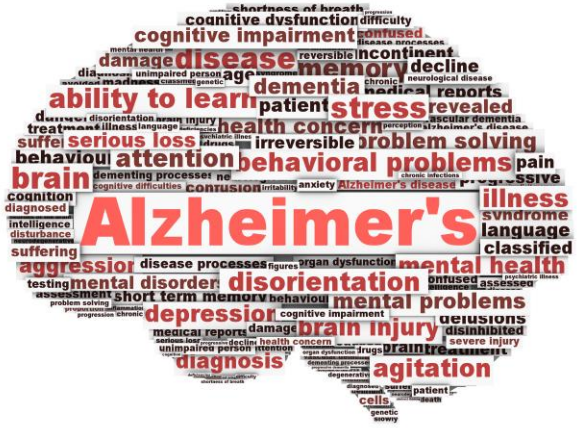

Search for early TTR-related biomarkers in a transgenic AD mouse model

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Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, / /

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Bem, parece que está feito.

Abstract

Dementia is a very delicate disease that not only affects the patients, but also everyone around them. Nearly 36 million people live with dementia, and future does not appear to shine brighter since for the year 2050 the prognosis is that this number will triple. Alzheimer's disease (AD) is the most prevalent form of dementia (75% of all cases), independently of age, and is mainly characterized by the presence of senile plaques (SPs) and neurofibrillary tangles (NFTs), accompanied by progressive dementia. Transthyretin (TTR) has been shown, through *in vitro* and *in vivo* studies, to exhibit a neuroprotective role in AD, however, its underlying mechanisms are still vastly unknown. With this project, we proposed a dual investigation: first, the study of two distinct proteins – sortilin (Sort1) and synaptophysin (Syp) – that were suggested to be altered in AD, and thus, assess its potential as a biomarker; and second, evaluate TTR's role in disease and its effect on these prospective biomarkers. All the experiments were performed in a transgenic AD mouse model bearing different TTR genetic backgrounds (two copies of the mouse TTR gene – AD/TTR$^{+/+}$, and one copy of the mouse TTR gene – AD/TTR$^{+/-}$), which was previously described in our laboratory. All the results were obtained through Western Blot analysis, using 3- and 7-months old AD/TTR mice. From this analysis we show that Sort1 is decreased at both ages in AD/TTR$^{+/-}$ mice, in relation to AD/TTR$^{+/+}$, and suggest that due to its behavior, this protein could be used for early AD detection, even when β-amyloid (Aβ) deposits are absent, and follow-up of therapies. Still regarding Sort1, female gender appears to be more affected since it showed a more accentuated decrease, compared to males. This is especially observed in older mice, thus showing the impact of aging in AD. As for Syp, we observed an increase in its expression for 3 months-old AD/TTR$^{+/-}$ mice, compared to AD/TTR$^{+/+}$, contrary to 7 months-old mice that showed no significant differences. Thus, we suggested that this alteration was due to an overlapping effect of aging over TTR reduction. Nonetheless, we also propose that Syp should be considered for further studies as an early AD detection biomarker. Alterations observed for both Sort1 and Syp were not restored in AD/TTR$^{+/-}$ mice treated with iododiflunisal (IDIF), known to stabilize TTR and shown to improve AD features, namely Aβ levels and deposition in the brain, and cognition in this mouse model. This indicates that Sort1 and Syp are dependent on TTR quantity and that its stabilization was not sufficient to reverse the effects of the TTR genetically reduced levels.

Keywords: Alzheimer's disease; biomarker; transthyretin; sortilin; synaptophysin; transgenic mouse model.

Resumo

Demência é uma condição bastante delicada que afeta não só o paciente, como também todos aqueles que o rodeiam. Aproximadamente 36 milhões de pessoas vivem com esta doença e o futuro não se apresenta brilhante, dado que o prognóstico para 2050 é de que este número irá triplicar. A Doença de Alzheimer (DA) é a forma mais prevalente de demência (75% de todos os casos), independentemente da idade, e é principalmente caracterizada pela presença de placas senis e emaranhados neurofibrilares, acompanhados de uma demência progressiva. À proteína transtirretina foi associado um papel neuroprotetor na DA, através de estudos *in vitro* e *in vivo*, porém, os mecanismos moleculares responsáveis por este papel são ainda imensamente desconhecidos. Através deste projeto, propusemos uma investigação bi-objectiva: em primeiro lugar, estudar duas proteínas distintas – sortilina (Sort1) e sinaptofisina (Syp) – que se verificaram estar alteradas na DA, e assim, averiguar o seu potencial como possível biomarcador; e em segundo lugar, avaliar o papel da TTR nesta doença e o seu efeito nas proteínas atrás referidas. Todas as experiências foram realizadas usando um modelo de murganho transgénico para DA, com diferentes genótipos de TTR (duas cópias do gene TTR de murganho – AD/TTR$^{+/+}$, e uma cópia do gene TTR de murganho – AD/TTR$^{+/-}$), previamente descrito no nosso laboratório. Todos os resultados foram obtidos através de análise por Western Blot, usando murganhos AD/TTR de 3 e 7 meses de idade. Desta análise surgiu que a Sort1 se apresenta diminuída em ambas as idades, nos murganhos AD/TTR$^{+/-}$, em comparação com os AD/TTR$^{+/+}$, sendo possível sugerir que, dado o seu comportamento, esta proteína poderá ser usada na deteção precoce de DA, mesmo quando é ausente a deposição de β-amiloide. Ainda sobre a Sort1, observou-se uma diminuição mais acentuada dos seus níveis no sexo feminino, em relação ao masculino, sugerindo então que o primeiro se encontra mais afetado. Esta diminuição encontra-se especialmente demarcada nos murganhos de 7 meses, o que demonstra o impacto do envelhecimento na DA. Em relação à Syp, observámos um aumento da sua expressão em murganhos AD/TTR$^{+/-}$ de 3 meses de idade, em oposição ao observado para murganhos de 7 meses, onde as diferenças não foram consideradas significativas. Sendo assim, sugerimos que esta alteração do comportamento de expressão da Syp é devida a um efeito do envelhecimento, que aparenta anular qualquer efeito proveniente da redução genética da TTR. No entanto, propomos que futuros estudos sobre a Syp (enquanto biomarcador) sejam realizados, uma vez que esta parece apropriada para a deteção de estádios precoces na DA. As alterações de expressão na Sort1 e Syp não

foram restauradas em murganhos AD/TTR$^{+/-}$ tratados com iododiflunisal (IDIF), um composto que promove a estabilização da TTR e o melhoramento das características da DA neste modelo animal (nomeadamente, níveis de Aβ e deposição no cérebro, e cognição). Tal indica que a Sort1 e a Syp são dependentes da quantidade de TTR e que mesmo a sua estabilização não é suficiente para reverter os efeitos da redução genética da TTR.

Palavras-chave: Doença de Alzheimer; biomarcador; transtirretina; sortilina; sinaptofisina; modelo de murganho transgénico.

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Abbreviations

ABCA1 – ATP-binding cassette A1

ABCB1 – ATP-binding cassette B1

Aβ – Amyloid-beta

AChEI - Acetylcholinesterase inhibitors

AD – Alzheimer's disease

AD/TTR$^{-/-}$ – AD mice, knockout for transthyretin

AD/TTR$^{+/-}$ – AD mice, hemizygous for transthyretin

AD/TTR$^{+/+}$ – AD mice, wild type for transthyretin

AICD – Amyloid intracellular domain

α2M – α2-Macroglobulin

ApoA-I – Apolipoprotein A1

ApoE – Apolipoprotein E

ApoJ – Apolipoprotein J

APP – β-Amyloid precursor protein

BACE-1 – β-site amyloid precursor protein-cleaving enzyme 1

BAI – Brain amyloid imaging

BBB – Blood-brain barrier

CCVs – Clathrin-coated pits and vesicles

CDK5 – Cyclin-dependent kinase 5

CERP – Cholesterol efflux regulatory protein (also known as ABCA1)

CK1 – Casein kinase 1

CLU – Clusterin

CR1 – Complement component (3b/4b) receptor 1

CSF – Cerebrospinal fluid

CTF – Carboxi-terminal fragment

EOAD – Early-onset Alzheimer's Disease

ER – Endoplasmatic reticulum

FAD – Autosomal dominant Alzheimer's Disease

FAP – Familial amyloidotic polyneuropathy

FDA – Food and drug administration

GSK3β – Glycogen synthase kinase 3β

IAPP – Islet amyloid polypeptide

IDE – Insulin-degrading enzyme

IDIF – Iododiflunisal

IVIG – Intravenous immunoglobulins

KPI – Kunitz protein inhibitor

LDL – Low density lipoprotein

LOAD – Late-onset Alzheimer's Disease

LRP1 – Low-density lipoprotein receptor related protein 1

MAP – Microtubule-associated protein

MAPKs – Mitogen-acivated protein kinases

MARVEL – MAL and related proteins for vesicle trafficking and membrane link

MCI – Mild cognitive impairment

MRI – Magnetic resonance imaging

NCT – Nicastrin

NEP – Neprilysin

NFT – Neurofibrillary tangles

NGF – Nerve growth factor

NSAIDs – Non-steroid anti-inflammatory drugs

PEN2 – Presenilin enhancer 2

PET – Positron emission tomography

P-gp – P-glycoprotein (also known as ABCB1)

PHF – Paired helical filament

PI-3K – Phosphatidylinositol-3-kinase

PiB – Pittsburgh Compound B

PICALM – Phosphatidylinositol binding clathrin assembly protein

PKA – Protein kinase A

Pro-NGF – Nerve growth factor's precursor form

PrP – Prion protein

PSEN1 – Presenilin 1

PSEN2 –Presenilin 2

RAGE – Receptor for advanced glycation end products

sAPPα – (extracelular) Soluble APP alpha

sAPPβ – (extracelular) Soluble APP beta

SEM – Standard error of the mean

SFs – Straight filaments

sLRP1 – Soluble LRP1

SNARE – Soluble NSF attachment protein receptor

Sort1 – Sortilin

SPs – Senile plaques

Syp – Synaptophysin

T_4 – Thyroxine

TGN – Trans-Golgi network

tPA – Plasminogen activator

TTR – Transthyretin

Introduction

"If any one faculty of our nature may be called more wonderful than the rest, I do think it is memory. There seems something more speakingly incomprehensible in the powers, the failures, the inequalities of memory, than in any other of our intelligences. The memory is sometimes so retentive, so serviceable, so obedient; at others, so bewildered and so weak; and at others again, so tyrannic, so beyond control! We are, to be sure, a miracle every way; but our powers of recollecting and of forgetting do seem peculiarly past finding out."

by Jane Austen, *Mansfield Park*

All around the world, dementia is one of the major concerns for society, independently of the socio-economic status. Nearly 36 million people live with dementia, and the prognosis is that by 2050 this number will triple (115 million)[2]. Within the cases of dementia, Alzheimer's disease (AD) occupies a special place, counting up to 75% of all cases[3, 4]. Different kinds of dementia, in addition to AD, have been characterized and within this list we can find vascular dementia, dementia with Lewy bodies, mixed dementia, Parkinson's disease, and Creutzfeldt-Jakob disease[5], among others. One of the major problems of AD, and other dementias, is the lack of early diagnosis techniques, whereas, in more late-stages AD is identified quite accurately by most clinicians[6]. In effect, definite AD (considered a dual clinicopathological entity[7]) is only diagnosed after postmortem evidence of extracellular amyloid (or senile) plaques and intracellular neurofibrillary tangles[8], presented with progressive dementia. They are considered pathognomonic signs (characteristic for a particular disease – from the Greek: *páthos* meaning "disease", and *gnõmon*, meaning "judge") for AD and so, after autopsy, their presence is used to verify the diagnosis.

Therefore, the uncertainty of the underlying diagnosis is a tremendous hurdle in the development of new therapies[6]. Despite of all the efforts, AD is still an incurable neurodegenerative disease.

Introducing Alzheimer

Alois Alzheimer (Figure 1) was born on 14 June 1864, in Marktbreit, Bavaria, and is considered one of the founding fathers of neuropathology. He was attributed the credits for discovering and describing a so-called "*presenile dementia*", which would be later named after him, in 1910, by his colleague Emil Kraepelin[9, 10]. With the simplest sentence: "The clinical interpretation of this Alzheimer's disease is still unclear." of the famous Textbook of Psychiatry (Psychiatrie: Ein Lehrbuch fur Studierend and Aerzt)[11] Kraepelin immortalized Alois Alzheimer. Alzheimer made fundamental contributions to understand other diseases such as vascular dementia, Huntington's disease, syphilis, brain tumors and epilepsy. He died from rheumatic endocarditis[9], curiously at the age of 51.

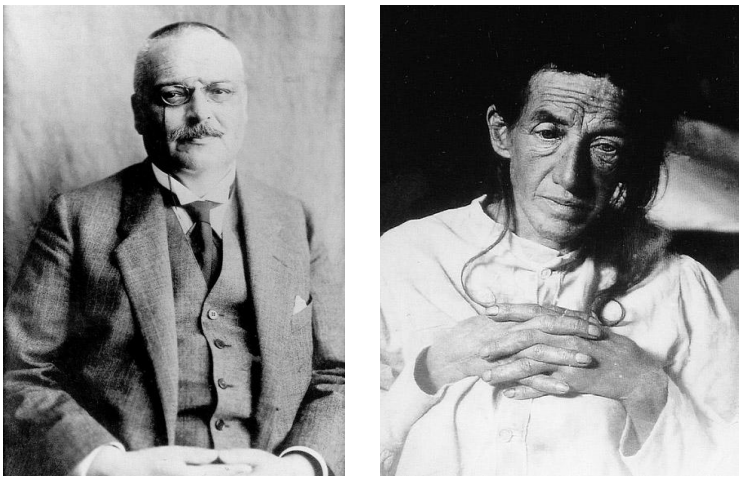


Figure 1. Alois Alzheimer (left) and its first patient, Auguste Deter (right).

Alzheimer's disease was first described in the 1907's paper entitled "Uber eine eigenartige Erkankung der Hirnrinde", by Alois Alzheimer. In it, the author described the behavior of a 51-year-old female patient (Figure 1) of the insane asylum of Frankfurt am Main. She (Auguste Deter) presented several symptoms that caught Alzheimer's attention, apart from the central nervous system anatomical characteristics. Among them, time and space disorientation, rapid loss of memory and mood swings were the most prominent symptoms[12]. In relation to pathological features, the observation of "thick bundles"[12] of fibrils (later known as senile/amyloid plaques and neurofibrillary tangles[13]) made AD a unique condition, distinguishing it from the other neurological conditions known[14].

After the initial work by Alois Alzheimer, scientists have been successively and continuously motivated to acquire the necessary knowledge to comprehend and unveil the

mysteries that surround this intriguing disease. And so, due to the outstanding work made by Alois Alzheimer's "followers", advances have been made, leading them closer to a possible cure. Amyloid-beta (Aβ) immunotherapy[15, 16], gene therapy[17], and deep brain stimulation[18, 19] are good examples of scientists' determination (in the most distinct fields) to achieve the ultimate goal, the cure for Alzheimer's disease.

By the eyes of Alzheimer

Alzheimer's disease is a progressive neurodegenerative disease and the most common case of dementia[20], covering a heterogeneous group of disorders[10] with increasing prevalence after the age 65[14]. Although AD is seen as an elderly disease due to its higher prevalence in the older population, it is also the most frequent form of dementia under the age of 65[21, 22]. More recently, in 2011, the National Institute of Aging and the Alzheimer's Association recommended new diagnostic criteria and guidelines, proposing three different stages for AD: (1) preclinical Alzheimer's disease; (2) mild cognitive impairment due to Alzheimer's disease; and (3) dementia due to Alzheimer's disease[5]. Genetically, AD is usually divided in two forms: autosomal dominant familial AD (FAD; Mendelian inheritance predominantly of early-onset – EOAD[23]) and sporadic AD (also called late-onset AD – LOAD), counting the latter as 95% of all AD cases[7].

In FAD, autosomal mutations capable of triggering the disease were identified, mainly, in three distinct genes: amyloid precursor protein (APP)[24, 25] gene, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes[26], in chromosomes 21q, 14q and 1q, respectively. Together, these mutations (more than 200 mutations known) are responsible for less than 1% of all cases of AD (http://www.molgen.vib-ua.be/ADMutations/). Contrary to FAD, sporadic AD does not exhibit autosomal-dominant inheritance but up to 60%-80% of this form of AD is genetically determined[23]. Thus, genetic risk factors are extensively studied, being the apolipoprotein E (ApoE) gene, in chromosome 19, an excellent example.

1. Increasing the risk

ApoE exists as three isoforms ε2, ε3 and ε4, with ε3 having the highest prevalence, and it plays an important role in AD, since the risk of developing disease is increased in carriers of the ApoE-ε4 allele. In 1993 the group of E.H. Corder stated that individuals with one and two copies of the ε4 allele have, respectively, a 45% and 50–90% probability of developing AD[27], and that a double dose of ApoE-ε4 allele was nearly enough to cause AD by age 80[28]. Despite the broad molecular evidence about ApoE's role in AD, its genetic variation is also present in other kinds of neurological disorders including Parkinson's disease and multiple sclerosis[23]. In 2009, three novel AD genes were identified, presenting high degree of association: CLU (clusterin or apolipoprotein J – ApoJ), CR1 (complement component (3b/4b) receptor 1), and PICALM (phosphatidylinositol binding clathrin assembly protein)[23].

As Stephen King wrote in *The Gunslinger:* **"Time's the thief of memory"**. Thus, in addition to the genetic risk, a well established (and intuitive) risk factor is aging, since in every species age brings a slowing of brain function[29]. It is considered the most important factor specially due to the increasing of life expectancy worldwide, in addition to the increasing of population, which in turn can be attributed to the postwar "baby boom".

Other risk factors, such as: diabetes mellitus[30], obesity, hypertension, metabolic syndrome, hypercholesterolemia[31], Down's syndrome[32], traumatic brain injury[33], gender, education[34] (female gender and low educational level with increased risk), social engagement, and diet, have been increasing evidence. Contrasting with the previous risk factors, wine consumption, coffee consumption, the use of non-steroidal anti-inflammatory drugs (NSAIDs), and a good balance of metal ions [35] are associated with reduced risks, thus showing some protective effects[34].

2. Symptoms and afflictions

In terms of symptoms, it is possible to divide and group them in three simple categories: **(1)** cognitive deficits that affect memory (amnesia and agnosia), speech (aphasia), and motor function (apraxia)[29]; **(2)** various psychiatric symptoms and behavioral disturbances such as depression, social withdrawal[8], personality changes, delusions, hallucinations, and misidentification[7, 20]; **(3)** difficulties with the daily living activities, such as driving, using the telephone, dealing with money and, later in the disease, all the basic needs (feeding, dressing, toileting)[20]. As one would expect, with disease progression the intensity of the symptoms increases and also, patients start to

become increasingly more dependent on others to do their every day chores. Hence, this disease does not just affect the life of patients but also the life of their caregivers.

3. How does it work?

All visible symptoms arise from the alteration and loss of structural complexity of our brain cells [29], which can begin as many as 20 years before symptoms appear[5]. Thus, all the above symptoms can be related to a series of pathological processes that appear to be altered in this dysfunction. AD is a complex multifactorial disorder in which protein alteration, oxidative stress, immune deregulation, neuronal inflammation, synaptic loss[36], defects in neurotransmission, disruption of neural network activity, and reduction of energy metabolism [19, 37] are considered triggering factors for neuronal degeneration. To increase the complexity of AD, the balance among these may vary from patient to patient[38]. Interestingly, the early symptoms of amnesia, if in the absence of any other clinical signs of brain injury, suggest that something is intermittently interrupting the function of synapses that help to encode new declarative memories, agreeing with the hypothesis that Alzheimer's disease is a synaptic failure[39].

Neuroimaging enabled the identification of the areas of the brain that were undergoing morphological and volumetric structural changes. The major areas suffering from these alterations are the entorhinal cortex and hippocampus, showing some correlation between the extent of alteration and cognitive symptoms/disease severity[6, 40]. Despite the vast knowledge acquired along the past century, the molecular pathway for AD origin is still mostly unknown.

"Aβ and Tau – cause or consequence?"

Different lines of thinking try to explain the molecular pathogenesis of AD, yet none has already been completely proven. Among them, two hypothesis stand out, giving rise to long and hard arguments between their supporters. The central foundation of these two theories relies on one question: Are amyloid plaques or neurofibrillary tangles the cause or a consequence of AD? First of all, the definition of two fundamental terms, and their inherent concepts, is necessary to understand this complex pathology.

1. Amyloid definition

The term amyloid (or a so-called amyloid state) was introduced by Virchow, in 1854, to denote a macroscopic tissue abnormality that exhibited a positive iodine staining reaction[41]. Currently, it is used to sort a class of proteins with a propensity to undergo conformational changes and share specific structural traits, resulting in insoluble fibril formation[41]. According to the Nomenclature Committee of the International Society of Amyloidosis, amyloid consists in extracellular depositions of protein fibrils with characteristic appearance in electron microscope, typical X-ray diffraction pattern (β-sheet)[42], and affinity for thioflavin dyes[43] and Congo red[44] (producing an apple-green birefringence). On electron microscopy, amyloid consists of rigid, linear, non-branching, aggregated fibrils that are 7.5 – 10.0 nm in width and of indefinite length[45].

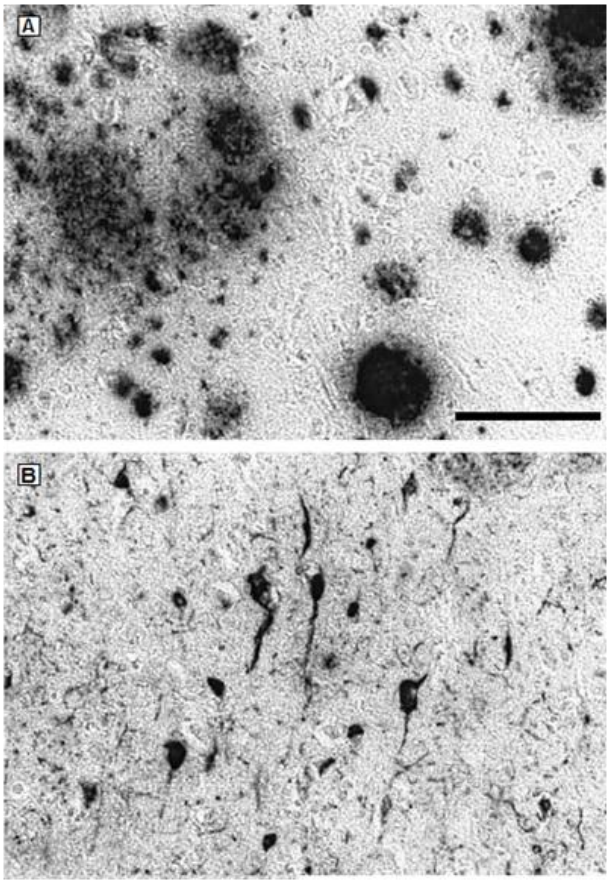


Figure 2. Senile plaques and neurofibrillary tangles. Inferior temporal cortex immunolabeled for abundant amyoid plaque deposits (A), and abundant neurofibrillary tangles (B) (bar=10 μm). (Adapted from Bennet et al., 2004)[1].

The deposition of amyloid fibrils is a consequence of the intermolecular hydrogen bonding of extended polypeptide strands that arise as a consequence of protein misfolding[46]. Out of curiosity, fewer than 25 amyloid-forming proteins have been identified and associated with a unique clinical syndrome, such as: Aβ with AD, transthyretin (TTR) with familial amyloidotic polyneuropathy (FAP)[47], islet amyloid polypeptide (IAPP) with diabetes type 2, and prion protein (PrP) with the spongiform encephalopathies[48]. For the present work, we are only interested in amyloid deposits composed by Aβ peptide.

Senile Plaques – Hallmark #1

To Aβ amyloid deposits (Figure 2) was attributed the nomination of **senile plaques** (**SPs**), and they can be distinguished in different plaques subtypes, including neuritic, diffuse, primitive, compact, cored and cotton-wool[14] depending on their composition. Despite of the variety, neuritic and diffuse plaques are considered the two major subtypes in AD. Neuritic plaques are constituted by

the 40– and 42–amino acid (aa) β-amyloid ($A\beta_{40}$ and $A\beta_{42}$, respectively) peptides, of about 4 kDa, surrounded by dystrophic neurites (axons and dendrites), microglia (monocyte- or macrophage-derived cells that reside in the brain), and reactive astrocytes[49, 50]. Diffuse deposits are mainly composed of $A\beta_{42}$[51] and lack the neuritic and glial components[52], but evolve over time with formation of discrete niduses that eventually become neuritic SPs[53].

2. APP and Aβ peptide: introducing concepts

The β-amyloid precursor protein (**APP**) is a transmembrane receptor (Figure 3) expressed ubiquitously in both neuronal cells and extra-neuronal tissues[54]. In humans, the APP gene is located in the chromosome 21 and is composed of 18 exons[55]. Three major isoforms are expressed by alternative splicing: APP770 (full length), APP751 (lacking exon 8), and APP695 (lacking exon 7 and exon 8)[56, 57], comprising mRNAs ratio of 1:10:20, respectively, in human cortex[54]. It belongs to a highly conserved family of type 1 transmembrane glycoproteins that extends also to invertebrate species, including the homologous: APL-1 (*Caenorhabditis elegans)*, APPL (*Drosophila)*, appa and appb (zebrafish), and APLP1 and APLP2 (in mammals, besides APP)[58]. APP770 and APP751 isoforms are expressed in most tissues and contain the Kunitz Protein Inhibitor (KPI) domain while APP695 isoform is mostly expressed in neurons and lacks this domain[57]. An interesting observation is that AD brain samples show increased levels of KPI-containing APP isoforms, thus suggesting that the balance between the KPI- and non-KPI-containing isoforms may be an important factor influencing Aβ deposition[59].

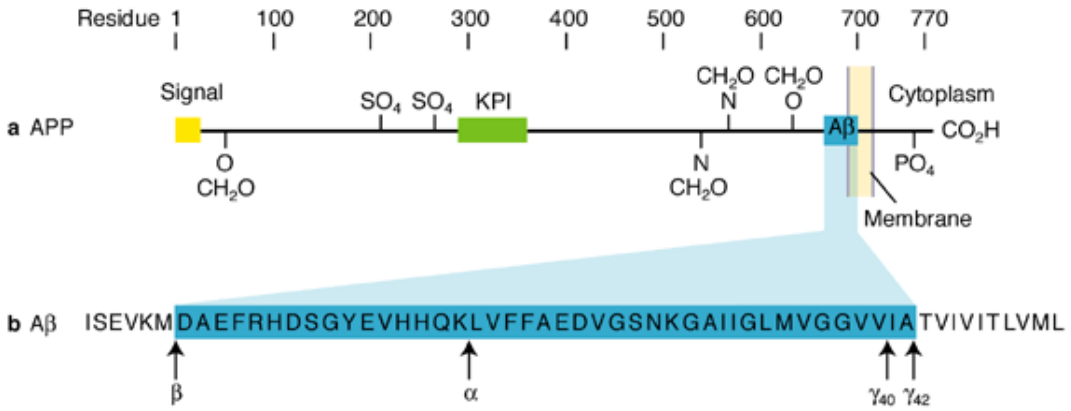


Figure 3. Schematic diagram of the amyloid precursor protein (APP) and its cleavage to give b-amyloid. (a) APP is an integral membrane, proteoglycan-like molecule of 700 amino acids (full length isoform); sulphation (SO_4), phosphorylation (PO_4) and carbohydrate attachment (CH_2O) sites, the Kunitz-type protease inhibitor domain (KPI) and the secretory signal sequence ('Signal') are shown. **(b)** The protein is proteolytically processed by secretases in several different pathways. Cleavage of APP at the β and γ sites generates Aβ sequences of 40 or 42 residues (amino acids in single-letter code). The most common cleavage by α-secretase precludes Aβ formation. (Chen and Schubert, 2002)[60].

The 4 kDa **Aβ peptide**, originated by the sequential cleavage of APP[61] (Figure 3), was first isolated and sequenced by Glenner and Wong, in 1984[62], and can be found in the plasma and cerebrospinal fluid (CSF) of healthy humans and other mammals[63]. It was described as a 24 aa peptide but later sequencing revealed that the peptide may actually comprise 36-43 aa[64], being the two major species $A\beta_{40}$ and $A\beta_{42}$. In healthy individuals, these two forms make up 90% and about 10%, respectively, of the Aβ peptides that are normally produced by brain cells[49]. Despite the little variation between forms, they differ greatly in properties. For example, $A\beta_{42}$ is more hydrophobic, thus, more prone to aggregation (compared to the less hydrophobic $A\beta_{40}$). In fact, it readily aggregates *in vitro,* being considered the more amyloidogenic and hence pathogenic species[65].

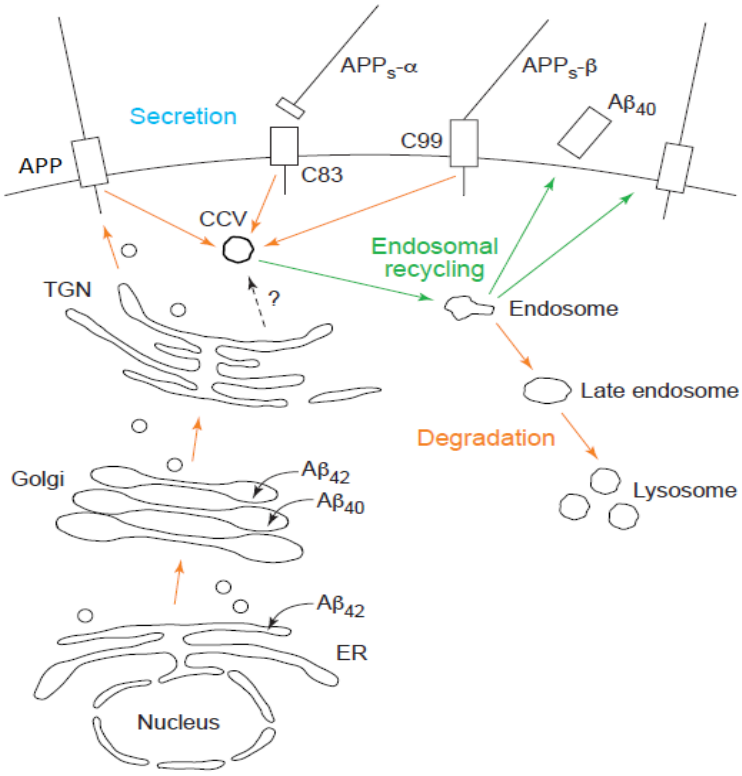


Figure 4. A simplified diagram of some of the principal routes of trafficking of the amyloid precursor protein (APP). After synthesis on ribosomes, APP is co-translationally translocated into the endoplasmic reticulum (ER) by its signal peptide and trafficks through the secretory pathway to the trans-Golgi network (TGN). A small portion of APP molecules reaches the plasma membrane, where the secretase cleavages can occur, generating soluble APP, α and β. Some cell surface holoproteins that remain uncleaved can be re-internalized via clathrin-coated pits and vesicles (CCVs) and enter the endosomal system. Here, they can be recycled to the cell surface, or enter late endosomes and lysosomes, presumably for degradation. $A\beta_{40}$ can be generated in part during endosomal recycling and released at the surface. $A\beta_{42}$ can be generated in considerable part in ER vesicles, and Golgi vesicles appear to contain both $A\beta_{42}$ and $A\beta_{40}$. However, our understanding of all of the sites in the cell for Aβ generation remains incomplete. (Adapted from Selkoe, 1998)[63].

3. APP processing

APP is co-translationally translocated into the endoplasmic reticulum (ER) (Figure 4) by its signal peptide and matures through the central secretory pathway, with only a small percentage of holoproteins reaching the cell surface[63]. During and after this trafficking through the ER, Golgi and trans-Golgi network (TGN), APP suffers specific endoproteolytic cleavages[63] that will originate several APP metabolites, among them the Aβ peptide. After reaching the membrane surface, APP can still undergo clathrin-mediated endocytosis and then rapidly recycle to the surface again[66], during which Aβ can also be produced[63].

Towards Amyloidogenicity or Non-amyloidogenicity?

APP processing can originate different metabolites (with different functions) depending on the proteolysis pathway initiated. Whether the amyloidogenic or non-amyloidogenic pathway is followed (Figure 5) is defined by the protease that initially cleaves the Aβ precursor.

The **non-amyloidogenic pathway** includes cleavage of APP by α-secretase, a zinc metalloproteinase of the ADAM family[57], followed by the action of γ-secretase[56], a high molecular weight complex of four proteins: presenilin 1 or 2 (PSEN1, PSEN2), nicastrin (NCT), anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2)[31, 67]. The cleavage by the α-secretase at Lys687 abrogates the production of Aβ since the cleavage is within the Aβ domain, resulting in the release of a large soluble ectodomain of APP called sAPPα (~100 kDa)[57], leaving behind a 83-residue carboxi-terminal fragment (CTFα, of ~10 kDa[68]). Then, γ-secretase acts in the CTFα, liberating the extracellular p3 peptide and the 50 aa APP intracellular domain[64] (AICD, of ~6 kDa)[69].

On the other hand, well suggested by its name, the **amyloidogenic pathway** originates Aβ peptide and consists of two sequential cleavages, first by the β-secretase (beta-site APP–cleaving enzyme 1 – BACE-1), and then by γ-secretase[36], after which Aβ may first appear in soluble form either within neurons or in the extracellular space[70]. The first protease, β-secretase, cleaves APP at Met671, releasing a large soluble ectodomain of APP called sAPPβ[71] (similarly to what happens in the non-amyloidogenic pathway). The remainder 99 aa CTFβ (of ~13 kDa[68]) is then cleaved by the γ-secretase, which occurs in the middle of the membrane and liberates, as said above, the Aβ peptide and the AICD[72]. This process generates different species of Aβ with variable

hydrophobic C-termini (due to the various proteolysis sites of γ-secretase) (Figure 6) that present different propensity to oligomerize[72] and, consequently, to form SPs. As previously referred, PSEN1 and PSEN2 mutations are highly correlated with AD: these membrane proteins, mainly localized to the endoplasmic reticulum and Golgi, are components of the γ-secretase complex, thus AD-linked mutations selectively enhance γ-secretase cleavage after residue 42 of Aβ[63].

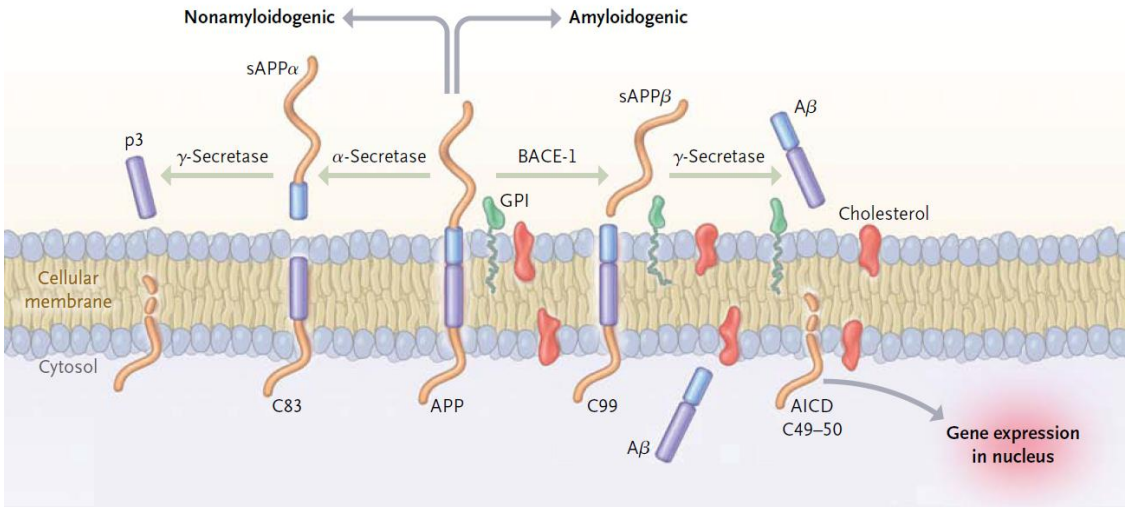


Figure 5. Processing of Amyloid Precursor Protein. The cleavage by α-secretase, interior to the Aβ sequence, initiates non-amyloidogenic processing. A large amyloid precursor protein (sAPPα) ectodomain is released, leaving behind an 83-residue carboxy-terminal fragment. C83 is then digested by γ-secretase, liberating extracellular p3 and the amyloid intracellular domain (AICD). Amyloidogenic processing is initiated by the β-secretase beta-site amyloid precursor protein-cleaving enzyme 1 (BACE-1), releasing a shorter ectodomain, sAPPβ. The retained C99 is also a γ-secretase substrate, generating Aβ and AICD. AICD is a short tail (approximately 50 amino acids) that is released into the cytoplasm after cleavage by γ-secretase. AICD is targeted to the nucleus, signaling transcription activation. (Adapted from Querfurth and LaFerla, 2010)[64].

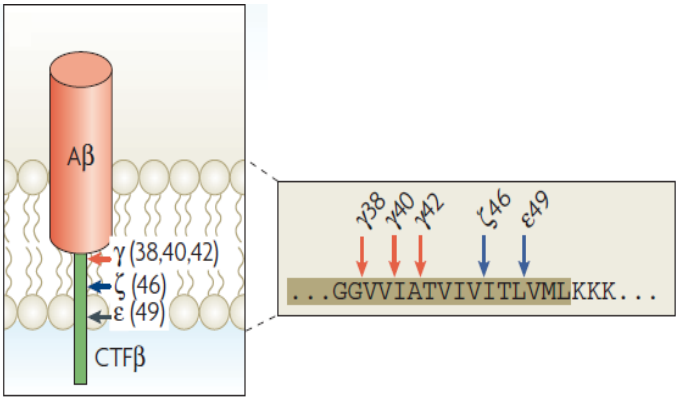


Figure 6. Various proposed sites of intramembrane proteolysis by γ-secretase. The amino-acid sequence around the cleavage sites of APP is shown (numbers refer to the sequence of Aβ; shaded amino acids are in the transmembrane domain). γ-secretase cuts its substrates several times and thus the cleavage sites are referred to as ε, ζ and γ (from the C- to N-terminal). The γ-site is variable and can occur at least after amino acids 38, 40 and 42. This cleavage is highly relevant for the subsequent aggregation propensity of Aβ. Some γ-secretase-modifying drugs shift the cleavage at Aβ42 to amino acid 38, and the resultant peptide aggregates much less readily. (Adapted from Haass and Selkoe, 2007)[72]

APP metabolites

In contrast to Aβ, the sAPPα metabolite has an important role in neuronal plasticity and survival[73] and acts as a protector against neuron insults (excitotoxicity and metabolic and oxidative insults)[74]. Interestingly, expression of sAPPα is sufficient, by itself, to rescue the abnormalities of APP-deficient mice, implying that most of APP's physiological function is influenced by sAPPα levels[75]. Although sAPPβ only differs from sAPPα by lacking the Aβ:1-16 region at its carboxyl-terminus, sAPPβ was reported to function as a death receptor 6 ligand and to mediate axonal pruning and neuronal cell death[57]. The function of the AICD is unclear, nevertheless, it has been shown to translocate to the nucleus, forming a transcriptionally active complex with Fe65 and the chromatin-remodeling factor Tip60[58]. Concerning the small p3 peptide, despite existing evidences of its role as pro-inflammatory agent, its function has not been established[76]. This process is the basis of "amyloid cascade hypothesis", which will be discussed forward.

4. Aβ clearance

Accumulation of Aβ is intimately related to the progression of neurodegeneration in AD and it may be seen as the rate of its generation versus clearance (elimination). This elimination process is achieved by two major pathways: **proteolytic degradation** and **receptor-mediated transport** from the brain[77] (Figure 7). In addition, and as curiosity, soluble Aβ can also be removed slowly, via interstitial fluid bulk flow, into the bloodstream[78]. However, this is responsible for the clearance of only 10–15% of the total Aβ in the brain[78].

Proteolytic degradation

Aβ is degraded by several peptidases, principally two zinc metallo-endopeptidases referred to as neprilysin (NEP) and insulin-degrading enzyme (IDE)[77].

NEP, possesses a catalytic site exposed extracellularly, which makes it a prime candidate for peptide degradation at extracellular sites of Aβ deposits[78]. *In vivo* studies revealed that inhibition of NEP protein or disruption of the NEP gene results in a defect in degradation and subsequent increased levels of Aβ[79, 80]. This suggests that age-related decrease of NEP could lead to increased brain concentrations of Aβ, plaque formation, and AD[80]. IDE (similar to NEP) hydrolyzes several regulatory peptides, apart

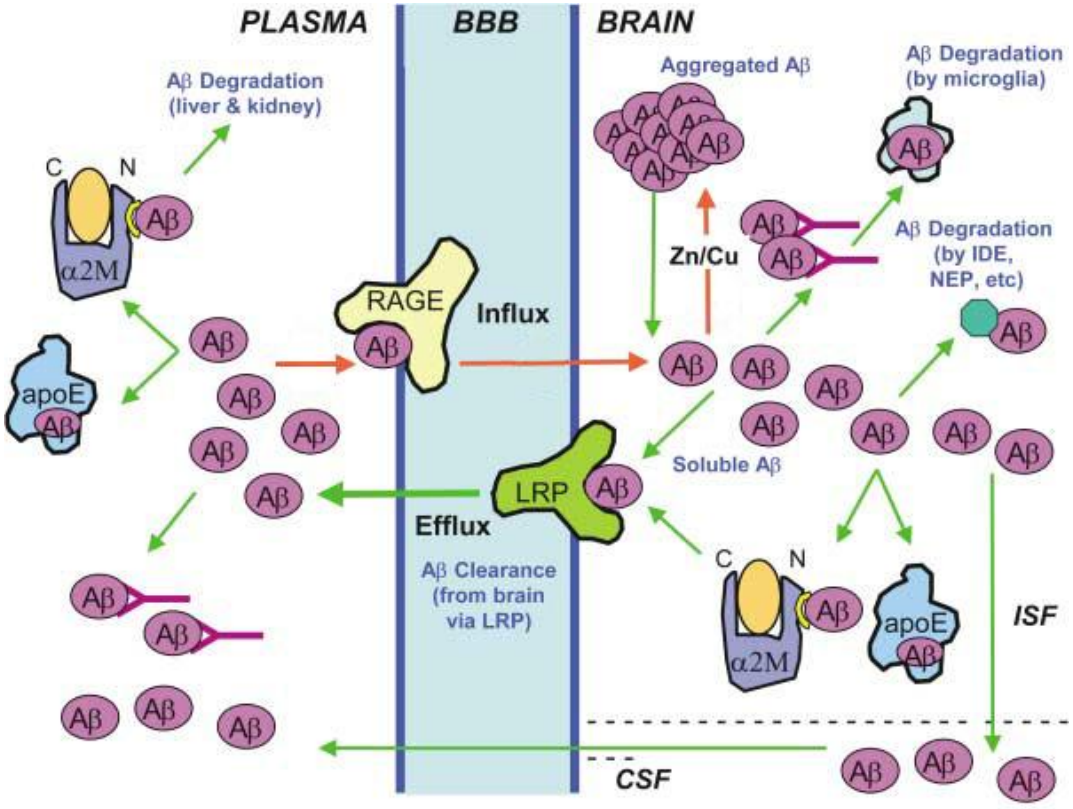


Figure 7. Pathways involved in removal of brain Aβ. Soluble Aβ in the parenchyma of the brain can undergo two basic fates. It can aggregate into fibrillogenic species that can be ultimately deposited as β amyloid, fostered by interaction with heavy metals (zinc, copper, among others). Soluble Aβ can be removed from the brain via two basic pathways: **(a)** enzymatic degradation or **(b)** receptor-mediated clearance. **(a)** Soluble Aβ can be degraded by specific peptidases, such as IDE and NEP, and, in addition, Aβ can also be internalized and degraded by activated micoglia in the brain. The amyloid vaccine has been speculated to promote this activity. **(b)** In an alternative Aβ clearance pathway, the peptide can be transported across the BBB and exported out of the brain into the blood stream either by direct binding to LRP (and P-gp, not showed) or by first binding the LRP ligands/Aβ chaperones apoE and α2M. Once Aβ enters the bloodstream, it can reenter the brain via the RAGE receptor or be delivered, via chaperone molecules such as apoE or α2M, to peripheral sites of degradation, such as liver and kidney. Another proposed mechanism for Aβ clearance is one in which antibodies to β amyloid bind Aβ in the blood stream and prevent reentry back into the brain. Green arrows signify pathways that might be pharmacologically enhanced, while red arrows and slashed circles indicate pathways that might be blocked as potential therapeutic ap proaches for the treatment and prevention of AD. (Adapted from Tanzi et al., 2004)[77]

from Aβ. A very convincing evidence of IDE's role in Aβ degradation came from a study in IDE *knockout* mice that revealed increased levels of Aβ (>50% decrease in Aβ degradation) and AICD peptides in the brain[81]. Increasing the evidence, epidemiological studies suggest that the gene encoding IDE in chromosome 10q, possesses genetic linkage for both LOAD and type 2 diabetes mellitus[78].

Receptor-mediated transport

✓ Efflux: LRP1/P-gp combination

Aβ clearance from brain to blood has to be a two-step process. First it has to pass through the abluminal (brain side) and then the luminal (blood side) plasma membranes of the brain capillary endothelial cells that comprise the blood-brain barrier (BBB)[82]. The first step is suggested to be held by the low-density lipoprotein receptor related protein 1 (LRP1), while the second still bears some doubts[82].

LRP1 is the major efflux transporter of Aβ across the BBB[83]. It is a member of the low density lipoprotein (LDL) receptor family and functions both as a multifunctional scavenger and signaling receptor, and as transporter and metabolizer of cholesterol and ApoE-containing lipoproteins[84]. LRP1 is localized predominantly on the abluminal side of the cerebral endothelium and is suggested as the major protein responsible for Aβ endocytosis and transcytosis across the BBB[85]. LRP1, in addition to Aβ and ApoE, binds several other ligands (approximately 40) such as: α2-Macroglobulin (α2M), tissue plasminogen activator (tPA), proteinase-inhibitors, APP, blood coagulation factors, growth factors, among others[85]. However, through *in vitro* ligand-binding affinities assays, LRP1 was found to preferentially bind Aβ peptides as compared to other ligands[86]. It appears genetically linked to AD in epidemiological studies and is negatively regulated by Aβ levels[78].

P-glycoprotein 1 (P-gp, also known as ATP-binding cassette B1 (ABCB1)) is an ATP-dependent efflux pump that, as well as mediating the removal of ingested toxic lipophilic metabolites[83], was suggested to be also an important (second step) active transporter of Aβ[87]. The conjugation of two results: a demonstration of direct interaction between Aβ and P-gp, and the post-mortem analyses of AD brain samples showing a negative correlation with Aβ deposition[88]; suggest that P-gp in directly involved in the clearance of Aβ. Another member of its family, cholesterol efflux regulatory protein (CERP, also known as ABCA1), has also been suggested to take part in this process. Contrary to P-gp, CERP controls Aβ clearance indirectly, via an ApoE dependent manner, thus enhancing its clearance from the brain[87].

This suggests that cooperation between LRP1 and P-gp is necessary for the efficient efflux of Aβ, thus, LRP1 should not be regarded as the only intervening.

✓ Influx: RAGE-mediated

The receptor for advanced glycation end products (RAGE), is a multi-ligand and cell surface receptor that binds soluble Aβ, and a major transporter of pathophysiologically relevant concentrations of plasma Aβ across the BBB[78]. RAGE expression has been found to be increased in brain endothelial cells and vascular smooth muscle cells in animal models of aging as well as in AD patients[89]. Contrary to LRP1, RAGE expression is positively correlated and sustained at an elevated level by excess amounts of Aβ, through a positive-feedback mechanism[78].

"Sink" hypothesis

The continuous removal of Aβ from the brain, blood and organs is essential for the regulation of Aβ brain levels. At the moment, a three-step process, dubbed as the "sink" hypothesis, is proposed to explain Aβ homeostasis. (1) Aβ binding to LRP1 at the cell membrane initiates rapid Aβ clearance across the BBB into the blood in vivo, followed by (2) circulating plasma soluble LRP1 (peripheral "sink" for brain Aβ) binding to and sequestering (>70% of) free Aβ in plasma, thus promoting continuous removal of Aβ from brain[89]. sLRP1 is the truncated extracellular domain of LRP1, after β-secretase cleavage of its β-chain[85]. Finally, (3) LRP1 localized to hepatic cells binds to and systemically clears circulating Aβ. In addition to the liver, sLRP1-Aβ complexes and free Aβ are also eliminated through the kidneys[89]. Sagare et al. showed that in AD patients the levels of sLRP1 were lower than in controls, plus, there was a huge increase in oxidized sLRP1 with very little affinity towards Aβ[86]. This will increase the Aβ free fraction promoting the influx from blood to brain.

5. Tau protein: introducing concepts

Tau protein was discovered in 1975 by the group of Marc W. Kirschener and belongs to the microtubule-associated protein (MAP) family[90, 91]. It is manly considered an axonal protein expressed in mature neurons[92] and defined as an essential protein for microtubules assembly and stability[90] and vesicle transport[64]. Tau can be found in many animal species such as *Caenorhabditis elegans*, *Drosophila*, goldfish, bullfrog, rodents, and human[93]. It is present as a single-copy gene (over 100kb)[94], localized on the long arm of chromosome 17q21[95] (MAPT gene[32]), and contain 16 exons (three of which are never present in mRNA of brain tissue – 4A, 6 and 8)[96]. In the central nervous system, alternative splicing of tau primary transcript generates six isoforms with an

apparent molecular weight between 60 and 74 kDa[97] and a natively unfolded conformation[98].

Tau – just medium phosphorylated

Tau is a component of microtubules, which represent the internal support structures for transport of nutrients, vesicles, mitochondria and chromosomes from the cell body to the ends of the axon and back[99]. It binds to microtubules through repetitive regions in their C-terminal part encoded by exons 9-12[93] and is considered a highly soluble protein that shows hardly any tendency to assemble under physiological conditions[100]. The different states of tau phosphorylation result from the activity of specific kinases and phosphatases[93], thus, an imbalance between these two classes of proteins will affect tau's biological function. In a hyperphosphorylated state, tau changes its native conformation and loses its affinity toward microtubules[101], thus being released in a soluble form[102]. Then, newly soluble tau proteins can be targeted for post-translational modifications (not necessarily just phosphorylation) that directly or indirectly alter tau conformation, promoting tau aggregation and paired helical filaments (PHFs) formation[97]. The longest form of tau (441 aa) possesses 85 putative phosphorylation sites (Figure 8)[103] (serine, threonine and tyrosine residues), which are available to numerous kinases, such as casein kinase 1 (CK1 – considered the major kinase of tau due to the)[97, 104], mitogen-acivated protein kinases (MAPKs), glycogen synthase kinase 3β (GSK3β), and cyclin-dependent kinase 5 (CDK5)[105]. Abnormal phosphorylation is not the only cause of tau's conformational change. Mutant tau proteins may also have diminish affinity for microtubules and promote, consequently, tau aggregation into PHFs, specially when this occurs inside the microtubule-binding domain[100]. More than 30 mutations of tau gene have been described, nevertheless, tau's mutations are not observed in AD[64]. Other mechanisms that promote tau aggregation have been proposed to involve several posttranslational modifications (such as ubiquitination, glycation, glycosylation, and transglutamination), the neuronal redox potential and the presence of cofactors (ApoE, and aluminium)[93].

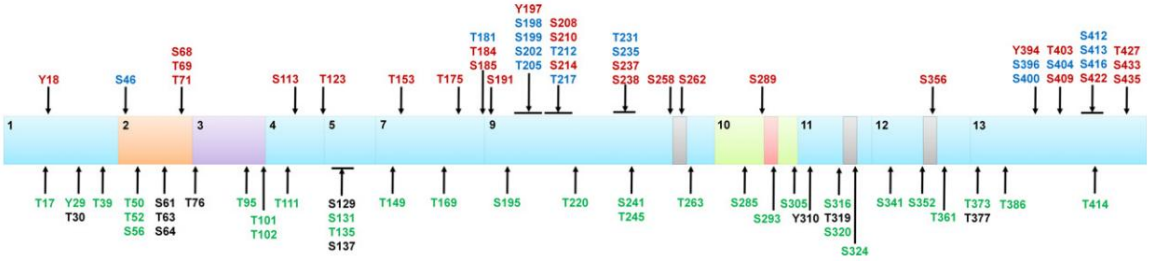


Figure 8. Tau phosphorylation sites. Tau phosphorylation sites found in AD brains (in red), those found in normal brain (in green) and those present both in normal and AD brains (in blue) are indicated according to the longest tau isoform tau. Putative phosphorylation sites that have not yet been proven to be phophorylated *in vitro* or *in vivo* (in black). (Adapted from Martin et al., 2011)[97]

Neurofibrillary tangles: Hallmark #2

Neurofibrillary tangles (**NFTs**) are filamentous inclusions (intracellular lesions), preferentially observed in pyramidal neurons, composed of filamentous aggregates of abnormally hyperphosphorylated microtubule-associated protein tau[64, 106]. NFTS, are constituted by PHFs and by a minor class that does not exhibit the marked modulation in width of PHFs[107] – straight filaments (SFs). Like SPs, NFTs are hallmarks of AD and responsible for other neurodegenerative disorders termed tauopathies[108] (e.g. Pick's disease, progressive supranuclear palsy, amyotrophic lateral sclerosis/parkinsonism–dementia complex of Guam, and some frontotemporal dementias)[92, 93].

Resuming to the explanation of AD pathogenesis, various hypotheses have been proposed with very different and plausible molecular mechanism, backing it up. Two hypotheses stand out, the "amyloid cascade hypothesis" and a so-called "tau and tangle hypothesis", very likely due to the fact that they are centered in the two hallmarks of AD.

6. Amyloid cascade hypothesis

First of all, it is of great importance to mention that APP processing is a normal metabolic event and that Aβ is a normal product of cellular metabolism throughout life and circulates as a soluble peptide in biological fluids[109]. Plus, Aβ deposition can also be found in the brain of non-demented elderly people.

The most persuasive theory is the "amyloid cascade hypothesis"[110] (Figure 5) and it suggests that amyloid deposition is the first step of a cascade of processes that ultimately culminate in disease[1, 25]. More concretely, it is based on the effects that the highly insoluble forms of Aβ peptide (as SPs or as toxic oligomers) have in terms of neurotoxicity, due to a dysregulation in APP processing or Aβ clearance, early in the

disease process[111]. It was first suggested that this dysregulation would increase the $A\beta_{42}/A\beta_{40}$ ratio, in other words, promote the production of the most neurotoxic form ($A\beta_{42}$). This would lead to aggregation and to SPs' formation, which in turn would be responsible for the subsequent pathology (including tau aggregation, phosphorylation, neuronal attrition and clinical dementia)[111]. Nonetheless, amyloid fibrils are not the only form of Aβ possible to observe. Various species, including monomers, oligomers, and protofibrils (usually shorter and thinner than amyloid fibrils)[112], with different characteristics, are gaining interest as to explain the toxic effects of Aβ. The relationship between SPs and clinical manifestations or neurodegenerative changes is quite controversial. Thus, more recently, the attention has been deviated from the harmfull effects caused by SPs, giving prevalence to the toxic Aβ oligomer hypothesis. Perhaps due to a greater capacity for diffusion and larger collective surface area for interacting with neurones and glial cells[61]. Some suggest that Aβ toxicity functions in a plaque-independent manner[113], stating that oligomeric intermediates present higher toxicity to the cell and, in addition, this is not related to a specific prefibrillar aggregate (dimer, trimer, and so on) but rather to the propensity that each species has to grow and undergo fibril formation[114].

Several observations consistent with the amyloid cascade hypothesis are continuously being found, e.g. intraneuronal accumulation of Aβ oligomers can activate signalling pathways which cause tau hyperphosphorylation[61]. This particular discovery strengthens the hypothesis on one hand, and on the other discredits the "tau and tangle hypothesis" (discussed forward). Other serve as supplement for this hypothesis and can be grouped together as Aβ-related hypotheses. It is the case of biometals (Zn(II) and Cu(II)) involvement with Aβ, microglia-derived toxicity, or membrane permeabilization by Aβ oligomers. Concerning the first, several contradictions in the application of the amyloid hypothesis can be removed by considering the role of redox-active metals in plaques as primary toxic agents and biometals as the trigger of Aβ fibrillization, in the case of sporadic AD[115]. As for the second, the inflammation hypothesis states that active phagocytic microglia, triggered by Aβ oligomers, is the primary cause of early toxicity[61]. However, the role of the different Aβ forms inducing the microglial phagocytosis, generation of oxidative stress, and inflammatory response remain unclear[116]. Finally, membrane permeabilization by amyloid oligomers (after formation of discrete pores or single channels – "channel hypothesis")[43], leading to an increasing in intracellular calcium concentration, has been proposed as the primary mechanism of pathogenesis[117]. Nevertheless, there is some disagreement as to the mechanism by which amyloid oligomers increase intracellular calcium[43].

Karl Herrup, in 2010, proposed a new model for AD build on a 3 key event: (1) a precipitating injury (head trauma, vascular events, illness or stress could initiate a protective response) that may not cease due to age-related failure of the normal homeostatic mechanisms), triggers (2) chronic inflammation, which in turn leads to (3) major physiologic shift in neurons[29].

A more consensual vision about Aβ is that it possesses a dual role: a neurotrophic and a neuronal degeneration action (if in high concentrations) in mature neurons. This is not a theory too difficult to accept since in Nature everything that is exaggerated brings some degree of harm. Its neuroprotective role was suggested to act against excitotoxic death by activating the phosphatidylinositol-3-kinase (PI-3K) pathway, serving as a double prooxidant/antioxidant and shown to bind and remove harmful substances by blocking them in plaques[118].

There are still some that defend a more controversial hypothesis, the “alternate hypothesis”, which opposes the amyloid cascade hypothesis by proposing that Aβ is not as a harbinger of death but rather a protective response to neuronal insult[119]. Despite all the advances made, the source of Aβ toxicity still remains elusive.

7. Tau and tangle hypothesis

“Tauists”, defend a collection of related ideas that maintain the primacy of NFTs formation as the AD-causing event, which Mudher and Lovestone designated as the “tau and tangle hypothesis” (Figure 9)[111]. It has emerged due to solid evidence that SPs do not account for the complex pathophysiology of AD[38]. It argues that in AD the normal role of tau is impaired and that NFTs accumulate to occupy much of the neuron and apparently result in neuronal death, as extracellular tangles in the shape of neurons are abundant in late stages of disease[111]. Maccioni et al, postulated a much more embracing tau hypothesis, in which, a series of damage signals (Aβ oligomers, oxygen free radicals, iron overload, cholesterol levels in neuronal rafts, LDL species and homocysteine, among other) trigger, by innate immunity, the activation of microglial cells with the consequent release of pro-inflammatory cytokines that modify neuronal behavior through anomalous signaling cascades, which finally, promote tau hyperphosphorylation[38]. As described in the tau section, hyperphosphorylation leads to tau oligomerization and production of NFTs that, after neuronal death, are released to the extracellular environment (“ghost tangles”, remaining characteristically stable[105]), contributing to activation of microglial cells and stimulating the deleterious cycle, leading to progressive neuronal degeneration[38].

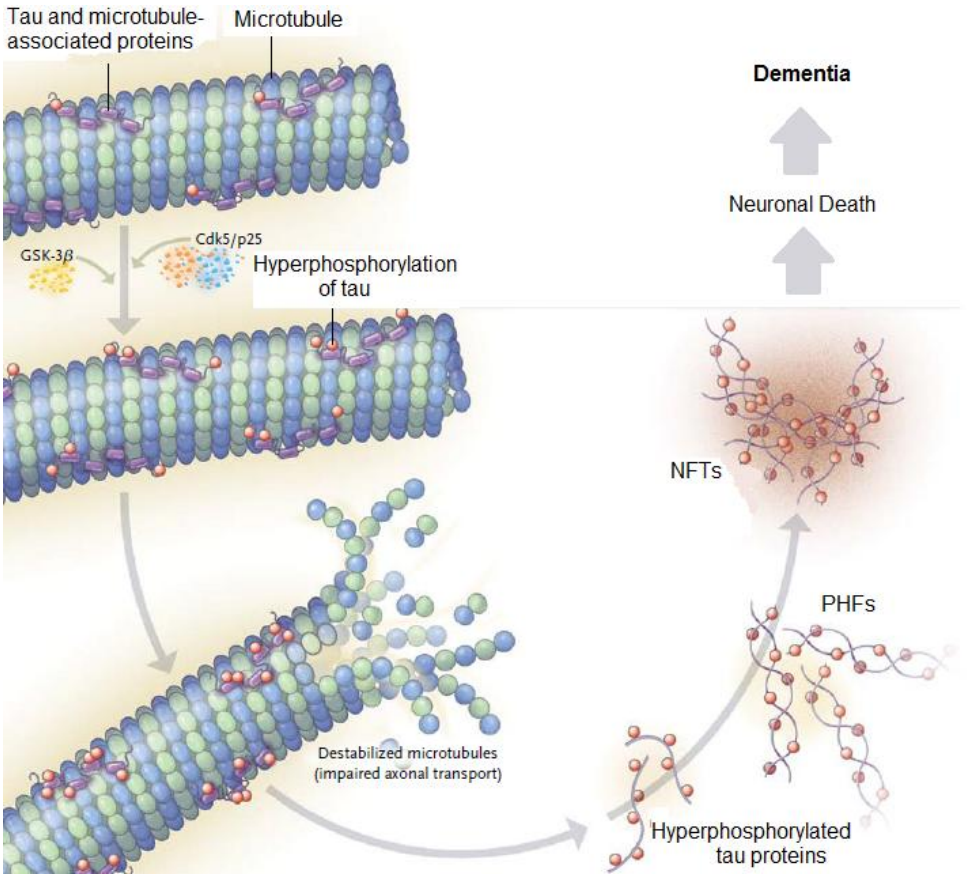


Figure 9. Tau Structure and Function. Normal phosphorylation of tau occurs on serine and threonine residues. These amino acids can be phosphorylated by a series of kinases, such as: glycogen synthetase kinase 3 (GSK-3β), cyclin-dependent kinase (cdk5) and its activator subunit p25 (shown), mitogen-activated protein kinase (MAPK), Akt, Fyn, and protein kinase A (PKA) (not shown). Tau binding promotes microtubule assembly and stability. Excessive phosphorylation of tau leads to decreased tau binding to microtubules, increasing free tau, which, under the appropriate conditions, will self-aggregate to form insoluble PHFs (paired helical filaments). Loss of tau binding is predicted to result in loss of microtubule function. All this process leads to neuronal death, which might result in dementia. (Adapted from Querfurth and LaFerla, 2010)[64].

The degree of tau phosphorylation in the AD brain is reasonably well correlated with the severity of AD symptoms. However, fetal tau, a much more phosphorylated form of tau than adult tau, does not induce AD-like pathology. In summary, there is no direct evidence for the neurotoxicity of hyperphosphorylated tau[105] (as in the case of Aβ toxicity).

Whilst discovering what and how is causing this complex AD pathology is fundamental, the ultimate goal for every scientist is finding the cure, or if not possible, finding a suitable temporary treatment.

Finding the treatment

Due to the complexity of AD, a vast number of targets and pathways may be chosen to intervene. Cholinergic degradation inhibitors, immunotherapy, secretase inhibitors, anti-inflammatory drugs, tau- and Aβ-deposition interfering drugs, are a few examples of huge classes of drugs that are being tested at the moment[120]. A few options for therapies will be listed next, however, it is important to notice that they only aim to treat symptoms and not the cause of the disease.

The first drugs developed for AD, acetylcholinesterase inhibitors (AchEI), aimed at increasing acetylcholine levels, previously demonstrated to be reduced in AD [7]. Currently, 5 drugs (FDA approved) are used for the "treatment" of AD in the initial stages: 4 AchEI (Donepezil, Rivastigmine, Galantamine and Tacrine) and 1 NMDA receptor antagonist (Memantine) (http://www.alzforum.org). As referred above, they are not effective, so other targets must be searched.

The first study to prove target engagement by a disease-modifying drug in living humans was reported by Rinne and colleagues, in 2010, using the monoclonal anti-Aβ antibody bapineuzumab[31, 121]. It revealed a reduction of fibrillar amyloid in the brain of AD individuals, but did not improve cognition or function[122]. Crenezumab is another antibody being used in pre-symptomatic treatment trials of Colombian mutant PSEN1 kindred[31]. These are just two examples of an immense list of antibodies that are being studied at the moment. Intravenous immunoglobulins (IVIG) have been proposed as potential treatment based on the hypothesis that IVIG contains naturally occurring antibodies that specifically promote clearance of Aβ peptides from the brain[123]. Secretase modulators[31], tau deposition modulators (e.g., methylene blue[64]) and molecules addressing oxidative damage[7] are also potential drugs under study. Unfortunately, not everyone responds positively to drugs that halt the progression of the disease and, when they do, the protective effect runs off over time. Recently, the "return" of electric shock therapy – deep brain stimulation – by the group of Dr Lozano, from Toronto, gave some hope to the society[124]. Not only did it stop the progression of the disease as also, in less affected patients, suggests a likely improvement in condition[18, 19]. Nevertheless, further work of this approach will be necessary.

A general recommended therapy is Diet and Lifestyle, so that cardiovascular risk factors can be controlled. This will decrease cerebrovascular events, which, in turn, will lead to a reduction in both vascular dementia and the poorly understood contribution of vasculopathy to AD[31].

Since no effective treatment has been developed, the best scenario is an early stage intervention. One common expression can be used to define the treatment approach in AD (and the rest of diseases): the sooner, the better, meaning that the sooner you discover the disease, the greater the odds of treating it. Thus, the search for proper (highly sensitive and specific) biomarkers is on constant demand, allowing a more effective and early stage intervention.

Diagnosis and Biomarkers

The search for early AD biomarkers has been highly targeted over the last years, as investigators believe that the generation of an effective treatment for AD is only possible if the disease is detected at very early stages.

According to Phelps and colleagues, in 1998, the sensitivity of the clinical diagnosis of AD is 93% and the specificity is 55% (which varies with age)[125]. It is a high value but when used in combination with other characterizing techniques (as biomarkers) it is possible to predict/diagnose AD with a greater confidence. By definition, and according to the International Programme on Chemical Safety biomarker is “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease”[126]. In AD, biomarkers are used to early diagnose the disease, by predicting who is going to develop AD from mild cognitive impairment (MCI)[127].

Neuroimaging, has recently been given some evidence in diagnosis (with improvement in PET and MRI spectroscopy resolution) due to the possibility of using specific tracers, such as a derivate of thioflavin T that crosses the BBB and binds selectively to Aβ (C11-labeled Pittsburgh Compound B – PiB[128]) that allow the identification of amyloid deposition in the brain *in vivo* [6] . In 2002, Klunk and colleagues reported a “definitive” diagnosis technique for AD – brain amyloid imaging (BAI) – using the PiB compound[128]. By 2010, the combination of increased BAI signal, low CSF $A\beta_{42}$, and high CSF tau in a subject with dementia was recognized as diagnostic for AD, and patients with MCI and appropriate BAI and CSF profiles could be predicted to progress to frank dementia with high degree of confidence[31]. However, further studies showed the existence of some conflicting reports, since it was not always possible to differentiate symptomatic AD from asymptomatic controls with amyloid plaques[14]. The combination

of neuroimaging and biomarker profile increases the predictive value of AD diagnosis and may lead to a correct characterization of persons at risk, prior to the development of clinical symptoms[6].

There has been an increase in the search for solid AD biomarkers, starting with those who seem to be altered in this condition when compared to normality. Hansson and group stated that the combination of CSF total-tau, phospho-tau and $A\beta_{42}$ yielded good sensitivity and specificity for detection of AD in patients with MCI[40, 127]. More recently, a model based on $A\beta_{42}$ and total-tau levels was developed that could accurately discriminate AD from controls by means of a discrimination line. After autopsy validation the model revealed a sensitivity of 100% and specificity of 91%[129]. Another obvious candidate is the major susceptibility gene for AD, ApoE-ε4. When grouped on the basis of CSF tau and Aβ markers, the association of ApoE-ε4 with AD was twice as strong as compared to when classifying patients according to clinical status[130]. CSF BACE-1 (β-secretase) is also being studied, demonstrating that (despite the small number of subjects) AD patients had increased BACE-1 activity compared with non-demented[131]. Levels of CSF sAPPβ, when combined with CSF tau, have also been reported to be useful in predicting cognitive decline in MCI cohorts[132]. Transthyretin (TTR) in CSF has also been proposed as a biomarker and revealed a significant (and selective for AD) negative correlation between TTR CSF levels and disease severity in AD[133]. Other studies came to contradict this idea suggesting that TTR potential as biomarker raises some doubt since its levels appear to fluctuate substantially within a single individual over a 2-week interval[132].

CSF biomarkers are very promising, although its collection is invasive and thus difficult to be a regular procedure in AD diagnosis. Plasma-derived biomarkers, such as $A\beta_{42}/A\beta_{40}$ ratio may also be useful in the identification of increased risk for developing MCI or AD[134]. Other are under investigation, and for instance, TTR plasma levels also showed a negative correlation between with AD [27, 135], supporting the observations reported for CSF.

Transthyretin – FAP and then AD

1. From component X to transthyretin

TTR was described for the first time by Seibert and Nelson, in 1942, as an X component "which is slightly more mobile than albumin"[136], and thus called prealbumin. Its current name is derived from its primary function, **trans**port of **thy**roxine (T_4) and **retin**ol, through the binding of retinol binding protein (RBP)[137]. TTR is a 55 kDa homotetrameric protein synthesized mainly by the liver and the choroid plexus[138] (corresponding to 20% of total protein synthesized[139]) and secreted into plasma and CSF, respectively. TTR is a single-copy gene mapped in chromosome 18 and its mRNA codifies a 147 aa peptide, corresponding to the TTR-monomer[140]. It is an evolutionary conserved protein and it is found in many vertebrates' species[140] The four monomers within a TTR tetramer, form an open channel where T_4 binds (Figure 10) while retinol interacts with only one of the dimers, at the surface[27].

Yet another TTR function was discovered: Liz et al. also established TTR as a cryptic protease of apoliprotein A1 (ApoA-1)[141] and later showed that TTR may affect HDL biology and the development of atherosclerosis by reducing cholesterol efflux and increasing the apoA-I amyloidogenic potential[142]. Thus, the possible protease role of TTR in the proven interaction with Aβ was addressed and it was observed that TTR was, indeed, able to proteolytically process Aβ in vitro [137]. In addition, cleaved Aβ peptides showed lower amyloidogenic potential than the full length counterpart[137].

2. TTR as a disease factor

TTR is the key protein in familial amyloidotic polineuropathy (FAP), firstly described as "peculiar form of peripheral neuropathy", in 1952 by the Portuguese professor Corino de Andrade[143], and associated to a deposition of TTR protein in 1978, by Costa et al.[144]. FAP is a hereditary autosomal dominant neurodegenerative disorder characterized by the presence of amyloid fibrils (Figure 10), especially through the peripheral nervous system, that leads to organ dysfunction and ultimately, death[145]. Several TTR mutations (over 100) have been related to provoke amyloid deposition and disease[146], the most frequent being the substitution of a valine residue for a methionine at position 30 (V30M)[147]. Other mutations should also be referred: T119M (substitution of threonine for methionine at position 119), a non-pathogenic variant presenting high binding affinity for T4 as compared to normal TTR[148]; and L55P and Y78F (substitution of leucine for

proline at position 55 and tyrosine for phenylalanine at position 78, respectively), two very aggressive pathogenic mutations that alter significantly TTR conformation[149, 150].

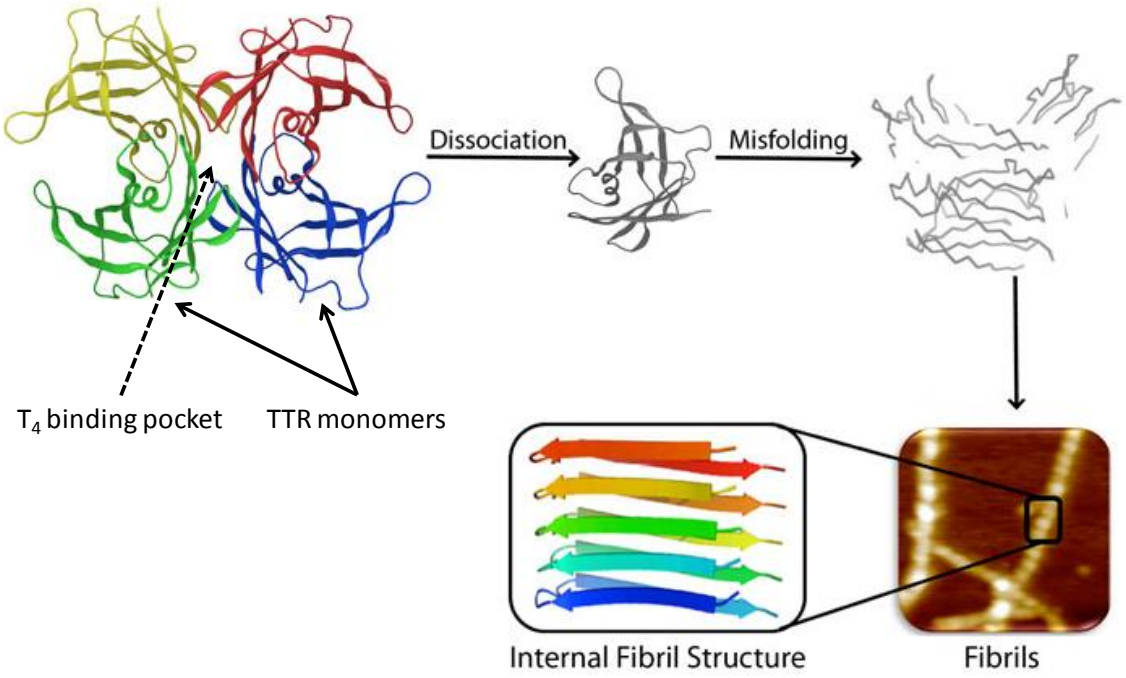


Figure 10. Transthyretin (TTR) structure and amyloidogenesis cascade. TTR is an homotetramer, with each monomer bearing 147 amino acid residues. The 4 monomers together form an open channel where T_4 can bind. For amyloidogenesis to occur, the TTR tetramer must first dissociate into four folded monomers and undergo partial denaturation. These pieces then subsequently misassemble into a variety of aggregate structures including toxic amyloid fibrils. (Adapted from http://www.scripps.edu/newsandviews/e_20110905/diagram.html)

It is believed that the amyloidogenic potential of the TTR variants is related to a decrease in tetrameric stability [151] and that the dissociation of the tetramer into monomers is the basis of a series of events that lead to the formation of TTR amyloid [152, 153]. Thus, TTR stabilization has been proposed as a key step for the inhibition of TTR fibril formation and has been the basis for FAP therapeutic strategies [154, 155]. Such stabilization can be achieved through the use of small compounds sharing molecular structural similarities with T_4, mostly belonging to the NSAIDs and binding in the T_4 central binding channel [156-160].

3. TTR as a protective molecule in AD

The first report that associates TTR to Aβ and AD in the context of a protective molecule is from Schwarzman et al. which describes the capacity of normal CSF to inhibit amyloid formation[161]. Prior to this finding, TTR was found associated to SPs, NFTs and microangiopathic lesions[162]. Although it was already known that other proteins such as

ApoE, ApoJ, gelsolin[163] and APP are able to sequester Aβ, contributing for the prevention of AD, Schwarzman and colleagues concluded that TTR was the major Aβ binding protein in the CSF[161]; TTR was also able to decrease the aggregation state of the peptide and to avoid its toxicity. The sequestration hypothesis was raised, suggesting that normally produced Aβ is sequestered by certain extracellular proteins, thereby preventing amyloid formation and Aβ cytotoxicity; when sequestration fails amyloid formation occurs[164]. The observation that TTR is reduced in the CSF of AD patients further supported the idea of a TTR protective role in this pathology[165]. Mammalian models have been used to mimic AD features but were never completely successful: AD transgenic mice did not show neurofibrillary tangles (NFTs) and demonstrate little or no neuronal cell loss[166-171]. However, in some of the models, animals showed increased TTR expression in the hippocampus; TTR was then described to be a survival gene[171] and although this work is controversial because TTR expression is thought to be confined to the choroid plexus and meninges (in the case of the brain), authors further showed that when a chronic infusion of an antibody against TTR was applied into the hippocampus of mice expressing human APP, an increase of Aβ, tau phosphorylation, neuronal loss and apoptosis was observed[172]. Underlying these observations is, according to authors, sAPPα that leads to increased expression of protective genes, such as TTR, to confer neuroprotection[172]. Other studies, using transgenic APP mice hemizygous for endogenous TTR showed accelerated Aβ deposition[67], while double transgenic mice for APP and TTR presented lower deposition[173]. However, in other models, TTR was described to have the opposite effect and was associated with increased vascular Aβ deposition[174]. More recently, Oliveira et al, reported findings on an APP/PSEN transgenic mouse model in different TTR backgrounds. In this study, it was stated that mice with genetic reduction of TTR showed increased Aβ brain levels, and that higher Aβ deposition was found in females, compared to males[175]. This work provided evidence for a gender-associated modulation of brain Aβ levels and brain sex steroid hormones by TTR, and suggests that reduced levels of brain testosterone and 17-estradiol in female mice with TTR genetic reduction might underlie their increased AD-like neuropathology[175].

Regarding the nature of TTR/Aβ interaction, different researchers confirmed TTR binding to Aβ[176-178], not only to the monomer but also to Aβ oligomers and fibrils, raising the hypothesis that TTR may be involved in the formation of senile plaques[137]; TTR was also able to inhibit and to disrupt Aβ fibrils. However, which TTR conformation binds Aβ peptide is still controversial. Du and Murphy claim that Aβ monomers bind more to TTR monomers than to TTR tetramers[179]; in this work, studies performed with WT TTR, T119M TTR and a double mutant (F87M/L110M TTR), which is a stable monomer

but a non-natural occurring mutation, authors showed that TTR tetramers interact preferably with Aβ aggregates rather than Aβ monomers enhancing Aβ aggregation, whereas TTR monomers arrest Aβ aggregate growth. Although interesting from a scientific point of view, the existence of functional biological active TTR monomers *in vivo* is far from established. Other studies indicated that amyloidogenic and unstable TTR mutants bind poorly to Aβ peptide [178, 180], suggesting that this interaction depends on the presence of the TTR tetramer. Very recently, genetic stabilization of TTR, through the presence of the T119M allele, which renders a more stable tetramer, has been associated with decreased risk of cerebrovascular disease and with increased life expectancy in the general population [181], further demonstrating the importance of the TTR tetramer in the protein biological activity.

The discussion on the TTR interaction with Aβ and consequent inhibition of aggregation and toxicity reduction raised the hypothesis that mutations in the TTR gene or conformational changes in the protein induced by aging, could affect the sequestration properties. A studied was conducted with the aim of identifying mutations in the TTR gene in the AD population but no correlation was found[138]. More recently, polymorphisms in the TTR gene were associated to hippocampal atrophy although the study could not associate this alteration to AD[182]. Nevertheless, destabilization of the protein may result from other events, such as metal ions concentration and interaction with other proteins.

Supporting the hypothesis that TTR might be destabilized in AD is, on one hand, the observation that TTR is early decreased in CSF and plasma of MCI and AD patients, and on the other hand, the lower levels of T_4 transported by TTR in these groups of patients[27], raising the hypothesis that TTR destabilization in AD accelerates its clearance, thus explaining the lower levels found. Moreover, *in vitro*, it is possible to restore the ability of TTR amyloidogenic/destabilized mutations to bind to Aβ peptide through the use of NSAIDs[183]. Importantly, *in vivo* administration of iododiflunisal (IDIF), one of the drugs shown to strengthen TTR/Aβ interaction, to APP/PSEN transgenic female mice in a TTR hemizygous background (model characterized and described by Oliveira and colleagues) resulted in decreased Aβ brain levels and amyloid burden, amelioration of the cognitive function and lower Aβ plasma levels[184]. This consolidated the notion that TTR stabilization is an important factor in TTR protection in AD, and suggested that TTR promoted Aβ clearance from the brain and from the periphery [184].

Although a growing body of evidence suggests TTR as an important modulator of AD pathogenesis, the mechanism underlying the effects described in the literature is incompletely understood; proteolytic degradation of the peptide, sequestration and

promotion of its clearance either by promoting its efflux from the brain or its uptake by the liver, influence in APP processing, and the effect of sex hormones have already been hypothesized and need to be further explored. It is also possible that TTR protection in AD is also conferred by interference in other pathways/molecules known to be altered in AD, such as APP trafficking and synaptic formation, although not yet addressed. In this line of thoughts, the experimental work presented in the next sections aimed at investigating the behavior of sortilin and synaptophysin, in AD transgenic mice in different TTR backgrounds. The above mentioned proteins will be next described, and have been proved to affect APP/Aβ circulating levels and neurotransmitter liberation, respectively. In addition, and because it relates to APP/Aβ levels, we also evaluated APP expression and processing.

Sortilin and Synaptophysin in AD

1. Sortilin

Sortilin (**Sort1**) is a member of the recently discovered family of Vps10p-domain receptors (of approximately 94 kDa)[185], and is expressed in neurons of the central and the peripheral nervous system, but also in extra-neuronal tissues including liver and fat[186]. It is an essential component for transmitting pro-neurotrophin–dependent death signals, and thus promotes apoptosis[187]. Agreeing with the latter, an important role of Sort1 in neurodegenerative disease has been proposed, by Al-Shawi et al., due to the observation of an age-related increase in its expression levels. Increased Sort1 levels, combined with also increased levels of proNGF (uncleaved precursor form of the nerve growth factor protein), suggest an influence of Sort1 in neuronal atrophy and cell death, in their older mice model[188]. After analysis, the authors observed no differences in Sort1 expression between AD patients and age-matched controls, however, this results show that the role of Sort1 in aging should not be despised[188]. Another group has shown, in their analysis of AD post-mortem brain tissue, increased levels of Sort1 (compared to controls), and suggested a possible role in the development of AD-related pathological changes[189]. Then, Sort1 was shown to be a binding protein of APP, and so, its influence in the evolution of AD pathogenesis, positive or negative, started being investigated.

Recently, more precisely in January of 2013, two interesting papers were published by the same journal. Gustafsen et al., suggested the role of Sort1 as a sorting protein in APP

processing. They were able to observe, *in vitro*, that when Sort1 was decreased, the levels of sAPPα were also decreased, suggesting an involvement in APP processing[190]. Thus, the proposed interaction of Sort1 with APP, in neurites, promotes α-secretase cleavage of APP (inhibiting Aβ production), and influences both production and cellular uptake of soluble forms of APP (leading to lysosomal degradation)[190]. The authors also commented that the previous findings from Finan et al., suggesting an increase in sortilin levels in AD patients, may be due to the use of a C-terminal tagging, which can affect the subcellular localization of Sort1. In the other publication, Carlo et al. denied the previous hypothesis and proposed Sort1 as a neuronal ApoE receptor, constituting a major endocytic pathway for clearance of ApoE/Aβ complexes[186]. Carlo's group observed, using ApoE- and Sort1-deficient mouse models, that the lack of receptor expression in mice resulted in accumulation of ApoE and Aβ in the brain, with aggravated plaque burden[186]. Thus, these two groups propose a negative correlation of Sort1 and AD progression.

A relationship between Sort1 and AD has been quite established, nonetheless, the exact mechanism underlying this involvement is not fully resolved, thus yielding distinct, and even sometimes contradictory, hypotheses.

2. Synaptophysin

Synaptophysin (**Syp**), a 38 kDa integral membrane protein, member of the MARVEL (MAL and related proteins for vesicle trafficking and membrane link)-domain family[191], is the most abundant integral synaptic vesicle protein and, therefore, is often measured in attempts to quantify synapses[192]. When bond to synaptobrevin (a protein of the SNARE complex), Syp inhibits the binding of the latter to SNARE complex, thus preventing the SNARE assembly and vesicle fusion[192, 193] (Figure 11). Since AD is characterized by an accentuated synaptic loss, the analysis of Syp's expression and behavior in AD was accessed by several groups. Ishibashi et al. observed that synaptophysin was more abundant in AD brain cortex than in controls, but showed a somewhat irregular pattern of staining, since a marked decrease was observed in foci where oligomer Aβ accumulated[194], leading to loss of normal synaptic functions. Another group revealed a link between $A\beta_{42}$ accumulation and loss of synaptophysin in a transgenic AD mouse model, however the expression of Syp in their AD model was decreased, compared to control littermates[195], opposing the results from Ishibashi and group. Other agreeing studies reveal reduced average levels of Syp in human hippocampus, when comparing

AD to control samples, and a correlation between Syp decreased levels and cognitive decline in AD[196].

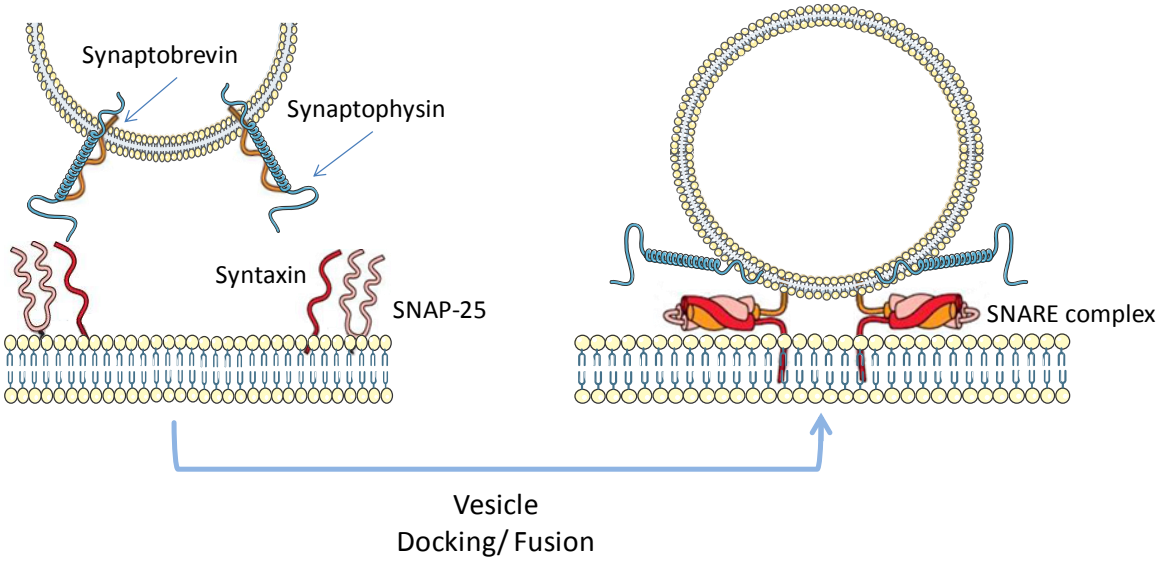


Figure 11. Synaptophysin involvement in vesicle fusion. Synaptophysin/synaptobrevin complex binds with syntaxin on the plasma membrane and forms a fusion pore. Then the tight formation of the SNARE complex disassociates synaptobrevin from sinaptophysin, thus weakening the synaptophysin complex and allowing the vesicle to fully fuse.

Objectives

The aim of this study was the search for early TTR-related biomarkers in a transgenic mouse model, constituted by AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ (bearing two copies of TTR and one copy of TTR, respectively) 3- and 7-months-old mice, by means of Western Blot analysis. For that we investigated:

(1) sortilin expression;
(2) synaptophysin expression;
(3) APP expression, through evaluation of:
    i. $APP_{full\ length}$
(4) APP processing, through evaluation of:
    i. CTFs levels
    ii. sAPP levels
(5) the influence of age in our mouse model, and in the expression of the above mentioned proteins.

Material and methods

Animals

The mouse model AβPPswe/PSEN1A246E/TTR used in this study was established and characterized in the Molecular Neurobiology Laboratory at IBMC, Porto. The colony was generated by crossing AβPPswe/PSEN1A246E transgenic mice[197] (B6/C3H background), purchased from The Jackson Laboratory, with TTR-null mice ($TTR^{-/-}$) (SV129 background)[198] as previously described[175]. Thus, we were able to generate AβPPswe/PSEN1A246E/$TTR^{+/+}$ (carrying 2 copies of the TTR gene), AβPPswe/PSEN1A246E/$TTR^{+/-}$ (carrying only one copy of the TTR gene) and AβPPswe/PSEN1A246E/$TTR^{-/-}$ (without TTR), hereafter referred to as AD/TTR+/+, AD/TTR+/- and AD/TTR-/-, respectively. Animals were housed in a controlled environment (12-h light/dark cycle; temperature, 22±2°C; humidity, 45-65%), with freely available food and water. All procedures involving animals were carried out in accordance with National and European Union Guidelines for the care and handling of laboratory animals.

In this study, we used two groups of cohorts of littermates. One group was composed by 3 months old male and female mice, as follow:

3 male and 3 female AD/$TTR^{+/+}$ mice;

3 male and 3 female AD/$TTR^{+/-}$ mice;

3 male and 2 female AD/$TTR^{-/-}$ mice.

The other group was composed by 7 month-old female mice, that underwent IDIF administration in a previous study[184]:

7 AD/$TTR^{+/+}$ control (not submitted to treatment) mice;

7 AD/$TTR^{+/+}$ treated (with IDIF drug) mice;

8 AD/$TTR^{+/-}$ control mice;

9 AD/$TTR^{+/-}$ treated mice;

3 AD/$TTR^{-/-}$ control mice.

These mice started IDIF treatment at the age of 5 months, before the onset of deposition, which lasted for 2 months and thus animals were sacrificed at 7 months of age, after the start of Aβ deposition. With regard to this group of mice, brain tissue homogenized in Tris Buffer Saline (TBS) and frozen at -80 ºC was already available in the laboratory.

Tissue Processing

Animals were sacrificed following anesthesia with a mixture of ketamine (75mg/kg) and medetomidine (1mg/kg) administrated by intraperitoneal injection. Efforts were made to minimize pain and distress; all animal experiments were carried out in accordance with the European Communities Council Directive. CSF was collected from the cisterna magna, assessed for blood contamination analysis as previously described[199] and stored at -80 °C. Blood was collected from the inferior vena cava in syringes containing EDTA as anticoagulant, followed by centrifugation at 1000 × *g* for 20 min at room temperature (RT). Plasma samples were then collected and stored at -80 °C. From each removed and dissected brain; hippocampus (divided in two halves) and cortex samples were collected and frozen immediately at -80 °C for biochemical analyses. As already described, tissue samples from mice that entered the IDIF study were already collected, corresponding to hemi-brains of each animal, thus the separation of the hippocampus was not possible at this stage. In the present study, only the hippocampus or all brain (for 3 and 7 months-old mice, respectively), were used and analyzed in the subsequent assays.

Sample preparation

Hippocampus samples were homogeneized in 300 µL of kinexus lysis buffer (20 mM MOPS pH 7.0; 2 mM EGTA; 5 mM EDTA; 30 mM sodium fluoride; 60 mM β-glycerophosphate pH 7.2; 20 mM sodium pyrophosphate; 1 mM sodium orthovanadate; 1% Triton X-100) and 1mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitors (PIs – stock at 100x). In relation to the other group of mice and since the all brain samples had already been collected and frozen in 500 µL of a different lysis buffer (TBS 50mM pH 7.4; 0.2% Triton X-100; 4mM EDTA; and PIs), it was necessary to prepare a kinexus lysis buffer 2x. By adding 500 µL of kinexus 2x (plus 2 mM PMSF and 2x PIs) we were able to equalize the conditions of the all brain and hippocampus samples. The homogenized samples were then centrifuged for 20 minutes at 14 rpm (4 °C), supernatants were collected and total protein concentration was determined using the Bradford method. After quantification, hippocampus and all brain samples were diluted to 2 mg/mL and 3 mg/mL, respectively. All samples were then boiled for 5 minutes with 1x SDS buffer (125 mM Tris pH 6.8; 4% SDS; 20% glycerol; 10% β-mercaptoethanol; and 0.08% bromophenol blue) and stored at -20 ºC for future analysis.

Western Blot

Proteins were separated by 10% SDS-PAGE (200V; 25mA; ~1.30h), and transferred (100V; 400mA; 2h) to a nitrocellulose membrane (Whatman™ GE Healthcare Life Sciences – Protran BA 83), using a wet system (Bio-Rad Criterion Blotter). Membranes were blocked using blocking buffer, 10% bovine serum albumin/nonfat dry milk (BSA/DM depending on the antibody) in phosphate-buffered saline containing 0.05% Tween-20 (PBS-T), for 1 hour at room temperature with gentle shaking. Alternatively, samples were separated using commercial gradient gels – Criterion XT Precast Gel, 4-12% polyacrylamide Bis-Tris, 18 well (#345-0124 Bio-Rad) using the recommended XT MES Running Buffer (#161-0789 Bio-Rad). After the electrophoresis (200V; 250 (maximum) mA; ~35min), proteins were transferred (100V; 400mA; 2h) to a nitrocellulose membrane (Whatman™ GE Healthcare Life Sciences – Protran BA 83), using a wet system (Bio-Rad Criterion Blotter). The membrane was dried, boiled 10 minutes with PBS, washed also with PBS, and followed the common protocol above specified.

Antibody incubation

After blocking, membranes were then incubated with primary antibodies against the proteins under study. After optimization of different variables such as dilution of primary and secondary antibodies, incubation conditions (solution and incubation time), type of gel and reference protein, the best conditions were established for each protein of interest and are summarized in Table 1. After the incubation with primary antibodies, membranes were washed 3 times for 10 minutes, followed by the suitable secondary antibody (anti-rabbit-HRP conjugated – AP311; The Binding Site – or anti-mouse-HRP conjugated – #31432; Pierce Antibodies) both diluted 1:5000, in 3% (1% when incubated with anti-α-tubulin) DM/PBS-T, for 45min at RT with gentle shaking. The blots were developed using Immun-Star™ WesternC™ Chemiluminescence kit (Bio-Rad) and proteins were detected and visualized using a chemiluminescence detection system (ChemiDoc, BioRad). When necessary, membranes were stripped using a commercial stripping buffer (Re-Blot Plus Solution (10x) – Millipore) during 20min at RT with gentle shaking, for re-utilization of the membrane, according to the manufacturer's instructions. Protein levels were normalized using the ratio between the protein of interest and α-tubulin.

Protein	Primary Antibody	Dilution	Incubation conditions	Protein loaded (µg) / type of gel
Sortilin	rabbit - ab16640; Abcam	1:1 000 / 2 000*	5% BSA/PBS-T, O/N at 4°C	30 / 10% SDS-PAGE
Synaptophysin	mouse - ab18008; Abcam	1:2 000 / 5 000*	5% DM/PBS-T, O/N at 4°C	30 / 10% SDS-PAGE
α-APP C-terminal	rabbit - A8717; Sigma	1:10 000	5% BSA/PBS-T, 1h at RT	50 / 4-12% PolyA Bis-Tris
β-Amyloid N-terminal	mouse - A5213; Sigma	1:10 000	5% BSA/PBS-T, 1h at RT	50 / 4-12% PolyA Bis-Tris
α-Tubulin	mouse - T8203; Sigma	1:10 000	5% DM/PBS-T, 1h at RT	30 / 50**

Table 1. List of antibodies used in Western Blot analyses. (*) Dilution of antibody suggested by the manufacturer / optimized dilution used in subsequent analysis. (**) The quantity of loaded protein varies according to the protein of interest being analyzed and type of gel necessary for this analysis.

Statistical Analysis

Quantitative data are presented as Mean ± SEM. Statistical analysis was carried out using Graphpad Prism 5 software. First of all, data was assessed whether it followed a Gaussian distribution by the Kolmogorov-Smirnov test. When found to follow Gaussian distribution, differences among groups were analyzed by one-way ANOVA (followed by Bonferroni's Multiple Comparison Test) and comparisons between two groups were made by Student's *t* test. In the cases of non-Gaussian distribution, differences among groups were analyzed by non-parametric Kruskal-Wallis test (followed by Dunns test). p values lower than 0.05 were considered significant.

Results

Previous work showed that the AD/TTR mouse colony established in our laboratory is a suitable model to study AD, in particular the neuroprotective role of TTR and gender differences in AD[175], as elevated brain levels of $A\beta_{42}$ were observed in particular in AD/TTR$^{+/-}$ female mice as compared to their AD/TTR$^{+/+}$ counterparts. AD/TTR$^{-/-}$ mice which are also generated in this colony, were also used in the present; however, and as previously described, the negative effects of the genetic reduction of TTR were not always observed in AD/TTR$^{-/-}$ animals compared to AD/TTR$^{-/+}$ and AD/TTR$^{+/+}$ littermates. This may be due to compensatory mechanisms generated by these animals as hypothesized by Oliveira and co-workers in the first characterization of this model[175]. Thus we choose not to present these data.

In the first characterization of this model, mice of 3, 6 and 10 months were evaluated for Aβ brain levels, as assessed by $A\beta_{40}$ and $A\beta_{42}$ peptides levels in brain homogenates, in two fractions: detergent-soluble fraction, corresponding to Aβ initial aggregates and oligomers; formic acid-soluble fraction, corresponding to higher ordered aggregates. In addition, Aβ burden was also evaluated by immunohistochesmistry, revealing that plaque formation started at the age of 6 months. Younger mice were only investigated for TTR levels in plasma, and compared to non-transgenic animals, revealing that TTR is early decreased, although its levels were raised in 10 months old mice, probably due to compensatory mechanism. However, the effects of TTR genetic reduction in pathways known to be altered in AD were not addressed in young mice, before the development of disease. As stated in the beginning of this thesis, Alois Alzheimer hypothesized that AD occurs due to neuronal failure, and thus this work focused on the search of AD biomarkers (proposed to be involved in neuronal failure) early affected by TTR, before Aβ deposition, using the AD mouse model described. Then, the results were compared to older mice, at an age known to already present Aβ deposition in the brain. In addition, mice that underwent treatment with IDIF, known to stabilize TTR and improve AD features, were also investigated to further validate the results, and to address the possibility of using these biomarkers for disease progression evaluation and follow-up of therapies. In particular we measured the levels of sortilin (Sort1), synaptophysin (Syp), APP expression and APP processing.

Sortilin: expression and quantification

To investigate if TTR affects Sort1 levels in the brain, we used western blot analysis of brain extracts. Based on literature, Sort1 protein is expressed all over the brain, without a preferred expression area. Nevertheless, it is necessary to take in account that AD is characterized mainly by alterations in the hippocampus, thus, the results obtained from all brain (7 months old mice, with and without IDIF treatment) must be compared to the ones in hippocampus, with caution.

Analysis of sortilin expression in 3 month-old mice

We started by analyzing Sort1 proteins levels in hippocampus of 3 months-old mice (Figure 12), and we found AD/TTR$^{+/-}$ mice presented significantly lower levels of sortilin when compared to AD/TTR$^{+/+}$ animals (p<0.01), (Figure 12B, left panel). Further analysis of the results by gender showed no significant differences between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$, either in male and female (Figure 12B, right panel). It is important to refer that these results are probably influenced by the small number of animals (n=3), in each group. Nevertheless, we can observe that the levels of Sort1 tend to decrease from AD/TTR$^{+/+}$ to AD/TTR$^{+/-}$ (male and female) and also vary in gender (lower levels of Sort1 in female).

Altogether, these results suggest that TTR influences Sort1 expression at this age, before Aβ deposition.

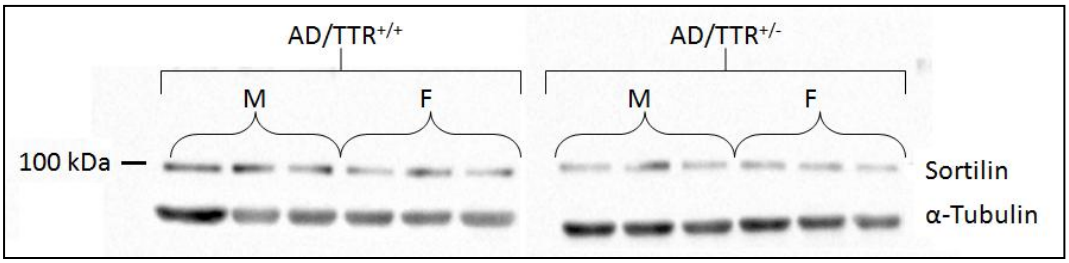
Analysis of sortilin in 7 month-old mice

To further understand if the effect of TTR genetic reduction on Sort1 levels was sustained overtime, we analyzed 7 months-old mice. Additionally, the study was also performed in brain tissue of 7 months-old animals that received IDIF, orally, for 2 months. As reported, IDIF administration resulted in lower Aβ deposition in the brain as well as cognitive improvements. Thus, we also intended at investigating the possibility of using Sort1 as a biomarker, both for disease progression and for follow-up of therapies. As already referred, brain tissue (all brain) was already available in the laboratory and originated only from females[184].

Our results (Figure 13) indicated that Sort1 was significantly decreased in AD/TTR$^{+/-}$ compared to AD/TTR$^{+/+}$ female mice (p<0.001). This indicates that, either the difference was accentuated with ageing, or that we could not detect statistic differences in 3 months

old mice due to the lower number of samples, as already suggested. Control and treated mice, from the same genotype, showed no significant differences in Sort1 levels (Figure 13B), indicating that TTR stabilization by IDIF did not affect sortilin.

A



B

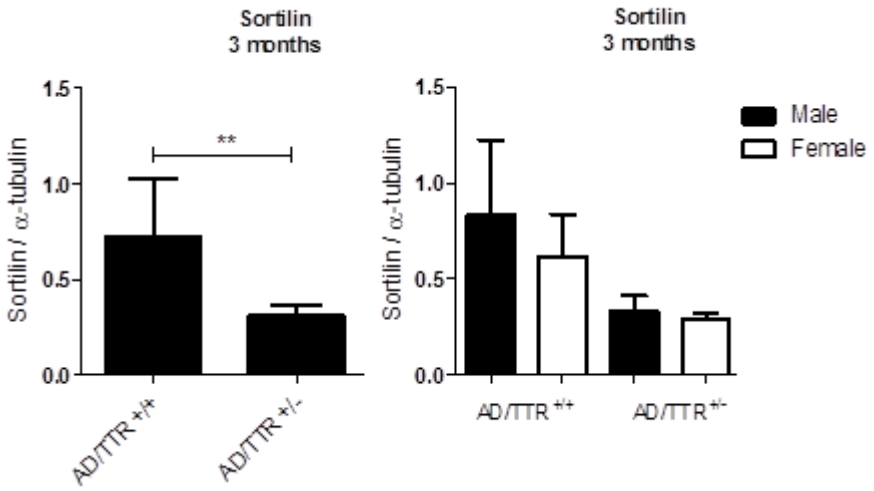


Figure 12. TTR influences sortilin expression in 3 months-old mice. Western blot analysis of sortilin expression (A) and respective quantification (B) grouped by genotype (left panel, n=6 in each group) and by gender (right panel, n=3 male/female in each group). Data represent the means ± SEM. Error bars represent SEM. $^{**}p < 0.01$ in a Student's *t* test.

Altogether, our results suggest that Sort1 is primarily affected by TTR quantity, and that TTR stabilization alone is not sufficient to recover Sort1 levels. In addition, it seems that Sort1 levels correlate positively with disease severity.

A B

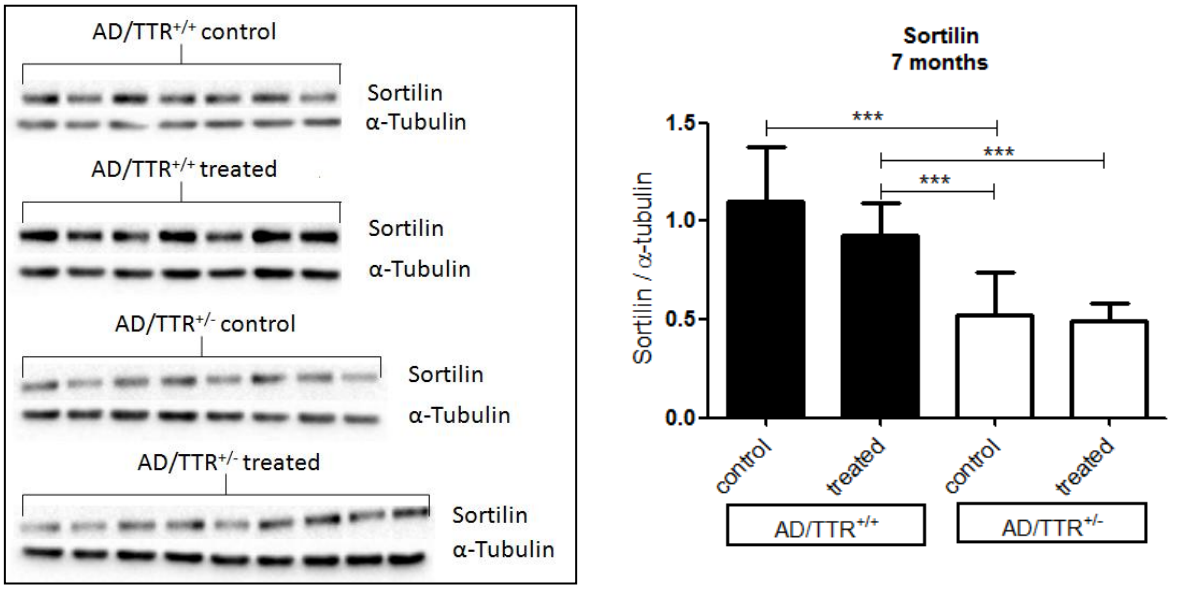


Figure 13. TTR influences sortilin expression in 7 months-old mice. Western blot analysis of sortilin expression (A) in 7 months-old mice, and respective quantification (B) of AD/TTR$^{+/+}$ control (n=7) and treated (n=7), and AD/TTR$^{+/-}$ control (n=8) and treated (n=9) groups. Data represent the means ± SEM. Error bars represent SEM. ***p < 0.001 in one-way ANOVA, with Bonferroni's post test.

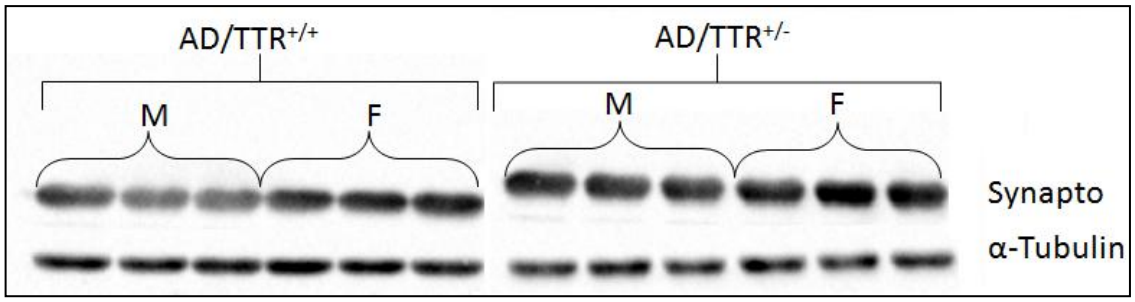
Synaptophysin: expression and quantification

Similar to Sort1 quantification, we performed western blot analysis to investigate if TTR affects synaptophysin (Syp) levels in the brain (Figure 14A). Based on literature, Syp protein is expressed all over the brain, not having a special area of expression. Again, comparison between data obtained for the hippocampus and for all brain must be done with caution since, as referred, AD affects in first instance the hippocampus.

Analysis of synaptophysin expression in 3 month-old mice

Our results indicated that Syp levels were significantly elevated in hippocampus from AD/TTR$^{+/-}$ compared to AD/TTR$^{+/+}$ mice (Figure 14B, left panel; p<0.05). In addition, it also seems that Syp's levels tend to be increased in female mice (Figure 14B, right panel). Again, the low number of animals in each group might explain the lack of statistical significance. Thus, Syp expression is suggested to be affected by the variation of TTR expression.

A



B

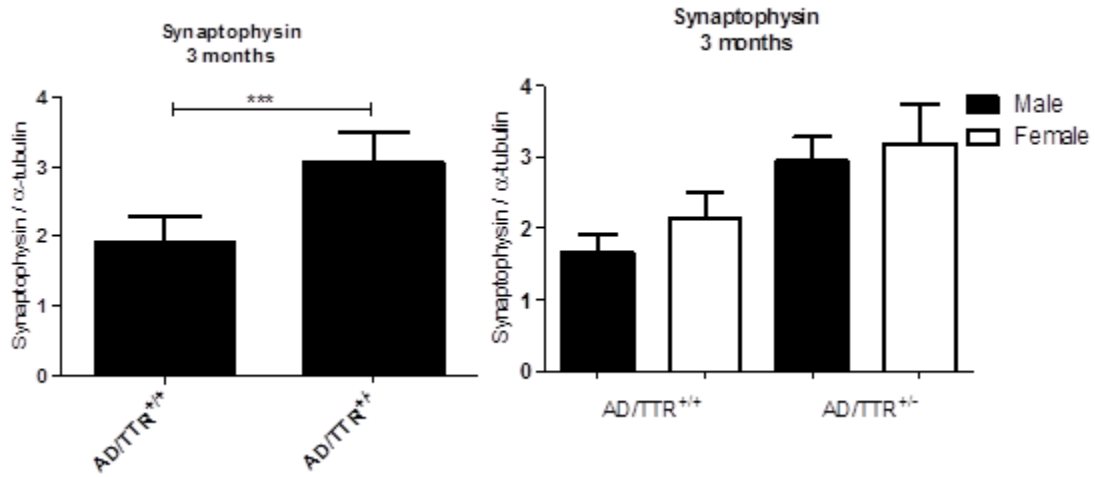


Figure 14. TTR influences synaptophysin expression in 3 months-old mice. Western blot analysis of synaptophysin (Synapto) expression (A) and respective quantification (B) grouped by genotype (left panel, n=6 in each group) and by gender (right panel, n=3 male/female in each group). Data represent the means ± SEM. Error bars represent SEM. ***p < 0.001 in a Student's *t* test.

Analysis of synaptophysin expression in 7 month-old mice

Contrary to the differences observed between TTR/AD$^{+/-}$ and TTR/AD$^{+/+}$ in 7 month-old mice for the expression of Sort1, Western Blot analysis (Figure 15A) of Syp in these animals did not show any significant differences between AD/TTR$^{+/-}$ and AD/TTR$^{+/+}$ female mice (Figure 15B), although a trend was observed. IDIF administration produced no significant effects on Syp expression.

Altogether, these observations indicate that the initial alterations in this protein were not maintained with ageing and its levels compensated. Curiously, this behavior might prompt Syp as an interesting biomarker allowing identification of early phases of disease

development, and distinguishing from advanced stages. Nevertheless, Syp will not serve as a biomarker for follow-up of therapies, at least the ones associated to TTR stabilization.

A B

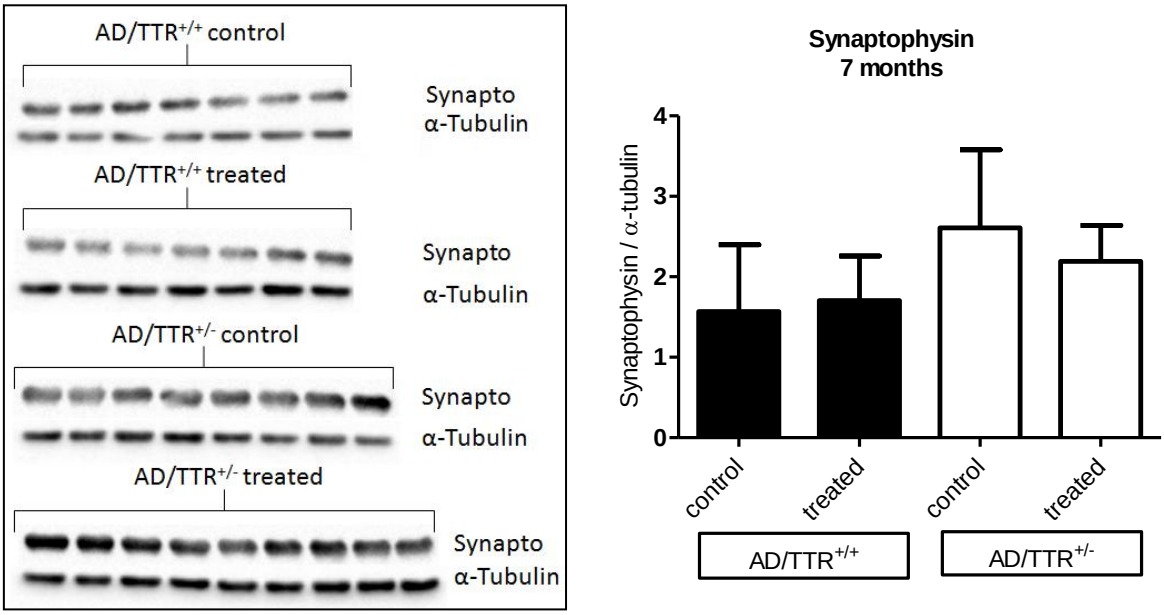


Figure 15. Synaptophysin expression in 7 months-old mice. Western blot analysis of synaptophysin (Synapto) expression (A) in 7 months-old mice, and respective quantification (B) of AD/TTR$^{+/+}$ control (n=6) and treated (n=7), and AD/TTR$^{+/-}$ control (n=8) and treated (n=9) groups. Data represent the means ± SEM. Error bars represent SEM.

APP expression and processing: C-terminal

Since Aβ, the key peptide in AD and thought to be the causative agent in this disorder, is generated upon APP processing, which in turn can be affected by sortilin, we then inquire whether APP expression and APP processing was altered by genetic decrease of TTR, using for the purpose, an anti-APP antibody, which is specific to the C-terminal of human APP_{695} (amino acids 676-695). This sequence is identical in APP_{751} and APP_{770} isoforms, corresponding to the last 20 aa, and thus enabling full lenght APP quantification. In addition to APP, it recognizes the C-Terminal Fragments (CTFs) – CTF-β (99 aa; MW ~13 kDa); CTF-α (83 aa; MW ~10 kDa); and CTFγ (or AICD; 57 aa; MW ~6.5 kDa). This will allow us to deduce about the effect of TTR in APP processing, through the quantification of each CTFs.

Analysis of APP expression and processing in 3 month-old mice

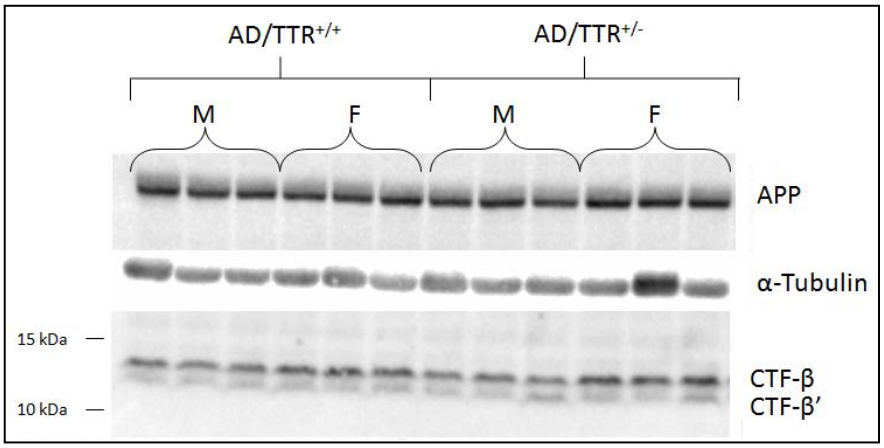
While analyzing the expression of APP in this cohort, we were not able to see significant differences between groups with different genotypes and genders (Figure 16A and B). Graphic analysis showed that the expression of APP is similar in the different groups, thus suggesting no influence of TTR in APP expression, as ascertained by Western Blot. In order to ascertain whether APP processing was influenced by TTR we started by analyzing the CTFs. Using the same blot membrane that was used for total APP expression (Figure 16A), but increasing exposure time, we were able to observe with higher resolution two bands, of which we identified the first as being the CTF-β-corresponding band. In addition, we suggest that the second band may be an N-terminally truncated APP CTF-β (CTF-β', composed by 89 aa), a product of β-cleavage of APP at residue 10. The results are depicted in Figure 16C and are presented as a ratio between levels of CTFs and full lenght APP. No differences were observed between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ mice, neither for CTF-β (Figure16C left panel) nor for CTF-β' (quantification data not shown) suggesting TTR does not influence APP processing, at this age.

Analysis by gender also did not show any significant differences, although a trend for increased CTF-β can be considered in female when compared to male, and in AD/TTR$^{+/-}$ when compared to AD/TTR$^{+/+}$ also in the female groups (Figure 16C right panel). If these results are confirmed, this indicates that in female and in particular in female AD/TTR$^{+/-}$, APP preferentially undergoes the amyloidogenic processing, explaining the higher degree of AD-like disease described in this model.

Analysis of APP expression and processing in 7 month-old mice

Next, the same full length APP and CTFs analysis assessed by Western Blot was performed for the 7 months-old mice brain samples (Figure 17A), to further characterize the influence of TTR in APP expression and processing, as disease develops. In terms of APP protein levels, we found no significant differences between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ groups (Figure 17B, upper panel), thus, suggesting that TTR had no effect on APP expression. Following the same line of thought – considering that the two bands correspond to CTF-β and CTF-β' (Figure 17A) – we were able to observe increased levels of both forms of CTFs in AD/TTR$^{+/-}$ female mice when compared to their littermates AD/TTR$^{+/+}$ (Figure 17B, lower panels). This suggests that TTR influences APP processing, and that TTR reduction stimulated the formation of both CTF-β, thus promoting the amyloidogenic pathway.

A



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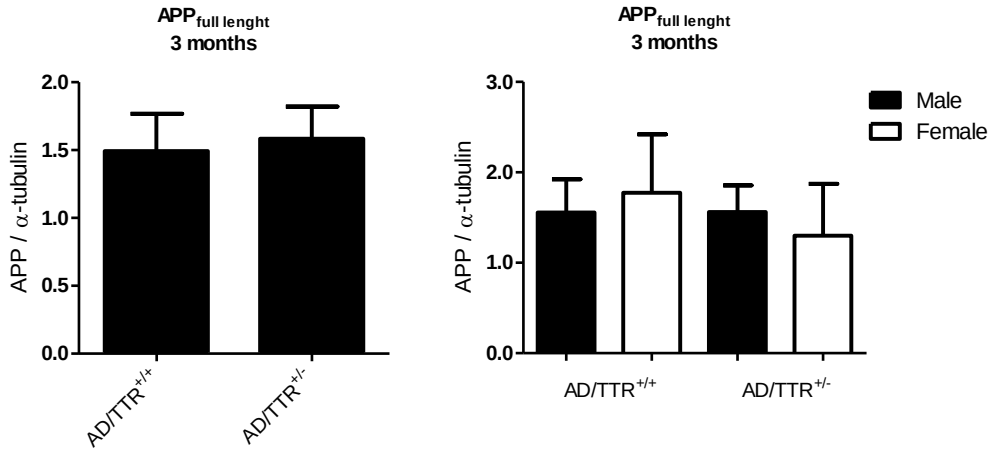


C

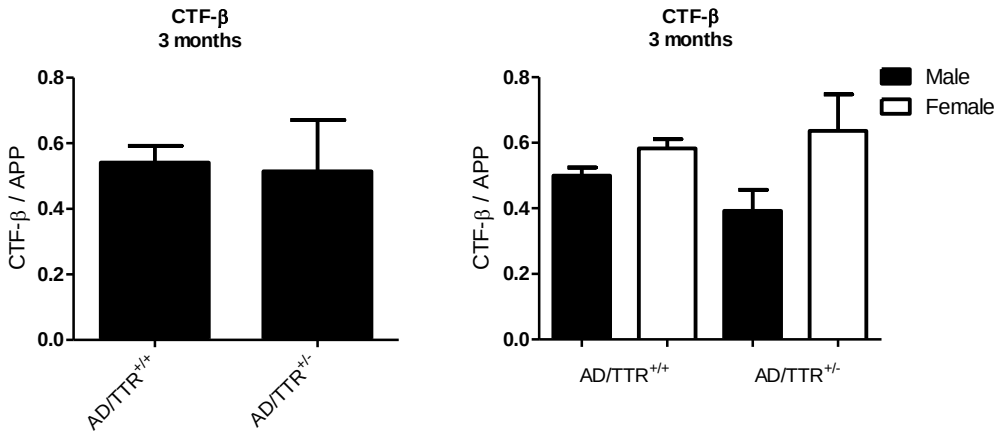


Figure 16. TTR effects on the amyloid precursor protein (APP) expression and processing in 3 months-old mice. Western blot analysis of APP and carboxi-terminal fragments (CTFs) expression (A) and respective quantification (B and C, respectively) grouped by genotype (left panel, n=6 in each group) and by gender (right panel, n=3 male/female in each group). Data represent the means ± SEM. Error bars represent SEM.

Analysis of IDIF treated mice samples revealed no differences when compared to the non-treated mice of the same age (data not shown), again suggesting that the quantity of TTR is determinant for its effects in APP processing, and stabilization of the protein per se does not compensate its genetic reduction.

APP expression and processing: N-terminal

APP processing also results in N-terminal fragments sAPPα or sAPPβ, depending if cleaved by α- or β-secretases, respectively. While sAPPα is considered neuroprotector and to induce the expression of survival genes, sAPPβ has been shown to act as a death receptor ligand, mediating neuronal death.

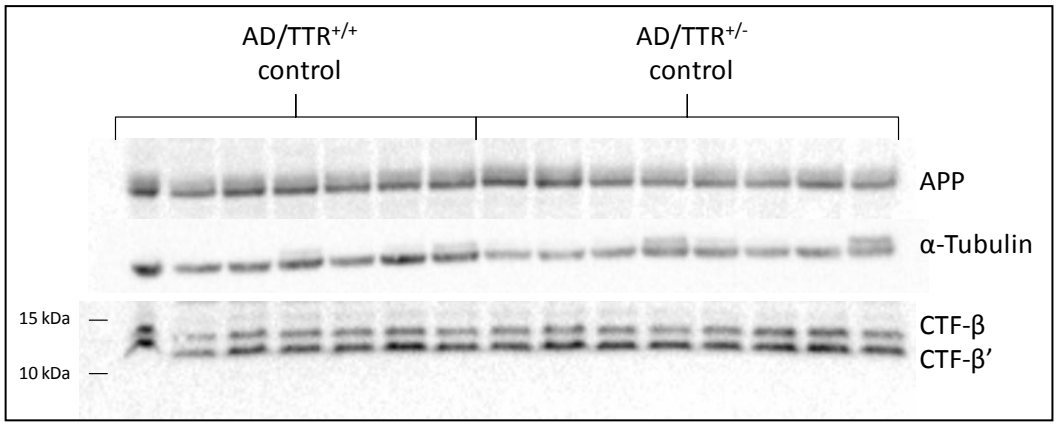
To assess the levels of sAPPα as well as the levels of Aβ peptide, we performed Western Blot analysis using a specific antibody that recognizes amino acid residues 1-12 of the Aβ peptide sequence. This antibody allows the recognition of sAPPα and Aβ peptide, as well as full lenght APP.

Analysis of APP expression and processing in 3 month-old mice

Despite using a specific antibody to detect the Aβ peptide, its corresponding band was not observed (data not shown), probably due to its normally low levels. However, we were able to detect and quantify the total APP and sAPPα bands (Figure 18A). While total APP levels were normalized using α-tubulin protein, sAPPα levels was again normalized using full lenght APP expression.

Differences between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ were not significant, neither for full lenght APP (Figure 18B, left panel) nor for sAPPα (Figure 18B, right panel), suggesting that TTR does not influence neither APP expression (as seen in the previous section), nor APP processing (leading to the formation sAPPα), at this age. Analysis by gender also did not demonstrate significant differences between none of the groups (data not shown).

A



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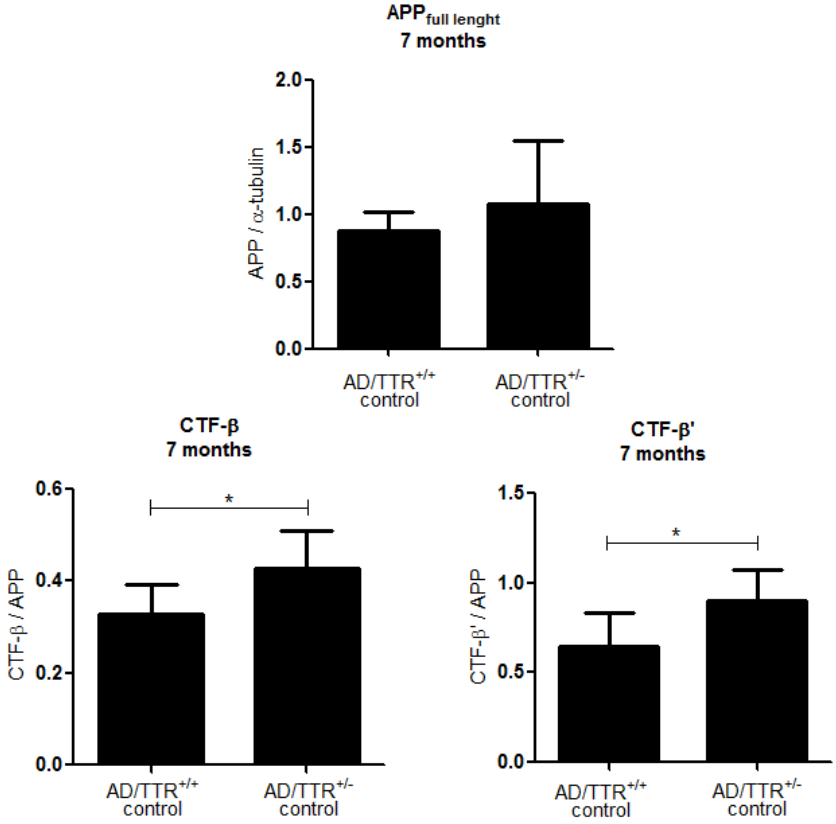
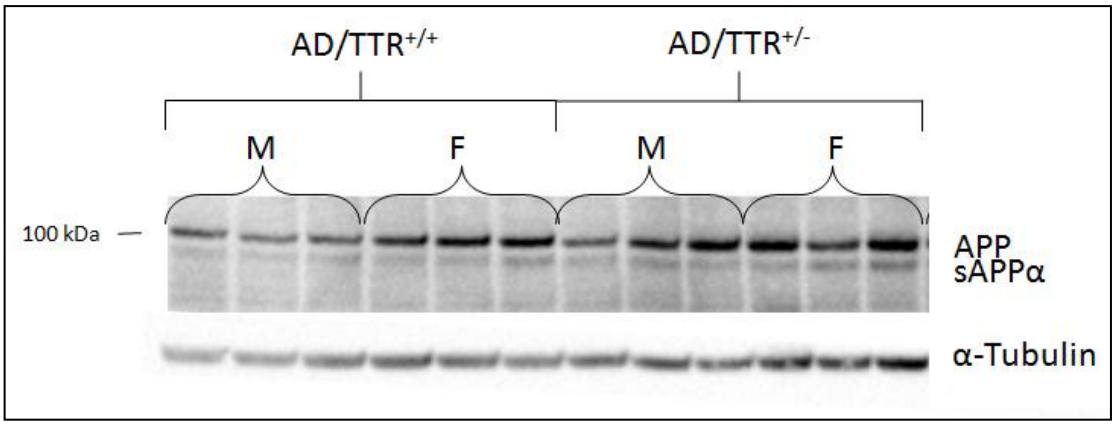


Figure 17. TTR influences APP processing, in 7 months-old mice. Western Blot analysis of 7 months-old AD/TTR$^{+/+}$ control (n=7) and AD/TTR$^{+/-}$ control (n=8) mice groups, in terms of full length APP and CTFs (between 15 and 10 kDa) expression. Data represent the means ± SEM. Error bars represent SEM. *p < 0.05 in Student's t test. CTFs values are show as a ratio between the quantification of the CTF-corresponding band and the quantification of APP-corresponding band.

A



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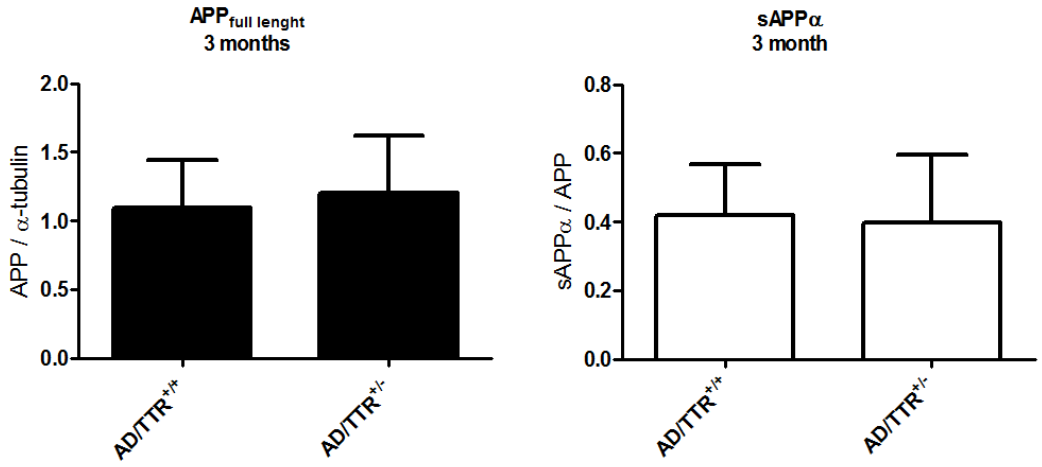


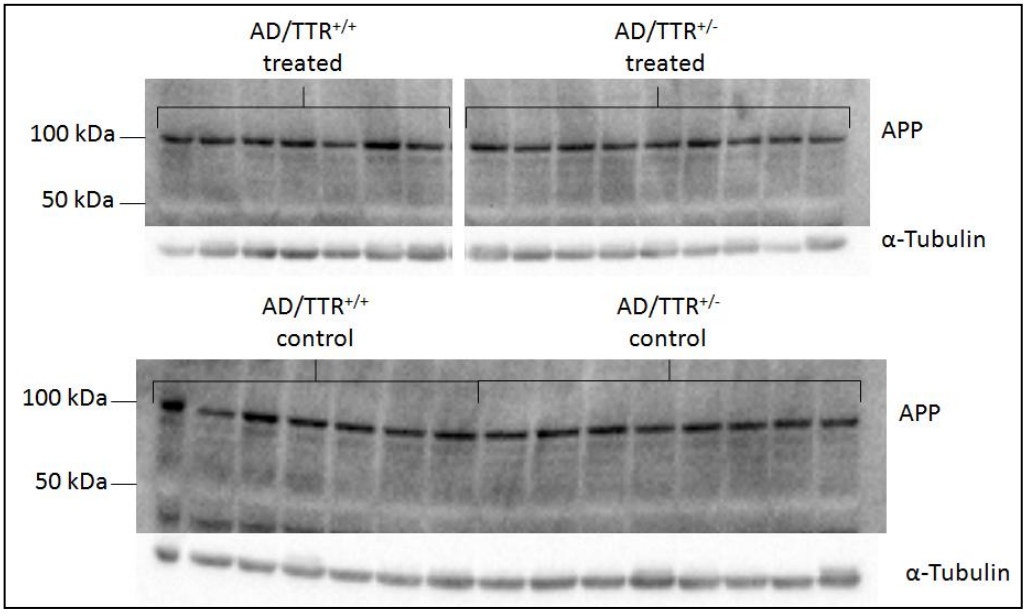
Figure 18. TTR effects on APP processing and expression in 3 months-old mice. Western Blot analysis of full length APP and sAPPα expression (A) and respective quantification (B, left panel and right panel, respectively) grouped by genotype (n=6 in each group). CTFs bands were not possible to identify. Data represent the means ± SEM. Error bars represent SEM.

Analysis of APP expression and processing in 7 month-old mice

The same full length APP analysis was performed in 7 months-old AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ mice samples (control and treated) (Figure 19A), and no significant differences were observed (Figure 19B). Curiously, and differently from the 3 month old mice, we could not detect the band corresponding to sAPPα (see Figure 19A). This may indicate that cleavage by α-secretase is decreased (in favor of β-secretase), thus explaining the increased Aβ brain levels found in these older mice and corresponding signs of AD-like disease. These findings are, apparently, unrelated to the TTR genetic reduction and to its conformational state (tetrameric stability), and appear to depend only on disease

progression, since we observed no differences neither between TTR/AD$^{+/+}$ and TTR/AD$^{+/-}$ nor between treated and non-treated mice. It is also possible that the age/disease progression effect is stronger that the effect of TTR reduction, and thus subtle differences between genotypes were not detected. In this case, a new analysis using a different, more sensitive, antibody or loading higher amounts of total protein in the gel, may help answering to this question.

A



B

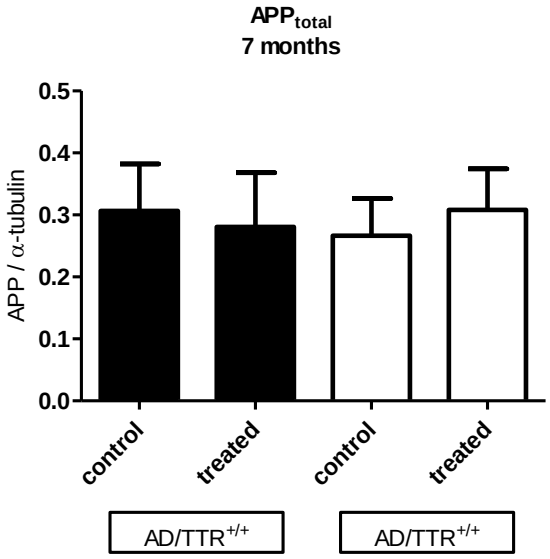


Figure 19. TTR effects on APP processing and expression in 7 months-old mice. Western Blot analysis in 7 months-old mice of full length APP expression (A) and respective quantification (B). The analysis included AD/TTR$^{+/+}$ control (n=7) and treated (n=7), and AD/TTR$^{+/-}$ control (n=8) and treated (n=9) groups. In addition to the CTFs bands not possible to be identified, sAPPα bands were not observed. Data represent the means ± SEM. Error bars represent SEM.

Discussion

Alzheimer's disease is the most prevalent form of dementia, worldwide. However, due to its complexity, most of the molecular mechanisms responsible for the pathological features remain unsolved. In addition to the little existing knowledge of molecular mechanisms, there are not any efficient drugs to treat AD, merely its symptoms. Therefore, a logical option is to discover this condition in its early stages, when the "treatment" can be more effective, and so it urges to find specific biomarkers that can differentiate an early stage AD patient, from a control. A variety of factors can be involved in the initiation and progression of AD and, among them, TTR has been shown to be an important modulator of AD pathogenesis, using mouse models. Thus, in this project, we intended to draw some conclusions about the influence of TTR on some proposed biomarkers, using a transgenic AD mouse model in different TTR genetic backgrounds (AD/TTR)[175], previously described by Oliveira and group. In this model, mice in a TTR hemizygous background are presented with a more severe form of AD-like disease, in particular female mice [175].

We started to investigate whether TTR had any influence in **sortilin** (**Sort1**) expression in hippocampus/all brain samples of transgenic AD mice, and if this effect was modified with aging. Our analysis showed a significant decrease in Sort1 expression in 3 and 7 months-old AD/TTR$^{+/-}$ animals, when compared to their AD/TTR$^{+/+}$ littermates. This suggests that TTR, indeed, influences Sort1 expression, in a way that its genetic decrease correlates with decreased Sort1 levels. Our observations agree with several recent works, namely with Finan et al. study from 2011, in which the authors showed decreased levels of Sort1 in AD post-mortem brain samples, compared to control [189]. In our work we did not use non-transgenic mice and thus we cannot assert differences in Sort1 expression levels between controls and AD-like samples. Nevertheless, we were able to compare its expression in AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ groups, establishing an inverse relation with disease progression, and thus, we hypothesized that Sort1 levels in control animals should be increased (agreeing with the literature). This is further supported by the observation that, in female, differences in Sort1 levels between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ are more pronounced in 7 months-old than in 3 months-old mice samples. Gustafsen et al. also stated a probable decrease of Sort1 expression in AD pathology, proposing Sort1 as an APP sorting receptor, which promotes the cleavage by α-secretase, inhibiting Aβ formation [190]. In addition, they referred that Sort1 also interferes with the production of soluble forms of APP and its cellular uptake, guiding it to lysosomal degradation. Thus, a

decrease in Sort1 levels would diminish would interfere with the above mentioned pathways, consequently promoting Aβ production and the progression of the disease. In our work, we showed that APP expression was not altered at any ages, contrary to its processing. Although in 3 months-old mice, no significant differences were obtained for total CTFβ levels (in which we consider both CTFβ and CTFβ'), when considering TTR background, a trend for increased CTFβs was observed in female, compared to males, in particular in AD/TTR$^{+/-}$. Importantly, significant differences were measured in 7 months-old animals as AD/TTR$^{+/-}$ presented higher levels of CTFβ and CTFβ' than AD/TTR$^{+/+}$ females, thus suggesting that besides age/disease progression, TTR also affects APP processing. In addition, in younger mice we were able to observe the sAPPα band, whereas in 7 months-old mice this band was absent. This meets the previous suggestion that a decrease of Sort1 would diminish α-secretase activity, and thus indirectly promoting the amyloidogenic pathway, showed by increased levels of CTFβ in AD/TTR$^{+/-}$ older mice. The presence of a visible sAPPα band in 3 months-old mice and its absence in 7 months-old mice, shows a consistency in results. Younger mice, present, in both TTR genotypes, the sAPPα band but do not show any difference between CTFβ levels; the opposite is observed for older mice. This shows that aging is an important factor that may overlap the influence of TTR reduction in APP processing, since both ages present decreased levels of Sort1 but only the 7 months-old mice suggest an unbalance in the amyloidogenic and non-amyloidogenic pathway. Another group also considered that Sort1 expression is diminished in AD, despite suggesting a different molecular mechanism for its relation with this disorder [186]. Carlo et al. suggest that Sort1 acts as a neuronal receptor for ApoE, thus being involved in ApoE/Aβ complex clearance from the brain; the lack of Sort1 receptor expression leads to increased ApoE and Aβ accumulation in the brain, resulting in disease escalation. Plus, they noticed a two-fold lower Kd for binding to Sort1, by ApoE ε3 (44 nM) versus ApoE ε4 (114nM)[186], which might be related to the different isoforms' risk in AD. In our work we did not assess ApoE levels, and thus we cannot infer on the mechanism underlying TTR/Sort1 relation. In the future, it would be interesting to investigate ApoE levels as well as a possible TTR/Sort1 interaction. In relation to the effects caused by the IDIF treatment, we show that no significant differences between control and treated mice, of the same genetic background, suggesting that despite TTR genetic reduction influenced Sort1 levels, its stabilization with IDIF was not enough to induce an alteration in Sort1 levels of treated mice. Thus we propose that the quantity of TTR, and not its stabilization state, is a major factor in the influence of Sort1. This also indicates that the beneficial effects of TTR stabilization by IDIF on AD features in this mouse model does not involve sort1, implying that TTR plays a role in AD pathogenesis via different pathways.

Data on the behavior of **synaptophysin** (**Syp**) expression is quite contradictory in the literature. While some suggest a decrease of Syp levels in AD, comparing to control[195, 196], others argue the opposite, despite acknowledging a negative correlation between Aβ accumulation and a decrease in Syp expression[194, 195]. Another study has shown that Syp is a probable γ-secretase-associated protein since its inhibition (using siRNA) resulted in a decrease of $A\beta_{40}$ and $A\beta_{42}$ levels[200], demonstrating a positive correlation between Syp's expression and Aβ levels. It goes without saying that this inconsistency is also the reflex of the lack of knowledge of Syp-related molecular mechanism in AD. Our results showed a significant increase in Syp expression in 3 months-old AD/$TTR^{+/-}$ mice, compared to AD/$TTR^{+/+}$ littermates, suggesting that TTR genetic reduction influences Syp expression; again, female mice showed a trend for higher Syp levels. As for the 7 months-old mice, no significant differences in Syp expression were observed between AD/$TTR^{+/+}$ and AD/$TTR^{+/-}$ female mice, although a tendency for increased levels of Syp was observed in the later. Because we did not perform a comparative study for the same TTR background at the two different ages evaluated, we were not able to distinguish if Syp levels increased in AD/$TTR^{+/+}$ or diminished in AD/$TTR^{+/-}$, comparing the 3 month to the 7 months-old animals. The inability to conclude on Syp behavior in our model is further complicated since we did not analyzed non transgenic animals, and therefore we did not ascertained Syp normal levels. In addition, the relative comparison between the two different ages evaluated is made between hippocampus and all brain, for the 3 and 7 months-old mice, respectively, which might have influenced the results. This limitation applies to all analysis performed and should be properly addressed in future experiments. Again, with regard to the effects of IDIF administration on the molecules under study, our data clearly indicated that TTR stabilization was not sufficient to restore their levels, and that TTR quantity is, at least in a first instance, a limiting factor, in opposition to effects on Aβ levels and deposition which were decreased in AD/$TTR^{+/-}$ IDIF treated mice when compared to non-treated[184]. In our opinion, AD-increased Syp levels are easily accepted, if one only looks at its molecular mechanism: if Syp expression ought to be increased, neurotransmission would be compromised, which would lead to the characteristic synaptic failure in AD. However, with the pathological evolution of AD (oxidative stress, SPs and NFTs formation, etc), the death of neurons and, subsequently, the destruction of synapses will lead to a natural decrease in Syp levels. In relation to the possible role of Syp as a γ-secretase-associated protein, we only observe a coherent behavior in 3 months-old mice[200]. Nonetheless, it is important to take into account that their study was performed *in vitro*, and that compensatory mechanism are triggered very often *in vivo*, especially in such complex diseases. Altogether, these observations prompt

Syp as a prospective and interesting biomarker that would allow the highly desirable detection of AD at its earliest stages.

In summary, our results showed differences in Sort1, Syp and APP processing dependent on the TTR background, further highlighting the importance of TTR in AD. Our observations also strengthened the *in vivo* evidence that this model is suitable for the study of the neuroprotective role of TTR and gender differences in AD as, in general, females showed more accentuated differences, thus recapitulating the trend observed for humans[201].

Conclusions

Two important notions to retain through the analysis of this work are: it is based on a single technique – Western Blot – which is a poor technique for the quantification of small changes, and on the analysis of hippocampus versus all brain, from mice of 3 and 7 months of age, respectively. The study performed in all brain can potentially result in the loss of specific alterations in the hippocampus, known to be particularly and early affected in AD. Future research should address this limitation and a higher number of mice hippocampus samples of different ages should be evaluated. In addition, future work should also include non-transgenic mice allowing the determination of Sort1 and Syp normal levels in the strain of mice used, in order to correctly conclude on the increase or decrease of these molecules in AD/TTR$^{+/+}$ versus AD/TTR$^{+/-}$ animals.

With regard to the influence of TTR in Sort1 and Syp expression and in APP processing, interaction studies between TTR and Sort1/Syp proteins are necessary to access whether their alteration is a direct or indirect effect caused by TTR genetic reduction. Cellular studies, in a more controlled environment, should also be engaged and would also enable us to confirm the effects of TTR in these molecules.

Sort1 showed to be influenced by TTR and presented some features that could allow Sort1 to be considered a biomarker for early detection of AD and for follow-up of AD therapies. As for Syp, it also showed to be influenced by TTR (in younger mice) and, interestingly, it showed to be highly affected by aging, independent of TTR genotype. This feature could allow Syp to be used as an early AD detection biomarker, prior to Aβ accumulation. The alterations in each molecule must be specific of AD and being AD such a complex disorder, association and combination of biomarkers will increase the chances of success.

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