neuropathological features such as amyloid deposition and/or NFTs in many other types of

dementia. With the new guidelines for the diagnosis of AD there is hope that incorporating scientific knowledge gained and technological will improve current diagnosis of AD and discriminate AD cases from other dementia cases.

1.1.3 - Risk Factors

Findings from epidemiologic and clinical studies suggest that various biological, behavioral, environmental, social and personal factors may contribute to the risk of cognitive decline and the onset of Alzheimer's disease (Hampel et al. 2011).

The most important risk factor associated with AD is ageing. As we age risk factors tend to increase and accumulate, while protective factors decrease. Our organism is more vulnerable to pathogens and biological processes become less efficient. Genetic factors also represent important risk factors. Family history is a very important aspect to determine the probability of developing AD (Tanzi & Bertram 2001). Other risk factors for AD were also investigated. Retrospective studies suggest that individuals with history of traumatic brain injury had a higher risk of dementia than individuals with no history of such injury. Moreover, postmortem and experimental studies support a link between these conditions (Reitz et al. 2012). Risk factors associated with cardiovascular disease are also being implicated in AD (Plassman et al. 2010). Cognitive reserve capacity of the brain, including reduced brain size, low education, low mental ability in early life, and reduced mental and physical activity during late life could also increase the probability of having AD (Mayeux 2003; Ballard et al. 2011). Smoking was also suggested to increase the risk of AD via several mechanisms (Anstey et al. 2007). A number of studies suggest that adopting a Mediterranean-style dietary pattern, especially rich in vegetables (Plassman et al. 2010; Gorelick et al. 2011), and keeping a high physical and intellectual activity may help to reduce the risk of cognitive decline and AD (Reitz et al. 2012; Hampel et al. 2011).

There is a large amount of studies about potential risk factors for this disease, but a lot of them are contradictory and/or do not show consistent data. Alcohol intake is a good example. In one study it is described to be a risk factor for AD (Anstey et al. 2009), while another study suggests that moderate alcohol intake, especially wine, could reduce the risk of Alzheimer's disease (Blennow et al. 2006). A recent study summarized 6907 full papers about risk factors in AD and compared all the information. The majority of the studies/risk factors showed "no consistent association" with AD development, like was the case for alcohol intake. This does not mean that these factors do not play a role in cognitive function, but known evidence is insufficient to draw a firm conclusion (Plassman et al. 2010). It is suggested that the current literature does not provide adequate evidence to make recommendations for pharmacologic strategies or lifestyle changes (Hampel et al. 2011). Critical improvements in research methods are

needed, such as precision, better validation, more standardized cognitive assessment measures across studies and studies of longer duration (Plassman et al. 2010). Nevertheless, a healthy lifestyle, with a strong emphasis on exercise and healthy food could be an important component in the prevention of dementia.

1.2. Tau and Neurofibrillary Tangles

Tau protein belongs to a group of proteins called Microtubule-Associated Proteins (MAPs). This protein promotes the assembly of tubulin into microtubules and microtubule stability. Microtubules are the major component of the neuronal cytoskeleton and are essential for the normal morphology, function and structure of neurons. The binding between tau and tubulin is regulated by phosphorylation through a balance between activity of kinases, like GSK-3 and CDK5 (Wyttenbach & Arrigo 2000), and activity of phosphatases, like PP2A (Chiu & Chuang 2010). Tau can be phosphorylated in 85 possible sites (Martin et al. 2011). Phosphorylation at position 181 is significantly enhanced in AD compared to controls (Hampel et al. 2011). In AD, there is an abnormal phosphorylation of tau that decreases the binding capacity to tubulin, due to conformational changes and misfoldings in the normal structure of tau, leading to microtubule disorganization and tau self-aggregation in the form of neurofibrillary tangles (NFTs) (Kolarova et al. 2012). NFTs are generally intraneuronal cytoplasmic bundles of paired, helically wound 10 nm filaments (PHFs) of tau, often interspersed with straight 10 nm filaments. These structures are mostly present in entorhinal cortex, hippocampal formation, amygdala, association cortices of the frontal, temporal, and parietal lobes, and certain subcortical nuclei that project to these regions (Selkoe 2011). Tau in NFTs is characterized by a high degree of phosphorylation on its 45 serines, 35 threonine and 5 tyrosine residues (Mandelkow et al. 2007) and by assuming a relatively insoluble form, contrary to its normally soluble form in the cytosol. There is an ongoing debate with regard to the extent to which phosphorylation and which specific phosphorylation sites are crucial for tau toxicity or tangle formation (De Strooper 2010). Tau aggregates are often complexed with ubiquitin (Selkoe 2011). If this ubiquitination represents an attempt to remove the tau filaments by way of degradation in the proteasome, it appears to be largely unsuccessful. As state above, in AD tau protein loses is function of keep the cytoskeleton well organized in the axonal process. This will affected the axonal transport of organelles, like mitochondria and endoplasmic reticulum, to the plus-end by kinesin. The absence of these organelles in the peripheral regions of the axons could generate a decrease in glucose, lipid metabolism and ATP synthesis, and the loss of Ca^{2+} homeostasis, that leads to a distal degeneration, process referred to as “dying back” of axons. Furthermore, phosphorylated tau protein has affinity to the kinesin and therefore is transported to the distal sites of neurons. How tau protein becomes

nonfunctional is not entirely understood. Hyperphosphorylation, acetylation, glycation, ubiquitination, nitration, proteolytic cleavage (truncation), conformational changes, and some other modifications have been proposed to cause the loss of normal function and the gain of pathological features of tau protein (Kolarova et al. 2012).

1.3. APP cleavage and Aβ peptides

1.3.1 – APP Cleavage

β-amyloid precursor protein (APP) is a type-I membrane protein with its amino terminus within the lumen/extracellular space and its carboxyl terminus within the cytosol/intracellular space (Haass et al. 2012). Three protease activities called α-, β- and γ-secretase are involved in processing this protein. Besides being the source of Aβ peptides, APP was shown to modulate cell growth, motility, neurite outgrowth, and cell survival in transiently transfected cell lines (Young-Pearse et al. 2007).

There are two major processing pathways for APP: the non-amyloidogenic or anti-amyloidogenic pathway, and the amyloidogenic pathway (Figure 3).

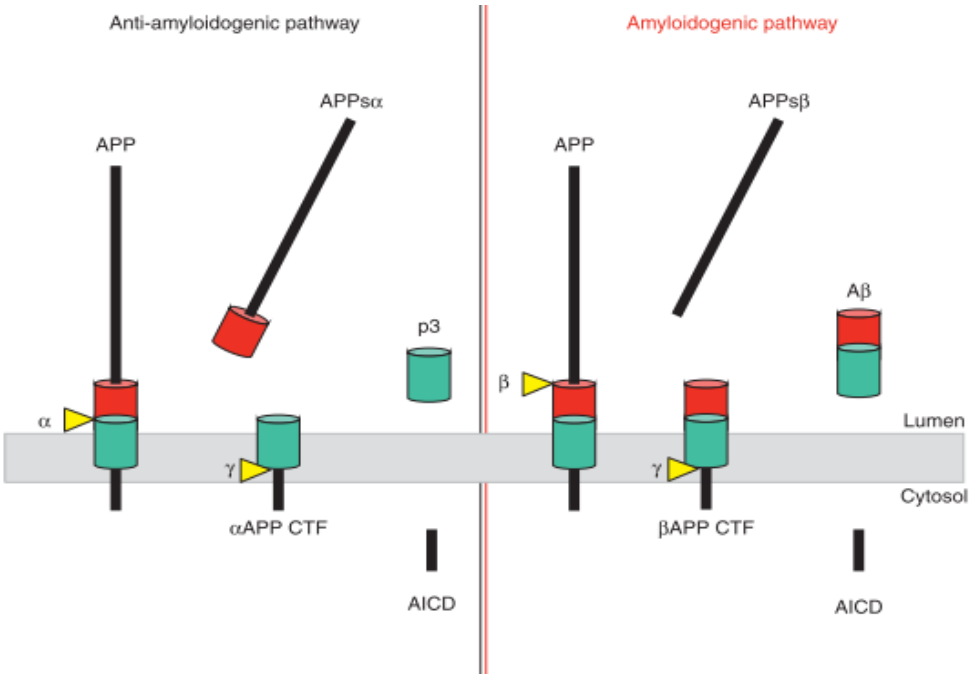

Figure 3 – Proteolytic processing of APP within the anti-amyloidogenic (left) and amyloidogenic (right) pathways (Haass et al. 2012).

1.3.1.1 - Non-Amyloidogenic Pathway

APP is constitutively cleaved in the anti-amyloidogenic pathway. Only 10% of the total cellular APP is cleaved in the amyloidogenic pathway (Murphy & Levine III 2010). The most common scission

happens between residues Lys-16 and Lys-17 of the Aβ region, by a set of proteases termed α-secretases. The soluble ectodomain region (APPs-α) is released from the cell surface, leaving in the membrane the carboxy terminal fragment of 83 amino acids (αAPP-CTF or C83). αAPP-CTF is further processed by the γ-secretase to generate a small peptide (p3) and the APP intracellular domain (AICD) (De Strooper 2010; Selkoe 2011). The p3 fragments have been found in diffuse amyloid plaques in AD brains. However very little is known about the toxicity or function of these p3 fragments (De Strooper 2010).

1.3.1.2 - Amyloidogenic Pathway

Amyloidogenic processing appears to be the preferential pathway of APP metabolism in neurons, largely because of the greater abundance of BACE1 (β-site APP cleaving enzyme 1), whereas non-amyloidogenic pathway is predominant in all other cell types (Haass et al. 2012).

The β-secretase activity initiates Aβ generation by shedding a large part of the ectodomain of APP, a truncated form of APPs (APPs-β). In the membrane remains APP carboxy-terminal fragment of 99-residues (βAPP-CTF or C99). βAPP-CTF can subsequently be cleaved by γ-secretase, releasing Aβ peptides and APP intracellular domain (AICD) (Haass et al. 2012; De Strooper 2010). Aβ peptides are released into vesicle lumens and into the extracellular fluid and AICD is released into the cytoplasm (Selkoe 2011).

The biological functions of APPs, APP-CTF, Aβ, and the AICD remain rather elusive. Aβ peptides seem to play an important role in synaptic physiology, regulating synaptic scaling and synaptic vesicle release (O'Brien & Wong 2011). βAPP-CTF was reported to be associated with neuronal degeneration in brain (Zheng & Koo 2006). Nikolaev and coworkers suggested that APPsβ is further cleaved by an unknown protease, producing a 35-kDa amino-terminal domain fragment that serves as a ligand for the death receptor DR6. When the fragment binds to DR6 receptor triggers the activation of caspase-6 and leads to axonal pruning during embryogenesis (Nikolaev et al. 2009). APPsα has been suggested to exhibit neuroprotective and synapse-promoting activities (Ring et al. 2007). It has been proposed that AICD, after being released into the cytosol, may have nuclear signaling functions (Von Rotz et al. 2004), but this remains controversial (Hébert et al. 2006).

1.3.2 – Secretases

1.3.2.1 - α-secretase

The proteases responsible for α-secretase activity are members of the "A disintegrin and metalloprotease" or ADAM family (Bandyopadhyay et al. 2007). There are two members of ADAM family that seem to have a more relevant α-secretase activity: ADAM 10 and ADAM 17. Although there are

others members, like ADAM 9, that were suggested to have α-secretase activity. Several studies show that down regulation or over expression of ADAM 9, 10, 17 and 19 leads to decreased or increased APPsα generation, respectively (Asai et al. 2003). However, disruption of individual genes that encode ADAM 10, 17 or 19 has no effect on α-secretase processing of APP, indicating that α-secretase activity is shared by a set of ADAM proteases (Weskamp et al. 2002). ADAM10 is the major candidate for constitutive α-secretase activity. This protease is crucial in several signaling pathways and has many substrates like N-cadherin, E-cadherin, β-catenin and Notch. ADAM17 has a vital role in the release of a series of membrane-bound proteins, including transforming growth factor-α (TGF-α), tumor necrosis factor-α (TNF-α), L-selectin, p75 tumor necrosis factor receptor (p75TNFR), and others. The evidence to support a role for ADAM17 in α-secretase processing of APP is not conclusive, but it seems that ADAM17 is responsible for the regulated fraction of the APP cleavage process (De Strooper 2010). Several studies suggested that the increase in activity of α-secretase could be an interesting anti-amyloidogenic therapeutic approach (Donmez et al. 2010; Bandyopadhyay et al. 2007). Stimulation of α-secretase activity should decrease Aβ formation and increase p3 and APPs-α secretion. However, unclarity about the specific functions of p3 and APPs-α complicates this approach.

1.3.2.2 - β-secretase

β-site APP cleaving enzyme 1 (BACE1) is the major β-secretase (De Strooper 2010; Murphy & Levine III 2010). There are two homologous forms of BACE, BACE1 and BACE2, which are >65% homologous. BACE1, is highly expressed in brain and pancreas, but the high pancreatic expression is currently not completely understood (Haass et al. 2012). BACE2 concentration is low in the brain, but it is present in most peripheral tissues at higher levels (Bennett et al. 2000). BACE2 is not involved in amyloidogenesis and may rather exert an anti-amyloidogenic activity in non-neuronal cells (Basi et al. 2003). Neurons from BACE1 knockout mice do not produce Aβ, confirming that BACE1 is the neuronal β-secretase (Cai et al. 2001). These proteases are membrane-bound aspartyl proteases with its active site in the lumen/extracellular space and optimally active at acidic pH (De Strooper 2010). BACE1 cleaves APP at the +1 (prior to amino acid 1) and +11 sites of Aβ. Increased BACE1 protein activity has been described in AD patient brains (Fukumoto et al. 2002).

BACE1 gene expression is affected by many factors: oxidative stress, energy deprivation, ischemia, hypoxia and traumatic injury (Chami & Checler 2012).

So far, only very few physiological substrates have been validated whose cleavage by BACE1 is associated with a clear biological function. BACE 1 knockout mice are viable and fertile and do not show any major behavioral, morphological, or developmental deficits (Cai et al. 2001). However more recently,

very high postnatal expression levels of BACE1 revealed a function of BACE1 in myelination, a process that occurs after birth. BACE1 knockout mice show a significant hypomyelination phenotype in the peripheral nervous system (Willem et al. 2006). Neuregulin-1 (NRG1) signaling pathway is responsible for the regulation of peripheral neuronal system myelination. NRG1 is a physiological substrate of BACE1 and its proteolytic processing facilitates its signaling activity. In knockout mice uncleaved NRG1 accumulates, confirming at least one of the roles of BACE1 in myelination. Since the neuregulin phenotype is a developmental phenotype, it is currently not considered to be a major issue regarding the development of inhibitors. BACE1 has also been shown to be involved in the regulation of voltage dependent sodium channels (D. Y. Kim et al. 2007). Moreover, other substrates such as Type II a-2,6-sialyltransferase, platelet selectin glycoprotein ligand-1, APP-like proteins, Aβ itself, and the interleukin-like receptor type II have also been shown to be processed by BACE1 (Willem et al. 2009). However, the physiological consequences of these cleavages are not always clear yet, and it is important to note that most substrates were identified based on overexpression of BACE1 and/or the substrate, which can possibly generate conditions allowing artificial substrate/protease interactions. Was recently observed BACE1 plays a critical role in retinal homeostasis and the utilization of BACE inhibitors can promote retinal damage and visual loss as a significant side effect (Cai et al. 2012). Clinical development of LY2811376 (Eli Lilly) was recently stopped due in part to retinal pathology in rats (May et al. 2011). With this new data, the utilization of BACE inhibitors should be viewed with caution as they could lead to retinal pathology (Cai et al. 2012).

Because of its crucial function in Aβ generation, BACE1 is a primary drug target for AD. However, the catalytic site of BACE1 is exceptionally long and it has been very difficult to develop small compounds targeting BACE1 in an efficient way in the past, because of several reasons: most inhibitors were large and lacked in vivo efficacy, had low stability and low BBB permeability (De Strooper 2010).

1.3.2.3 - γ-secretase

γ-secretase is a member of intramembrane cleaving aspartyl protease family consisting of multiple subunits. This protease cleaves residues within the transmembrane domain, in a process called regulated intramembrane proteolysis (RIP) (Steiner et al. 2008).

γ-secretase protease complex (230 kDa) consists of one copy of four proteins: anterior pharynx-defective 1 (Aph-1), presenilin enhancer protein 2 (Pen-2), presenilin (PSEN) and nicastrin (Nct) (Figure 4). PSEN is a major constituent of γ-secretase proteolytic activity (De Strooper 2010). PSEN1 encodes for presenilin 1 (PS-1) which contains nine transmembranar domains with a cytosolic amino terminus and a luminal carboxyl terminus. PSEN2 encodes for presenilin 2 (PS-2) and is a close homologue of PSEN1.

Mutations in this subunit affect the conformation of the protein (Oehlrich et al. 2010). Aph-1 is a seven transmembrane protein with a cytosolic carboxyl terminus, that is necessary for γ-secretase activity, but its precise function in the complex is not fully understood (Fortna et al. 2004). It may act as a scaffold protein for the initial binding of Nct and assembly of the complex (Haass et al. 2012). Pen-2 is a 101 amino acid protein with two transmembrane domains whose terminals are both in the lumen (Crystal et al. 2003). Apparently it facilitates PS endoproteolysis into its active heterodimeric state and stabilizes PS within the γ-secretase complex (Hasegawa et al. 2004). Finally Nct is a type 1 membrane glycoprotein with a large luminal domain and an ectodomain responsible for the complex maturation (Oehlrich et al. 2010). Nct is synthesized as a 110 kDa precursor protein that needs PS to leave the endoplasmatic reticulum and to reach the cell surface (De Strooper 2003). Nct has also demonstrated to be important in substrate recognition (Wolfe 2008), required as a size selecting factor for substrate recognition. The reason why PS requires three additional proteins for its activity remains unclear. However, each subunit influences the others stability and they are all a prerequisite for the complex assembly (De Strooper 2010; Wolfe 2008).

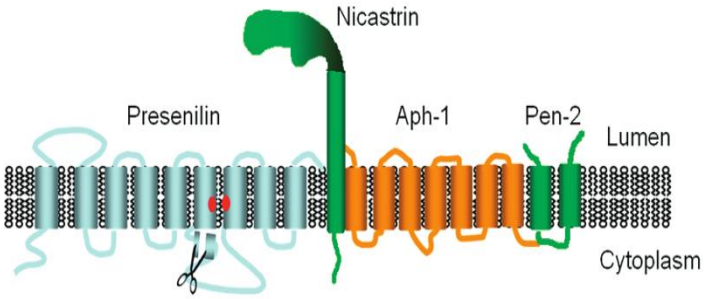

Figure 4 - γ-secretase complex consists of four subunits: presenilin, nicastrin, Aph1 and Pen2. In red the catalytic aspartyl residues are indicated, in the presenilin protein (Oehlrich et al. 2010).

The intramembrane processing of APP by γ-secretase is not restricted to a single site. Cleavage can occur at the ε-site (after amino acid 49 or 48), ζ-site (after amino acid 46 or 45) and γ-sites (mostly at amino acid 42 or 40 but also after amino acid 37, 38, 39 and 43). γ-secretase cleavage of APP is also not precise, changing with physiological conditions, at least between amino acid 37 and 43 of the Aβ domain (Haass et al. 2012). These differences are very relevant for the understanding of AD pathology and therapeutic modulation to selectively prevent Aβ42 generation can be a good approach.

γ-secretase is known to have more than 50 substrates in addition to APP. These include Notch, Jagged and Nectin-1α (Lleó 2008). The different pathways are activated after RIP cleavage, allowing the migration of intracellular domains to the nucleus. Due to the involvement of this secretase in the amyloidogenic pathway many inhibitors and modulators of γ-secretase activity have been investigated (Oehlrich et al. 2010).

1.3.3 – Aβ Peptides

Aβ is a 4 kDa peptide that results from the cleavage of its precursor protein APP by β-secretase and γ-secretase. There are different Aβ species, but those ending at position 40 (Aβ40) are the most abundant (80-90%), followed by Aβ42 (5-10%). The slightly longer forms of Aβ, particularly Aβ42, are more hydrophobic and fibrillogenic, and are the principal species deposited in the brain (Murphy & Levine III 2010). After being produced Aβ42 peptides have a higher capacity to auto-aggregate leading to the formation of dimers, oligomers, fibrils and finally senile plaques (SPs). There are several morphologically distinct types of amyloid plaques like diffuse plaques and neuritic plaques. Diffuse or pre-amyloid plaques contain skeins of insoluble amyloid fibrils and these are intermixed with a poorly defined array of nonfibrillar forms of the peptide. They are largely composed of Aβ42 that suggested to be more neurotoxic than Aβ40. These plaques appear to lack the surrounding dystrophic neurites and altered microglia and astrocytes which are features of the neuritic plaques. Diffuse plaques appear to be less bioactive (i.e., they lack significant surrounding neuritic and glial cytopathology) and they are present even in some cognitively normal people (Selkoe 2011).

It is still a matter of debate which aggregation state of Aβ is toxic. Some studies indicated that SPs might be the main toxic forms of Aβ. Conversely, other studies have suggested that dimers/oligomers of Aβ are toxic and plaques might represent a “protective form”. Aβ dimers/oligomers were shown to inhibit long term potentiation (LTP), which is necessary for learning, memory, and facilitate long term depression (LTD) and decrease dendritic spine density (Shankar et al. 2008). Moreover dimers/oligomers potently induce hyperphosphorylation of endogenous tau, followed by a progressive collapse of neuritic cytoskeleton (Selkoe 2011). In contrast, monomers and amyloid plaques did not impair LTP (Shankar et al. 2008). These data support the hypothesis that soluble oligomers of Aβ are sufficient to induce synaptic loss, tau hyperphosphorylation, neurofibrillary degeneration, and memory impairment, in the absence of amyloid plaques (Hardy & Selkoe 2002).

The Aβ peptides that are isolated from the brains of AD patients display a large variety. This diversity can be explained by some processes, like proteolysis, additional enzymatic modifications, chemical reactions that occur slowly during the years that Aβ peptides are in amyloid plaques or spontaneous reactions such as oxidation, hydrolysis, racemization and disulfide bond or ketoamine formation (Reissner & Aswad 2003). The most common alterations are amino-terminal truncations, cyclized Glu residues (pyro-Glu-3 or pyro-Glu-11) (Miravalle et al. 2005), and isomerization of Asp (iso-Asp) residues (De Strooper 2010). These modifications make the peptide more resistant to proteolytic degradation and/or more hydrophobic as a consequence of the loss of the amino-terminal charge (Russo et al. 2002). pGlu derivates are particularly neurotoxic (Russo et al. 2002) and the glutaminyl cyclase that catalyzes the modification of glutamate (Glu) to pyroglutamate (pGlu) works at low pH

(Schilling et al. 2008). The majority of Aβ peptides are produced in the acidic environment, so modification of Aβ peptide by glutaminyl cyclase may be relevant. Yet, it remains unclear whether glutaminyl cyclase can act intracellularly, or only later, when the Aβ peptide has been secreted. Both pGlu and iso-Asp modifications have deep effects on the biophysical properties of Aβ peptide, and targeting of these modifications has been suggested as possible targets for the treatment of AD. The use of compounds that specifically inhibit glutaminyl cyclase showed benefits in a transgenic AD mouse model by decreasing the amyloid plaque load (Schilling et al. 2008). Clearly, more work is needed to explore this hypothesis. Amino-truncated Aβ species were found to aggregate at the earliest stages of Alzheimer pathology (Delacourte et al. 2002). The full-length Aβ peptides represented 37 ± 7% of all Aβ species in brain. Taken together, truncated variants thus accounted for more than 60%, among which 17 ± 7% and 20 ± 4% corresponded to truncated species starting at residues 4, 5 and 8, 9 and 10, respectively. In AD patients' brain, there are fewer species of amino-truncated Aβ-40 than amino-truncated Aβ-42 (Sergeant et al. 2003). Truncated Aβ peptides exhibit an especially high aggregative and toxic potential and their accumulation was supposed as an early event in AD plaque forming (Bibl et al. 2012). It can therefore be hypothesized that Aβ-40 co-aggregates with Aβ-42 deposits being a late event of Alzheimer pathophysiology (Sergeant et al. 2003). This hypothesis is also supported by observation of the amyloidosis process in the brain of individuals affected by Down's syndrome. Aβ-40 deposits are mainly observed in the oldest Down's syndrome individuals whereas intraneuronal Aβ-42 is the earliest species to accumulate (Mori et al. 2002). Truncated species could play a decisive role as seeds for fibrillogenesis and amyloid deposition.

1.4. The Genetics of Alzheimer's disease

1.4.1 - Familial and Sporadic AD

Based on age at onset, two types of AD are defined: early-onset forms (EOAD) and late onset forms (LOAD) (Lambert & Amouyel 2011). EOAD usually develops between 35 and 60 years of age (Wyttenbach & Arrigo 2000) and is often linked to a genetic cause, like autosomal dominant mutations in APP, presenilin-1 (PSEN1) and presenilin-2 (PSEN2) genes (Murphy & Levine III 2010). This type of AD only affects < 5% of all patients (Rocchi et al. 2003). The majority of patients suffer from LOAD, that occurs sporadic manner (Reitz et al. 2012). Sporadic patients do not have a clear familial history of AD. Nevertheless, there is also a genetic component associated with this late form of AD.

AD is a genetically heterogeneous disorder, and several genes contribute to the disease risk (Papassotiropoulos et al. 2008). Mutations in APP, PSEN1, PSEN2 cause early onset AD while polymorphisms in ApoE (Apolipoprotein E) increase risk to develop AD (Selkoe 2011).

1.4.2 - Amyloid Precursor Protein

40 mutations in APP gene are identified, being 9 of this mutations linked with duplications of APP gene (http://www.molgen.vib-ua.be/ADMutations). These mutations are very informative on the pathogenic mechanisms of AD (Saunders et al. 1993). Two different mechanisms are known by which the APP gene can cause AD: overexpression of the APP gene or mutations causing an increase of amyloidogenic cleavages. The presence of an extra copy of chromosome 21 or microduplications within this chromosome can lead to the increased production of APP (Rovelet-Lecrux et al. 2006). In Down's syndrome the extra chromosome 21 (or part of it containing the APP gene) increase the APP expression thus increasing the Aβ production. Overproduction of these peptides is suggested to lead to the formation of difuse plaques that appear in patients starting at the age of 12 years (Mann et al. 1996). Besides duplication of APP, APP missense mutations exist that increase the cleavage of APP by β and γ-secretase leading to increased processing via the amylodoigenic pathway (Selkoe 2011). The AD-associated missense mutations in APP are mainly found in three different regions: near the β-secretase cleavage site, leading to elevated Aβ levels; mutation within the Aβ sequence leading to enhanced aggregation of this peptide; and mutations surrounding the γ-secretase cleavage site, leading to enhanced production of Aβ42 (Levy et al. 1990; Wolfe 2008). Recently, a coding mutation in APP gene was found to be a protective against AD. This APP variant was found to be common in elderly population. A single nucleotide polymorphism, close to the proteolytic cleavage site of β-secretase, probably impaired the BACE1 cleavage of APP (Jonsson et al. 2012). The protective effect of this mutation further proofs the principle that reducing BACE1 cleavage of APP may protect against AD. The discovery of these mutations has also provided strong support for the amyloid cascade hypothesis of AD pathogenesis.

1.4.3 - Presenilin 1 and Presenilin 2

PSEN1 and PSEN2 are also genes associated with familial AD. Autosomal dominant missense mutations in these genes lead to the development of AD with an early onset, between 35 and 60 years old (Selkoe 2011; Papassotiropoulos et al. 2008). These two genes code for two highly homologous (65% identity) transmembrane proteins called presenilins 1 and 2 (PS1, PS2). PS is a constituent of γ-secretase (Oehlrich et al. 2010), and is crucial in the processing of APP (Papassotiropoulos et al. 2008). PSs are also

involved in developmental morphogenesis (Rocchi et al. 2003). AD causing mutations in PS1 and PS2 enhance the processing of APP to form amyloidogenic Aβ (Hardy & Selkoe 2002). 197 PSEN1 mutations have been identified (http://www.molgen.vib-ua.be/ADMutations). Mutations in the PSEN1 gene are the most common cause of EOAD, accounting for up to 70% of the patients. 25 mutations in the PSEN2 gene are known (http://www.molgen.vib-ua.be/ADMutations). Mutations in this gene account for less than 5% all early-onset patients of the disorder (www.ghr.nlm.nih.gov).

1.4.4 - Apolipoprotein E

Apolipoprotein E (ApoE) is a protein involved in the mobilization and redistribution of cholesterol during neuronal growth after an injury. It is also involved in immunoregulation and activation of several lipolytic enzymes. There are three major isoforms of ApoE: ApoE2, ApoE3 and ApoE4 (three alleles: ε2, ε3, ε4) (Rocchi et al. 2003). These alleles are different from each other on the basis of two single-nucleotide polymorphisms (SNPs), resulting in two amino acid changes at positions 112 and 158 (Papassotiropoulos et al. 2008). The ε3 allele is the most frequent in the population – 72%, followed by ε4 – 17% and finally ε2 – 11%. The ε2 allele has been shown to have an impact on longevity and may confer protection against AD (Rocchi et al. 2003). Many studies have demonstrated that the presence of one or two ε4 alleles of the gene ApoE is the most prevalent genetic risk factor for AD (Selkoe 2011; Rocchi et al. 2003), being present in 30% of the patients. When compared to individuals with no ε4 alleles, the increased risk for AD is 3-fold in heterozygote and about 12-fold in homozygote (Kim et al. 2009). However, ApoE4 is neither necessary nor sufficient to cause AD (Tanzi & Bertram 2001). It has been suggested that ApoE4 allele leads to decreased clearance of Aβ and increased aggregation of this peptide when compared with ApoE3 (Evans et al. 1995; Rocchi et al. 2003). The diverse isoforms of ApoE have different affinities for Aβ and tau protein. ApoE4 binds to Aβ more rapidly than ApoE3 leading to the formation of monofibrils that precipitate into dense structures (Sanan et al. 1994) . On the other hand, ApoE3 and ApoE2, unlike ApoE4, bind to tau protein serving as protection against NFT formation (Weisgraber 1994; Rocchi et al. 2003).

1.5. Amyloid cascade Hypothesis of Alzheimer Disease

Given the cytological and biochemical complexity of the disorder, it has been difficult to come to agreement about the temporal sequence of events that lead to dementia and which steps are most amenable to intervention. The amyloid cascade hypothesis (ACH) proposes that accumulation of Aβ in the brain is the primary influence driving AD pathogenesis. The rest of the disease process, including

formation of NFTs is proposed to result from an imbalance between Aβ production and Aβ clearance (Hardy & Allsop 1991) (Figure 5). This hypothesis is strongly supported by genetic, pathological and pharmacological evidence. Patients with Down's syndrome, who possess an extra APP gene, have increased levels of APP protein, and develop AD by the age of 50 (Esler & Wolfe 2001). The description of a rare case of Down' syndrome in which the triplication of the *APP* locus was absent, and who showed no signs of dementia and no amyloid deposition in brain upon death at the age of 78 years (Prasher & Farrer 1998). Mutations in tau protein gene lead to the development of frontotemporal dementia with Parkinsonism. This disorder is characterized by severe deposition of tau in NFTs in the brain, but no deposition of amyloid. This indicated that tau alterations are not enough to induce amyloid deposition. Thus, in AD the NFTs should form after changes in Aβ metabolism (Hardy & Selkoe 2002). Transgenic mouse expressing high levels of human mutant APP show brain Aβ deposition and synaptic loss and gliosis (Armstrong 2011). Moreover, genetic risk factors such as ApoE can be linked to Aβ physiology as well. Experiments done in APP transgenic mice harboring the human APOE gene showed that ApoE was clearly involved in Aβ clearance, with ε2, ε3 and ε4 being increasingly less effective at clearance of Aβ peptides. This evidence support that an imbalance of Aβ clearance in the brain may be the underlying mechanism driving LOAD cases (Wang et al. 2001). EOAD caused by APP gene mutations also have greater number of NFTs, supporting a link between APP and cytoskeleton (Armstrong 2011). Cases linked to PSEN1 have a greater number of SPs and NFTs compared with cases of sporadic AD, suggesting that PSEN1 may increase tau deposition (Shepherd et al. 2004). Several studies indicate that genetic variability in Aβ catabolism and clearance may contribute to the risk of late-onset AD (Hardy & Selkoe 2002). All these findings support the hypothesis that Aβ accumulation is the initiating step in AD pathogenesis.

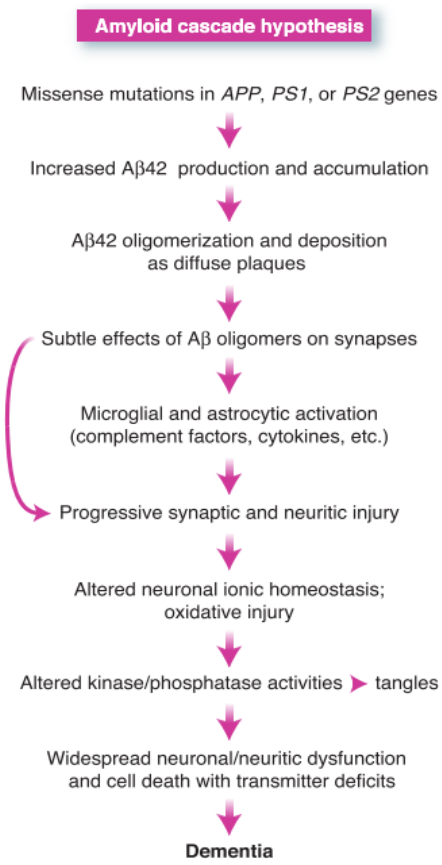

Figure 5 - The sequence of pathogenic events leading to AD proposed by the amyloid cascade hypothesis. The curved violet arrow indicates that oligomers may directly injure the synapses and neurites of brain neurons (Hardy & Selkoe 2002).

On the other hand, there are observations that are difficult to reconcile with the hypothesis. The number of amyloid deposits does not correlate well with the degree of cognitive impairment (Terry 1996). Furthermore Aβ plaques can be found in the cortex of apparently healthy aged subjects (Selkoe 2011). An additional main opposition that challenges this hypothesis is the fact that amyloid-directed

therapies all failed in the clinic until now, while they had shown a great potential in preclinical studies, leading to considerable amyloid burden reduction in the brain. As a result of their failure, there has been controversy on the value of the amyloid cascade hypothesis.

According to this hypothesis, a drug that inhibits the production or facilitates the clearance of Aβ from the brain should significantly slow, cure, or prevent disease progression in AD patients. Despite aforementioned evidence, such a drug is not available yet and currently, the amyloid cascade hypothesis remains clinically unproven (Hardy & Selkoe 2002).

1.6. Animal Models

Alzheimer disease is a human specific disorder. There are currently no animal models available that reflect every facet of AD. However, several specific aspects can be modeled and a wide range of animals are used in the research for new knowledge about AD. Invertebrate animals such Drosphila and C. elegans, mammals like mice, rats, dogs, cats and non-human primates are used as animals models in AD and are each useful due to their specific characteristics that allow specific questions to be investigated. For some mammalian models, Aβ accumulation develops naturally with age (nonhuman primates and dogs), while in other species genetic modifications have to be done to induce Aβ accumulation (mouse).

The use of mouse models offers several advantages. Mice are easy to manipulate and house due to their small size, they have an abundant progeny, they respond well in memory and learning tasks and the cost of their maintenance is lower compared with larger species. The major drawback is that mice do not spontaneously develop AD-like pathology. To induce plaque deposition, the introduction of human genes expressing mutations in APP or both APP and PS1 is required. In general, the incorporation of more mutations leads to acceleration of the pathology. The first transgenic mouse model for AD with plaque deposition was generated in 1995 by 10-fold elevation of mutant human APP expression and consequently the same increase in Aβ levels (Elder et al. 2010; Games et al. 1995). However, transgenic mice expressing a single APP mutation do not demonstrate all the hallmarks of the disease, especially the formation of NFTs and the neuronal and synaptic loss. A triple transgenic model with three mutant genes (APPswe, PSEN1 and TAU) that progressively develops Aβ plaques and NFTs was generated recently. Furthermore, these mice also show gliosis, synaptic damage and inhibition of LTP, decrease in learning ability and memory impairment (Oddo 2003). Nevertheless, limitations of mouse models remain, like the co-expression of human APP and murine APP and the lack of temporal order in the events of the disease (in most of transgenic mice cognitive deficits precede plaque deposition, whereas in human patients the

opposite temporal order occurs), prevent an accurate comparison with human pathology (Pype et al. 2003).

Animals in which Aβ deposition occurs naturally with age could be a more physiologically relevant model but they have their own limitations including a long lifespan and late development of pathology, and their use can also be complicated by ethical considerations and costs (Paul Murphy & Levine III 2010).

Nonhuman primates (NHP) have an identical Aβ sequence to human and nearly identical APP sequence, however they only develop limited AD-like neuropathology with age (Paul Murphy & Levine III 2010). They show low amounts of amyloid deposition in comparison to AD patients, and the Aβ peptides may be more soluble than in AD brain. Abnormal neurofilaments can be identified, but NFT pathology is not a typical feature of pathology in NHPs (Rosen et al. 2009).

Like NHPs, dogs naturally develop Aβ deposition and accumulate Aβ in the brain as they age which coincides with declines in learning and memory (Portelius et al. 2010). In recent times, the lifespan of dogs has greatly increased due to advances in medicine and nutrition. As a result, dogs now suffer from cognitive dysfunction in which neurodegenerative symptoms are exhibited. Cognitive deficit behavior in aged dogs includes disorientation while walking, active incontinence, sleeping during the day, but restlessness at night, development of impaired learning, depressed memory and reduced behavioral flexibility (Yu et al. 2011, Studzinski et al. 2005). Dogs are evolutionary closer to humans than rodents species, which is also reflected in a more similar brain structure and function (Portelius et al. 2010). Like in ageing human brain, dog brain also demonstrates oxidative damage, caspase activation, neuritic dystrophy, astrogliosis, cortical atrophy and decreased brain volume (Studzinski et al. 2005). Canine APP is 98% identical to human APP and is venerable to similar post-translational modifications (Satou et al. 1997). Aβ first accumulates in the prefrontal cortex and then distributes to the enthorhinal and parietal cortices, a pattern similar to that seen in AD patients (Head et al. 1998). Canines develop amyloid deposits by the age of ten years and these deposits correlate with cognitive dysfunction (Borghys et al. 2012). Deposits contain Aβ42, and occur almost always as diffuse deposits with no neuritic plaques or NFTs (Paul Murphy & Levine III 2010). Moreover, Aβ isoforms pattern in CSF from dogs resembles that in humans, making dogs a useful complementary model for assessing therapies that target the reduction of Aβ levels, which may be reflected in the CSF (Portelius et al. 2010; Borghys et al. 2012).

Beagles are a dog bread that is commonly utilized in AD studies. This bread already allowed to show that familial influence is an important determinant of plaque development, that deposits are age-dependent, and that neuritic plaques and NFTs are rare in the laboratory beagles (Russell et al. 1996). A study by Yoshino and co-workers compared density of SPs in the cerebral cortex of several dog breads. Beagle, Collie and Labrador retriever were the breads that showed a higher accumulation of SPs per square

millimeter (Yoshino et al. 1996). Beagles are small and docile dogs which is an advantage in housing and experimental manipulations.

1.7. Biomarkers

A biomarker or biological marker is an objective measure of a biological or pathogenic process that can be used to evaluate disease risk or prognosis, to guide clinical diagnosis or to monitor therapeutic intervention (Humpel 2011; Blennow et al. 2010). The sensitivity, specificity and ease-of-use are the most important factors that ultimately define the diagnostic utility of a biomarker (Humpel 2011). The cerebrospinal fluid (CSF) is the fluid is in close contact with brain and can reflect biochemical changes that occur in this organ, making this fluid an optimal source of AD biomarkers (Blennow et al. 2010). An important obstacle for the use of CSF biomarkers is the need for a lumbar puncture to collect some of this fluid. This is an invasive technique, which should only be executed by qualified physicians to reduce the chance of potential side effects, the most common being post lumbar puncture headache (PLPH). Therefore, considerable efforts are being made to discover new biomarkers in other, more easily accessible fluids, like blood, urine or saliva. Besides the biochemical biomarkers, imaging biomarkers represent a major field of AD research.

1.7.1 – Imaging Biomarkers

Current imaging biomarkers include imaging techniques to visualize *in vivo* aggregates of Aβ and measure brain metabolism and brain atrophy.

Both CSF Aβ42 and amyloid positron emission tomography imaging (amyloid-PET) are biomarkers of brain Aβ plaque deposition. The development of Aβ PET ligands, like Pittsburgh compound B (PIB), has allowed the direct visualization of fibrillar Aβ in patient brain (Jack et al. 2010; Blennow et al. 2010). PIB specifically binds to fibrillar Aβ, and not to soluble Aβ or to diffuse plaques (Jack et al. 2010) being a valuable tool as part of AD diagnosis.

FDG-PET (FDG is an analogue of glucose) is utilized to measure brain metabolism, which indicates synaptic activity (Attwell & Laughlin 2001). Decreases in FDG uptake correlate with greater cognitive impairment. FDG-PET studies in patients with AD show a specific pattern of decreased glucose uptake in a lateral temporal-parietal and posterior cingulate, precuneus distribution. Studies show a correlation between decreased FDG-PET uptake and both low CSF Aβ and increased CSF tau (Petrie et al. 2009).

Magnetic resonance imaging (MRI) can be utilized to measure brain atrophy, which is caused by loss of synapses and neurons and dendritic pruning (Bobinski et al. 2000). There is a correlation between the severity of atrophy and the severity of cognitive impairment in patients along the evolution of AD (Jack et al. 1992). Atrophy on MRI is not specific for AD, but the degree of atrophy correlates well with Braak staging at autopsy (Silbert et al. 2003). Furthermore, although CSF tau is predictive of future conversion from MCI to AD, the predictive power of structural MRI is greater (Jack et al. 2010).

1.7.2 – CSF Biomarkers

CSF biomarkers for AD can be divided into core and basic biomarkers. Basic biomarkers are used to identify conditions that coexist with AD, whereas core biomarkers have been developed to identify the central pathogenic process of AD (Blennow et al. 2010).

1.7.2.1 – Basic Biomarkers

Basic biomarkers include assays for blood–brain barrier (BBB) status and inflammatory processes in the brain. The ratio of albumin concentration between CSF and serum is the standard biomarker for BBB function. An increase in the ratio indicates that there is damage in the BBB and the albumin is crossing this barrier and goes into the brain. Unlike for infections, inflammatory diseases, brain tumors and cerebrovascular disease, in AD the albumin ratio is normal. This allows to distinguish AD from others disorders (Blennow et al. 2010).

1.7.2.2 – Core Biomarkers

The most important core biomarkers currently used for AD are: Aβ42, total tau (t-tau) and phosphorylated tau (p-tau). Enzyme-linked immunosorbent assay (ELISA) is the technique most commonly utilized to measure the different biomarkers in the CSF (Sämgård et al. 2010; Blennow 2004).

As mentioned before, Aβ42 is a product of APP cleavage through the amyloidogenic pathway that in certain conditions can aggregate leading to the formation of amyloid plaques in the brain. In the CSF of AD patients this peptide shows a significant reduction when compared to controls. This reduction is probably caused by its enhanced aggregation and plaque deposition in the brain, reduced clearance, diffusion of Aβ from the brain to the CSF and neuronal loss (Humpel 2011; Blennow et al. 2010). Low levels of CSF Aβ42 correlate with high ^{11}C-PIB biding, which supports the notion that CSF Aβ42 levels reflect fibrillar Aβ42 levels and amyloid plaques in the brain (Blennow et al. 2010). AD patients exhibit a

50% decrease in CSF Aβ42 levels on average when compared with age-matched healthy individuals (Blennow 2004).

Several data suggested that CSF t-tau levels reflect the intensity of neuronal and axonal degeneration in the brain. High CSF t-tau has been associated with rapid cognitive decline (Sämgård et al. 2010) and fast progression from MCI to AD (Blennow et al. 2010).The levels of t-tau increase with age from <300 pg/ml between 21 and 50 years old to <500 pg/ml in people of more than 70 years old. However, t-tau levels are significantly increased in AD patients as compared with age-matched control subjects (Humpel 2011), around 300% higher (Blennow 2004). Total-tau levels also allow to distinguish some disorders, for example in Creutzfeldt–Jacob disease (CJD) tau levels are strongly elevated (>3000 pg/ml) when compared with AD (>600 pg/ml). It might also be a prognostic marker for conversion from MCI to AD, because high CSF tau level has been found in 90% of MCI cases that later progressed to AD (Humpel 2011).

The detection of tau phosphorylated at position 181 is significantly enhanced in CSF of AD patients compared to controls (Humpel 2011). A significant correlation between CSF p-tau-231 concentrations and presence of NFTs in the brain has also been shown (Buerger et al. 2006). Additional to other approaches different phosphorylated sites can be used for differential diagnosis. For example, phospho-tau-231 and phospho-tau-181 can be used to distinguish AD from controls and FTLD, LBD and vascular dementia (VaD) (Grundke-Iqbal et al. 1986). High CSF p-tau 181 levels have been associated with a rapid progression from MCI to AD and with a quick cognitive decline in AD patients (Sämgård et al. 2010).

Besides Aβ and tau biomarkers, there are other possible candidates for CSF AD biomarkers under investigation but until now they do not reach the sensitivity and specificity of Aβ and tau. Few of these biomarkers are briefly discussed below.

BACE1, the main β-secretase responsible for APP cleavage, shows an increased expression and enzymatic activity in AD patient brains. This increase in concentration can be measured in CSF of AD (Barão et al. 2013) and prodromal AD patients (Fukumoto et al. 2002), suggesting that upregulation of BACE1 must be an early event in AD (Blennow et al. 2010). BACE1 levels in CSF show a good correlation with total-tau and hyperphosphorylated tau levels in the CSF, suggesting that the recorded alterations in BACE1 levels correlate with cell death and neurodegeneration (Barão et al. 2013).

Neuronal and synaptic proteins correlate with cognitive function and disease progression, so they are also investigated as potential biomarkers for AD. Visilin-like protein 1 (VLP-1) is a neuronal calcium sensor that is markedly increased in CSF of AD patients. It is also high in patients who carried ApoE4 and also negatively correlated with Mini-Mental state examinations scores (Lee et al. 2009).

Following neuronal damage, free radicals are produced. F2-isoprostane is formed *in vivo* from the free radical-catalyzed lipid peroxidation. Studies show that CSF F2-isoprostane levels are increased in AD patients when compared with healthy individuals of the same age (Montine et al. 2007) and is also increased in cognitively impaired individuals with prodromal AD.

1.7.3- Blood Biomarkers

Due to the invasive nature of the procedure for collecting CSF (lumbar puncture) and requirement of highly trained personnel, this method is incompatible with routine application. The advantage of a blood-based biomarker would be that collecting blood is cheap, rapid, less invasive, easier and can be performed repeatedly. Especially during clinical trials, it is necessary to evaluate the drugs' efficacy and bioavailability over time, which requires continuous sampling. So far blood biomarkers are not being used in the AD diagnosis, while they do have proven their value for monitoring treatments effects. Below several blood-based biomarkers under study will be discussed.

The levels of Aβ are increased in blood plasma from familial AD and Down's syndrome patients, but results are inconsistent for sporadic AD (Cedazo-Minguez & Winblad 2010). However, longitudinal studies have shown that high levels of Aβ42 in plasma is a risk factor for developing AD (Kuo et al. 2000) – see topic 9 "Plasma biomarkers for AD".

Blood platelet APP is processed by the same amyloidogenic and non-amyloidogenic pathways as in brain. Platelet APP processing in AD patients is altered compared to control subjects (Colciaghi et al. 2004). Increased levels of Aβ are produced by platelets from AD patients compared to normal control subjects due to increased β-secretase and decreased α-secretase activities (Tanga et al. 2006).

An unbalance between kinases like GSK3 and CDK5 and phosphatases like PP2A is probably contributing to the hyperphosphorylation of tau protein. GSK-3 activity is regulated by a wide variety of kinases and systems including serine/threonine kinase (Akt), protein kinase A (PKA), protein kinase C (PKC), MAP kinases, and the Wnt pathway (Chiu & Chuang 2010). PKC activity appears to be defective in sporadic AD. The activation of the α-secretase-mediated cleavage of APP is either direct by activation of PKC isozymes, or indirect through PKC activation of ERK1/2, or both (Lammich et al. 1999). On the other hand, Aβ peptides can inactivate PKC (Zhu et al., 2001). In patients who already have elevated Aβ concentration, this Aβ inhibition of PKC may act as a positive feedback, causing greater reduction of α-secretase activity and thus further reduction of Aβ. All these data supports the view that PKC could be an early predictive marker for AD (De Barry et al. 2010).

In the brain of AD patients, ubiquitin levels are elevated and the proteasome pathway fails leading to more Aβ accumulation and consequently more toxicity for the cell. It was recently shown that in peripheral blood mononuclear cells, the enzymes for proteasomal activity are changed. When compared to healthy subjects, the concentration of enzyme E1 was increased in peripheral blood mononuclear cells (PBMCs) of AD patients, whereas the concentration of the enzyme E2 was decreased (Ullrich et al. 2010). Changes in concentration of this two enzymes could also be a predictive marker for AD.

Throughout cell life time the telomere size decreases, leading to cellular senescence. However, it has been shown that PBMCs of AD patients have shorter telomeres than those in age-matched controls and are more sensitive to apoptosis (Reback et al. 2003). Exposure to Aβ40 peptide induced the expression of an unfolded p53 protein isoform in fibroblasts and it was hypothesized that low amounts of soluble Aβ induce early pathological changes at cellular level that may precede the amyloidogenic cascade. One of these changes is the induction of a novel conformational state of p53 in fibroblasts and that could be used as a marker of the early stage of AD (Lanni et al. 2007).

1.8. Pharmacotherapies of AD

There are currently no treatments available that stop, reverse or slow down the neurodegeneration in AD. Available drugs can only moderately and temporarily slow down cognitive symptoms.

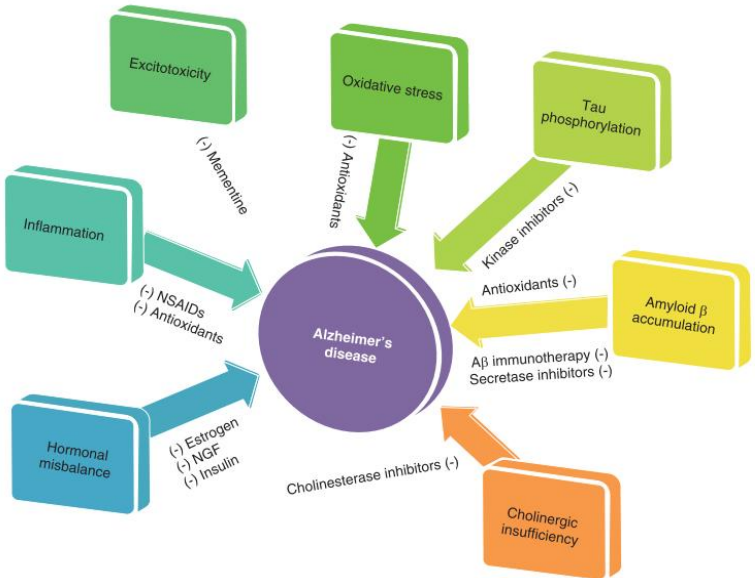

Figure 6 - Pharmacotherapeutic targets in AD (Chopra et al. 2011).

Several pharmacotherapeutic targets in AD (Figure 6) have been identified based on the many facets of this disease, like: cholinergic insufficiency, excitotoxicity, oxidative stress, inflammation, tau phosphorylation, hormonal misbalance and Aβ accumulation (Chopra et al. 2011).

1.8.1 - Symptomatic treatment

The only FDA-approved drugs for the treatment of AD patients are the acetylcholine esterase inhibitors (AchEIs) tacrine, donepezil, galantine and rivastigmine, and a non-competitive NMDA antagonist memantine (Biran et al. 2009).

1.8.1.1 - Cholinergic insufficiency

Studies have shown that AD patients have a reduced choline uptake, reduced acetylcholine (ACh) release and a presynaptic cholinergic deficit. According to the cholinergic deficit hypothesis, many symptoms of dementia can be explained by the lack of Ach (Van Marum 2008). The conventional pharmacotherapies for AD try to restore the cholinergic balance through increasing levels of Ach in the brain, in order to enhance cholinergic neurotransmission. Drugs known as acetylcholinesterase inhibitors (AchEI) were the first approved drugs for AD. Tacrine was the first widely used AchEI that inhibits both acetycholinesterase and butyrylcholinesterases (enzymes responsible for the degradation of Ach). However, it has a short half-life (requires four-time-daily dose) and has gastrointestinal and liver toxicity side effects. For these reasons, tacrine is rarely used nowadays (Chopra et al. 2011). Second-generation AchEI, including donepezil, rivastigmine and galantamine have fewer side effects. Donepezil is a noncompetitive, reversible AchEI with a relative long half-life that increased the preservation of cognition. Rivastigmine is also a noncompetitive, reversible AchEI that also inhibits butyrylcholinesterase. It has gastrointenstinal side effects, but some reports have shown excellent results as alternative previous cholinesterase inhibitors (Wentrup et al. 2008). Finally, galantamine is a weak competitive inhibitor of acetylcholine-esterase and modulates allosteric nicotine receptors. This drug has a short half-life but placebo-controlled trials demonstrated favorable effects on both cognitive and functional performance (Tariot & Federoff 2003).

1.8.1.2 - Excitotoxicity

Dysfunction of the glutamate neurotransmission system is also observed in AD brain, leading to excitotoxicity and therefore propagating cellular injury and apoptosis. Glutamate is the major excitatory neurotransmitter in the brain, which under normal conditions mediates learning and memory processes through NMDA receptors. Under abnormal conditions, such as in AD, increased glutamatergic activity can lead to sustained low-level activation of NMDA receptors, which may impair neuronal function and may be responsible for cell death observed in AD. Memantine is an NMDA antagonist that appears to restore the function of damaged nerve cells and reduce abnormal excitatory signals (MO & K. 2006). Thus, this

drug is being used as adjuvant to AchEI therapy. Targeting both glutamatergic and cholinergic pathways showed additive benefits in patients with moderate to severe AD by significantly improving measures of cognition, daily activities and behavior (Dantoine et al. 2006).

1.8.2 - Disease modifying therapies

1.8.2.1 - Oxidative stress

Oxidative stress is an important process associated with both aging and AD. Oxidative damage marked by lipid peroxidation, nitration, reactive carbonyls, and nucleic acid oxidation is increased in vulnerable neurons in AD. Markers of oxidative stress suggest that it precedes the formation of SPs and NFTs (Bonda et al. 2010). Aβ toxicity can be attenuated by antioxidants in vitro, whereas Aβ increases oxidative stress (Chopra et al. 2011). Curcumin, a pigment present in the rhizome of turmeric, and resveratrol, apoliphenol found in grapes, have anti-amyloidogenic, anti-oxidative, anti-inflammatory and neuroprotective proprieties (Ringman et al. 2005). Curcumin inhibits Aβ fibril formation whereas resveratrol reduced the levels of secreted and intracellular Aβ. Green tea catechins, ginkgo biloba extracts, ginseng extracts and bacopa monniera are other natural substances that may have neuroprotective and anti-inflammatory functions (Chopra et al. 2011).

1.8.2.2 - Tau phosphorylation and aggregation

Hyperphosphorylated tau is the principal constituent of NFTs which may promote neuronal network breakdown. It was reported that in AD phosphatases (PP2A) decreased (Chiu & Chuang 2010) and kinases (GSK-3 and CDK5) are increased (Wyttenbach & Arrigo 2000). Inhibiting drugs of kinases responsible for the phosphorylation of tau may be a good approach to reduce NFT formation. GSK-3 and CDK5 are the principal candidates (Biran et al. 2009). Lithium treatment already showed that by inhibiting GSK-3 tau pathology was reduced (Chiu & Chuang 2010).

1.8.2.3 - Hormonal misbalance

Compared with age-matched controls, AD patients show reduced CSF insulin (Craft et al., 1998). Insulin abnormalities and insulin resistance may contribute to AD pathophysiology and clinical symptoms. Insulin plays a role in memory function and it has been proposed that it can accelerate intracellular trafficking of Aβ and interfere with its degradation. It also may induce the hyperphosphorylation of tau

(Planel et al. 2007). Thus, abnormalities linked to this protein may influence Aβ and hyperphosphorylated tau levels in AD patient's brain.

1.8.2.4 - Inhibition of Aβ accumulation

According to the amyloid cascade hypothesis, aberrant Aβ metabolism is the underlying cause for neurodegeneration and dementia in AD. Therefore, there are three main strategies that can be followed to decrease Aβ peptides in AD patient's brain: modulate Aβ production, inhibit its aggregation or increase Aβ clearance.

i – Inhibition/modulation of Aβ production

To decrease the production of Aβ peptides three approaches can be followed: increase α-secretase activity, promoting the non-amyloidogenic pathway; inhibit/modulate β-secretase activity or inhibit/modulate γ-secretase activity.

Increasing α-secretase activity will not only diminish Aβ production but also increase the production of neuroprotective molecules like APPsα. It has been shown that muscarinic AchE-receptor agonists (e.g. NGX267) can promote α-secretase processing of APP (Biran et al. 2009). ADAM 10 overexpression in brain was shown to have protective effects in an AD mouse model, reducing BACE1 processing of APP, lowering amyloid deposition and improving several cognitive parameters (Postina et al. 2004). ADAM 17 is also a potential target but it is also involved in the release of proinflammatory TNF-α, which might lead to inflammation in AD patient brain (Kim et al. 2008).

γ-secretase inhibitors (GSIs) can be classified in three subgroups depending on where they bind to the complex: active site binding GSI, substrate docking-site binding GSI and alternative binding site GSI (Wolfe 2008). The first generation of GSIs (e.g. LY-450139) decreased Aβ production, but also showed undesirable side effects, the administration of this drug was associated with an increased risk of skin cancer and the phase II clinical trials stopped (http://www.alzforum.org). Gastrointestinal toxicity, increased susceptibility to infections and decline in cognition are some of the side effects associated with the use of GSIs, which are attributed to inhibition of Notch processing by γ-secretase (Searfoss et al. 2003). Second generation of GSIs showed to be more selective for APP over other subtracts, especially Notch (Wolfe 2008). BMS-708,163 was the first of these APP-selective GSIs to reach clinical trials, showing 30% decrease in CSF Aβ40 and Aβ42 following daily dose of 100 mg after 28 days and by 60% at daily dose of 150 mg. However this compound has discontinued in phase II/IIa/IIb (http://www.alzforum.org).

Furthermore, another concern associated with the use of GSIs is the presence of a rebound effect (an increase in the levels of Aβ when concentrations of the drug decrease) associated with GSI treatment. However, the exact reason for this effect is still unknown (Oehlrich et al. 2010).

An alternative approach is the utilization of γ-secretase modulators (GSMs). A GSM is a molecule that changes the relative proportion of Aβ isoforms while maintaining the rate at which APP is processed. Non-steriodal anti-inflammatory drugs (NSAIDs) were the first generation GSMs (Weggen et al. 2001). Sulindac sulfide and flurbiprofen were shown to reduce the production of Aβ42 and increase the levels of Aβ38, without changing Aβ40 levels. The inhibition of the Notch pathway is considered as a major problem for the utilization of GSIs. The GSMs bypass this problem by not affecting the Notch processing, which is a crucial advantage over the GSIs. However, the use of GSMs also faces some obstacles (Oehlrich et al. 2010). NSAIDs exhibit activity against cyclo-oxigenase 1 (COX1), inhibiting this protein. This leads to significant renal and gastrointestinal toxicity. However the COX1 activity was shown to be independent of the γ-secretase modulatory activity or vice versa (Weggen et al. 2001). Tarenflurbil is devoid of COX1 activity and shows γ-secretase modulatory activity, thus representing a second generation NSAID (Eriksen et al. 2003). This compound was tested in phase III in clinical trials, however in this phase it failed to show a beneficial effect on function and cognition. This was probably explained by low dosage, poor brain penetration or low potency that may have prevented the compound to exert some effect in the brain (Green et al. 2010). More efforts were subsequently done to improve brain penetration and increase the potency of GSM compounds. JNJ-40418677 was shown to selectively reduce Aβ42 both *in vitro* and *in vivo.* JNJ-40418677 displayed excellent brain penetration after oral treatment in mice and did not affect the formation of APP-CTF and AICD, Notch processing or the activity of COX enzymes (Van Broeck et al. 2011).

The modulation of β-secretase instead γ-secretase activity appears to have some advantages: its inhibition not only reduces Aβ levels but also prevents accumulation of the βAPP-CTF, that besides having poorly understood toxic effects also contains the entire Aβ domain and serves as the final substrate for Aβ production (Schenk et al. 2012). Moreover, interference with the Notch pathway can be avoided (De Strooper 2010). On the other hand, BACE1 is also responsible for the cleavage of other substrates besides APP. BACE1 knockout mice display reductions in myelin sheath thickness of axons of both peripheral sciatic nerves and optic nerves. Hypomyelination and increased seizures observed in $BACE1^{-/-}$ mice have raised concerns that therapeutic BACE1 inhibition may be associated with similar untoward effects in humans. However, whether the hypomyelination and seizure phenotypes in $BACE1^{-/-}$ mice are caused by the lack of BACE1 activity in the adult or during embryonic or postnatal development is currently

unknown (Vassar & Kandalepas 2011). Moreover a utilization of BACE inhibitors can promote retinal damage and visual loss as a significant side effect (Cai et al. 2012)

The development of BACE1 inhibitors turned out to be extremely challenging. First, BACE1 has an exceptionally long catalytic site making it very difficult to develop small compounds targeting BACE1 in an efficient way, because they need to cross the BBB (De Strooper 2010). Ideally, BACE1 inhibitor drugs should be of a molecular weight <500, orally bioavailable, metabolically stable, intrinsically potent, and highly selective for BACE1 over BACE2, cathepsin-D and other aspartic proteases. This latter point is relevant because BACE1$^{-/-}$ BACE2$^{-/-}$double knockout mice have enhanced postnatal lethality compared with BACE1$^{-/-}$single knockout mice (Dominguez et al. 2005) and cathepsin-D has a essential role in lysosomal function (Schenk et al. 2012) . Compounds must also be hydrophobic enough to penetrate both plasma and intracellular membranes to gain access to the compartment lumen where the BACE1 active site is localized. Finally, efficacious BACE1 drugs would need to efficiently cross the BBB (Vassar & Kandalepas 2011).

Despite these challenges, potent nonpeptidic small-molecule BACE1 inhibitors have shown success in lowering cerebral Aβ levels in several models. A nonpeptidic compound, TAK-070 that bound to full-length BACE1, showed to lower the brain levels of soluble Aβ after short-term oral administration in Tg2576 mice (Fukumoto et al. 2010). TC-1 is novel tertiary carbinamine BACE1 inhibitor that showed to be a potent inhibitor, that lowered brain Aβ levels in a mouse model. Intravenous infusion of TC-1 led to a significant but transient lowering of CSF and plasma in conscious rhesus monkeys because it underwent CYP3A4-mediated metabolism. Oral co-dosing of TC-1 with ritonavir, a potent CYP3A4 inhibitor, in rhesus monkeys led to a significant and sustained reduction of APPsβ, Aβ40 and Aβ42 in CSF and of Aβ40 levels in plasma (Sankaranarayanan et al. 2009). A small-molecule drug, CTS-21166 from CoMentis was announced to complete the first human phase I clinical trials (Vassar & Kandalepas 2011). However very little has been published on this molecule, and it is unclear if it is still in clinical development (Schenk et al. 2012). Eli Lilly has reported preclinical animal model and early stage clinical testing in humans with a novel BACE inhibitor, LY2811376. Significant reduction in brain and CSF Aβ levels was observed in preclinical models (mouse and dog) after oral administration. However, the development of this molecule was discontinued as a result of non-clinical non-target-related pathology findings (May et al. 2011). LY2886721 was other BACE1 inhibitor from ELI Lilly that reach phase II in clinical trials but was discontinued in the beginning of June 2013 due to liver abnormalities that showed up in 4 out of 45 patients during routine testing. In phase I of clinical trials, this compound showed to lower CSF Aβ40, Aβ42, and sAPPβ concentrations, while increasing sAPPα in healthy adults in both single and multiple dose studies (http://www.alzforum.org). MK8931 is a BACE1 inhibitor from Merck that is now in phase II/III of clinical trials. In phase I a single 100 mg dose of MK8931 showed to reduced CSF Aβ40 and Aβ42

more than 90% in healthy volunteers (http://www.alzforum.org). Now Efficacy and Safety of MK-8931 in Mild to Moderate Alzheimer's Disease will be assessed in phase II/III that is ongoing (http://clinicaltrials.gov).

Substantial preclinical data provide critical support for BACE1 as a tractable target for small-molecule intervention to test the amyloid hypothesis clinically.

ii – Enhancement of Aβ clearance

An alternative strategy is to target Aβ after it has been synthesized. This might also allow intervention later in the disease process.

Although many studies are focused on understanding the formation of Aβ peptides and neuritic plaques, the mechanisms of degradation of these peptides is another important field to explore how to decrease the concentration of Aβ in AD patient brain. The normal Aβ fractional clearance rate is estimated to be 8% per hour (Bateman et al. 2006). Many studies indicate that there are several proteases that are able to lower Aβ levels or prevent amyloid plaque formation. Neprilysin and insulin degrading enzyme (IDE) are believed to be responsible for most Aβ degradation. Neprilysin is a plasma membrane bound type II metalloprotease that is responsible for the extracellular degradation of a variety of peptides (Murphy & Levine III 2010). In CSF of early AD patients the level of this enzyme is decreased (Murphy & Levine III 2010; De Strooper 2010). IDE is a zinc protease that cleaves peptides like insulin, glucagon, and others (De Strooper 2010). This metalloprotease has approximately a 20-fold higher affinity for insulin than for Aβ, but hydrolyzes insulin at a much slower rate. Thus, insulin acts as an effective inhibitor of the IDE-dependent cleavage of Aβ, which may form the basis for a link between type II diabetes and AD (Murphy & Levine III 2010). However, there are more proteases related with Aβ clearance: endothelin converting enzymes 1 and 2 were shown to decrease accumulation in the brain; angiotensin converting enzyme whose gene polymorphisms have been associated with increased or decreased genetic risk for AD; matrix metalloprotease whose expression can be induced in astrocytes by stress like the presence of Aβ and unlike most other Aβ degrading enzymes can also degrade fibrillar Aβ; plasmin that is decreased in AD patients; and cathepsin B that is involved in Aβ-monomer and Aβ-fibril degradation (De Strooper 2010). Moreover receptors involved in cholesterol and lipid metabolism (LRP receptors) have been suggested to mediate Aβ efflux from the brain to CSF and blood (e.g. MDR1-P-glycoprotein), so its overexpression can increase Aβ clearance (Biran et al. 2009). Also mention RAGE receptors and their function.

Immunotherapy has been, by far, the most studied strategy to decrease amyloid burden, with several immunotherapeutic agents tested in clinical trials. Two ways of intervention can be followed:

active immunization with injection of Aβ peptides to induce an amyloid antibody response, and passive immunization, which consists of infusing anti-Aβ antibodies in blood circulation.

The first anti-amyloid vaccine to reach clinical trials, AN1792, was shown to efficiently clear amyloid plaques in Phase II clinical trial. However, this clearance did not seem to prevent progressive neurodegeneration and more importantly, severe side-effects (meningoencephalitis), resulting in the interruption of the trial (Bayer & Cappai 1999; Gilman & Koller 2005). The meningoencephalitis was related to a vigorous T-cell autoimmune response against the full-length Aβ peptide (Ferrer & Rovira 2004). Therefore, a second generation of amyloid vaccines was developed using smaller Aβ peptides rather than full-length (Maier & Seabrook 2006). CAD106 uses a shorter N-terminal (Aβ1-6) peptide segment that show to be safe and well tolerated and triggered a specific Aβ antibody response in two thirds of treated AD patients. Also in terms of safety, this approach seems to be beneficial. The fourth Phase II clinical trial is ongoing.

Bapineuzumab is an N-terminal humanized monoclonal antibody that targets amyloid beta. This antibody was already in phase III clinical trials when the drug failed to arrest cognitive decline. Miles and co-worker showed that Bapineuzumab was only capable of bind to Aβ peptides that assumed a helical conformation. Thus, this antibody was just binding to a population of peptide that adopts a helical structure, preventing the improvement of cognitive function (Miles et al. 2013)

Solanezumab is a humanized monoclonal antibody that binds specifically to epitopes located in the center of soluble Aβ sequence (Siemers et al. 2010). It is presumed to act on peripheral Aβ, altering the equilibrium between plasma and CSF amyloid and resulting in the efflux of amyloid from the CNS into a 'peripheral sink'. However, it is also able to cross the BBB and exert its action in the brain parenchyma (De Mattos et al. 2002). This monoclonal antibody is the first therapeutic drug to be evaluated in the Anti-amyloid Treatment in Asymptomatic Alzheimer's Disease (A4) prevention clinical trial. However like Bapineuzumab clinical trials were discontinued.

iii – Reduction of Aβ aggregation

The last strategy is preventing the aggregation of Aβ peptides. This protein tends to aggregate, leading to the formation of toxic forms. Zinc (Zn) and cupper (Cu) are involved in the Aβ aggregation. PBT2 is a chelators of Zn/Cu, that was tested in phase II of clinical trials and show a significant reduction of cognitive decline (Lannfelt et al. 2008). Clinical studies are ongoing (http://clinicaltrials.gov)

1.9. Plasma biomarkers for AD

As mentioned before, the wide spread use of CSF biomarkers is limited by the fact that a lumbar puncture is needed to collect samples. Lumbar puncture is a relatively low-risk procedure. Nevertheless, it remains an invasive intervention, which is an additional burden for the patients. Post-lumbar puncture headache (PLPH) is the most frequent complication that typically lasts for a couple of days and can be severe enough to immobilize the patient and to require therapy (Lavi et al. 2010). Besides recruiting enough patients for CSF sampling by lumbar puncture, it is even more difficult to find cognitively healthy people to undergo this procedure for the collection of control samples. For all the above-mentioned reasons, blood-based biomarkers might provide an interesting alternative for diagnosing and tracking the course of dementia, as well as for therapy monitoring. Advantages include procedures for acquiring blood which is cheaper, easier and less invasive allowing its routine collection even in elderly people.

Brain is the primary source of Aβ. However, Aβ can also be derived from other sources. BACE1 activity can be found at very high levels in the AD brain (Haass et al. 2012), but is also present at lower levels in peripheral organs such as skeletal muscle, liver, kidney and lung. Although the formation of Aβ species may be higher in the brain compared to other organs, it is possible that production of Aβ in peripheral organs may result in a significant contribution to the plasma Aβ pool (Oh et al. 2008). Platelets are another source of Aβ (Slemmon et al. 2007), though mostly Aβ40. This peripheral production of Aβ peptides is crucial to take into account when assessing plasma Aβ levels and raises the question whether Aβ in circulation reflects the pathology observed in the brain. Because brain is still considered to be the primary source of Aβ, it is also suggested to be the major determinant of the Aβ dynamic equilibrium between brain and periphery (Figure 7).

Like in CSF different Aβ peptides are present in plasma. Aβ37, Aβ38, Aβ39, Aβ40 and Aβ42 where characterized as regular constituents of human plasma (Bibl et al. 2012). Animal data suggest that approximately 10% of Aβ from the brain interstitial fluid (ISF) moves into blood stream via ISF bulk flow (Shibata et al. 2000). However, most of the movement of Aβ is thought to be dependent on transporters such as low-density lipoprotein receptor-related protein-1 (LRP-1) (Shibata et al. 2000; Deane et al. 2003) and receptor for advanced glycation end products (RAGE) (Deane et al. 2003) due to the presence of tight endothelial cell junctions at the BBB.

A correlation between CSF and plasma Aβ levels was found in healthy individuals (Giedraitis et al. 2007), however such correlation is not seen in AD patients. Also in MCI subjects no correlation between CSF and plasma Aβ levels could be observed (Giedraitis et al. 2007; Vanderstichele et al. 2000; Matsumoto et al. 2007; Mehta et al. 2001; Höglund et al. 2004; Hansson et al. 2010). Thus it seems that the equilibrium between CSF and plasma Aβ is disrupted early in AD pathogenesis (Giedraitis et al. 2007;

Lewczuk et al. 2010). On the other hand, there is a tight correlation between Aβ40 and Aβ42 isoforms in plasma as well as in CSF. Highly significant correlations were also found between full length and N-truncated Aβ40 and Aβ42 isoforms in plasma. Again no correlation between the levels of Aβ42 in CSF and plasma and only a very weak correlation between Aβ40 in CSF and plasma was measured (Hertze et al. 2010).

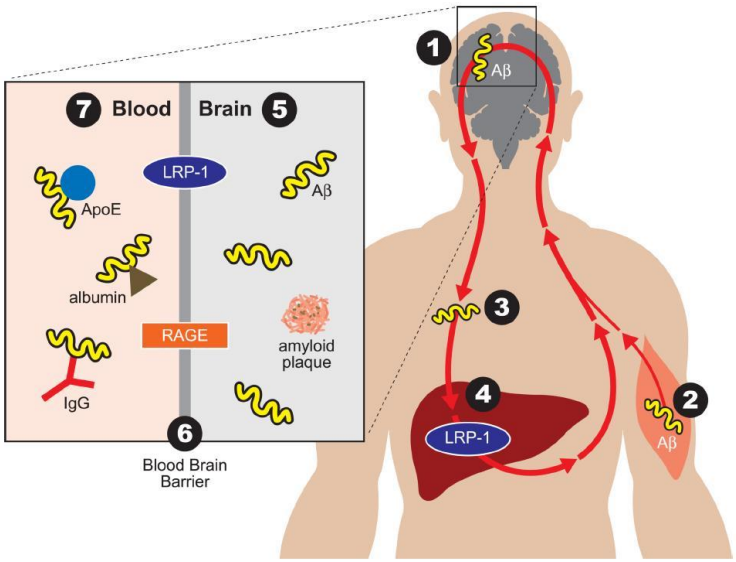

Figure 7 - Relation between CSF and peripheral compartments. Aβ peptides are synthesized in the brain (1), as well as in the periphery (2). Circulating Aβ peptides enter the blood stream (3) and are partly cleared by the LRP-1 receptors in the liver (4). Soluble extracellular brain Aβ (5) may accumulate in the brain parenchyma as amyloid plaques. Receptor mediated movement of the soluble Aβ through the BBB (6) is mediated by transporters such as LRP-1 for efflux, and RAGE for influx. Once in the blood, Aβ peptides are bound by numerous binding proteins (7). Aβ specific IgG is also able to bind Aβ peptide in the blood, and may induce efflux of Aβ from the brain to the blood via the "peripheral sink" mechanism. Aβ – amyloid-β peptide, BBB- Blood-brain barrier, LRP-1: low-density lipoprotein receptor-related protein-1, RAGE: receptor for advanced glycation end products (Oh *et al.* 2008).

Bilb and co-workers compared serum and plasma in order to understand which fluid is the more appropriate to measure Aβ peptides. The concentrations of peptides Aβ37, Aβ38, Aβ39, Aβ40 and Aβ42 were significantly higher in plasma than in serum. Aβ peptide levels were also more stable in plasma than in serum under conditions of storage at room temperature for up to 48h. Thus, plasma may be more appropriate than serum for analyzing Aβ peptides for routine purpose. This study also showed that the analysis should be done within 24 hours after storage at room temperature (Bibl, Welge, et al. 2012).

Like for CSF measurements, substantial variability is observed for when comparing study results and this could be attributed to several factors. The processing of the blood can alter the concentration of Aβ peptides in plasma. Processing of the whole blood to plasma would spin down the platelets which are known to contribute to the blood Aβ pool (Evin et al. 2003b). However, a study has demonstrated that over 90% of the Aβ appears to remain within the plasma component when compared to the Aβ levels in

the whole blood prior to processing (Slemmon et al. 2007). The lack of standard procedures both pre-analytical and analytical is another source of variability between the different studies. Efforts are being made to standardize the procedures in the measurement of plasma Aβ peptides. Differing results may well be related to assay configuration since antibodies are known to recognize different epitopes or conformational forms of Aβ (Blennow et al. 2008).

Accurate measurement of Aβ levels in plasma is complex due to a number of technical challenges. Firstly, Aβ binding proteins occur in plasma. Aβ peptides have amphoteric and amphipatic characteristics that give this molecule a great capacity to interact with a large number of plasma proteins, such human serum albumin (HSA), α2-macroglobulin (α2M), α1-antichymotrypsin, cellular prion protein, serum amyloid P (SAP), islet amyloid polypeptide (IAPP), complement proteins, transthyretin, apoferritin, apolipoproteins and various lipoproteins (Kuo et al. 2000; Stanyon & Viles 2012). HSA is the most abundant protein in blood serum. 90-95% of the Aβ found in blood plasma is bound to HSA (Stanyon & Viles 2012). HSA occurs in blood serum at a concentration of 640 μM, but has a markedly reduced concentration in the CSF of typically 3 μM. This may explain why Aβ plaques are mainly observed in the extracellular space of the brain and not the peripheral tissues. Nevertheless, HSA still represents the major protein component of the CSF and 40% of Aβ within the CSF will be bound to HSA (Stanyon & Viles 2012). Several studies show that HSA has a role in preventing the formation of fibrils due to the binding to Aβ monomers/oligomers. A reduction of the Aβ pool in plasma is suggested to, in turn, reduce Aβ levels in the CSF as Aβ is in a dynamic equilibrium and is able to cross the blood-brain barrier (Boada et al. 2009).

Reproducible and accurate measurement of Aβ in plasma is also a challenge because of the hydrophobic nature of the full length peptide, as well as the heterogeneity of different truncated Aβ fragments (Hansson et al. 2010).

Another problem associated with the measurement of Aβ peptides in plasma is the presence of endogenous antibodies in this fluid that can interact with the assay antibodies leading to inaccurate results. When utilizing immunoassays, possible interference of other than the target proteins or interactions of endogenous antibodies with the target protein or assay antibodies should be taken into account. These interactions can alter the result of the test and if not controlled for, can lead to a wrong diagnosis or poor evaluation of the effect of a drug.

Blood samples from human origin were shown to contain different endogenous antibodies (Koshida et al. 2010). There are two major types of endogenous antibodies, heterophilic antibodies (HA) and human anti-animal antibodies (HAAA). HA are antibodies produced against poorly defined antigens (Kaplan & Levinson 1999). These polyreactive antibodies are generally weak antibodies that recognize

antibodies from other species (Kaplan & Levinson 1999; Sehlin et al. 2010). HAAA are produced against a well-defined antigen (Kaplan & Levinson 1999) like human anti-mouse antibody (HAMA) that binds specifically to mouse antibodies (Dodig 2009). HAAA include human anti-mouse, -rabbit, -goat, -sheep, -cow, -pig, -rat and –horse antibodies. Human anti-mouse antibody (HAMA) is the most common type of anti-animal antibodies (Kricka 1999; Koshida et al. 2010). They can belong to the IgG, IgA, IgM, or rarely IgE class. These antibodies can be anti-idiotype (directed against the hypervariable region of immunoglobulin molecule) or anti-isotype (directed against the constant regions). Anti-isotype antibodies may be more common than anti-idiotype antibodies (Kricka 1999). These endogenous antibodies can arise from iatrogenic or noniatrogenic causes. Nowadays a wide range of diagnostic and pharmaceutical agents are derived from an animal source, like antibody-targeted imaging reagents (mouse source), antibody-targeted drugs (mouse source) (Grossman 1986) or even insulin (pig source). Blood transfusion is also associated with an increased incidence of anti-animal antibodies. Vaccination against infectious disease is another route for animal protein antigens to be exposed to the immune system and trigger antibody formation (Kricka 1999; Koshida et al. 2010). Moreover, patients diagnosed with cancer tend to acquire HAMAs more frequently than the ones without cancer (Koshida et al. 2010). Non-iatrogenic causes are associated with animal handling (agriculture, farming), keeping of animals as pets and maternal transfer across the placenta to the unborn child (Kricka 1999). The prevalence of anti-animal antibodies in the general population varies widely and can range from <1% to 80% (Kricka 1999; Koshida et al. 2010).

As mentioned HA and HAAA are present in large amounts in serum but they are also present in CSF (Sehlin et al. 2010) and they can interfere with the measurement of analytes in several immunoassays. There are different types of interference in immunoassays described: cross reactivity, the hook effect, antibody interference, and matrix effects. Cross reactivity is a non-specificity, whereby a substance in the sample with structural similarity to the analyte competes for the antibody binding. The hook effect is a state of antigen excess, in which very high concentrations of analyte saturate all the available binding sites of reagent antibodies without forming complexes. The hook effect can be eliminated if a wash step is included between the incubation of sample with capture antibody and the addiction of detection antibody (Miller 2004). Can also be that an optimization of antibody concentration is needed to solve this effect. The matrix of a sample is the environment of the analyte and includes properties like pH value, viscosity, ionic strength, and the protein and lipid concentrations of the sample (Lachno et al. 2009). Matrix effects are caused by variations in the reactivity of the analyte due to variations in its environment in the sample (Miller 2004). Antibody interference happens when endogenous antibodies like HA and HAAA bind to assay antibodies leading to false-positive or false-negative results (Miller 2004; Dodig 2009). HA and HAMA present in samples can interfere in clinical

assays by bridging between the mouse immunoglobulin capture antibody and the mouse immunoglobulin conjugated detection antibody leading to a false-positive result (Figure 8B). Negative interference occurs due to binding of HA or HAMA directly to the capture or detection antibody preventing the reaction with the analyte (Figure 8C and D, respectively) (Kricka 1999; Miller 2004; Dodig 2009). It has been reported that the risk of HA interference is likely to increase when using the same monoclonal antibody both as capture and detection antibody. Moreover, different assay antibodies and technologies can exhibit different sensitivities to the presence of interference(Koshida et al. 2010; Sehlin et al. 2010).

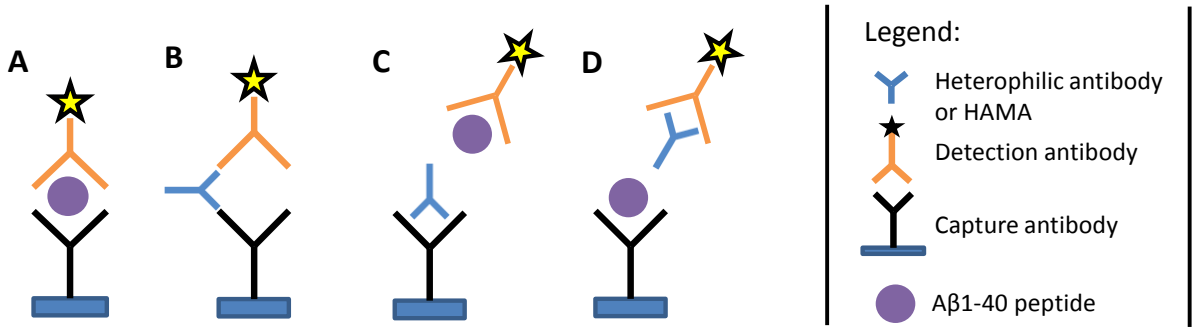

Figure 8 – Schematic representation of several different interference effects which may occur in immunoassays. (A) Assay with no interference. (B) A "bridge binding" by heterophilic antibody or by HAMA, resulting in a false-positive signal. (C and D) antibody interference where the interfering substance has anti-idiotypic binding qualities to the capture or the detection antibody, respectively, thus preventing the binding of the analyte. Both occurrences result in false-negative signals.

Several strategies exist to, on the one hand, prevent the development of HAAA, and on the other hand, to remove or block the interference caused by these antibodies in the different immunoassays. Immunosuppressive drugs can be used before and after the administration of mouse antibody agents and in this way minimize the development of HAMA (Kricka 1999). One way to reduce interference is to dilute samples prior to analysis. However, the low concentrations of Aβ found in human plasma samples do not permit extensive sample dilution before analysis (Sehlin et al. 2010). Therefore, other approaches have to be followed to block the remaining interference signals. A blocking agent can be included in the immunoassay (e.g. in the assay diluent) and the samples can be pretreated before the assay. There are some blocking agents available commercially like Immunoglobulin Inhibiting Reagent (IRR, Bioreclamation), Heterophilic Blocking Reagent (HBR, Scantibodies) and Heteroblock (Omega Biologicals) (Kricka 1999). HBR contains immunoglobulins with different characteristics. It is a unique formulation of immunoglobulins targeted specifically against heterophilic antibodies to neutralize their interference (http://scantibodies.com). Optimization of the reagent concentration is crucial as Koshida and co-workers

showed that a low dose of HBR rather enhanced the interactions instead of blocking them. However, with higher doses of HBR the interactions are reduced in a dose-dependent manner (Koshida et al. 2010).

HA, HAMA and other endogenous immunoglobulin interference should be taken in account when plasma samples are being analyzed in other to avoid false-positive or false negative-results. A good approach is the utilization of a blocking reagent in a sufficient amount to block this kind of interaction, allowing a more accurate measurement of the biomarker under investigation. Since obtaining plasma would be less invasive than CSF sampling, many strategies are under investigation to optimize plasma Aβ as a predictor of AD as well as to monitor the efficacy of Aβ modifying agents.

Chapter 6

References

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