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MODULATING FACTORS IN THE TRANSTHYRETIN/AMYLOID BETA INTERACTION

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De acordo com o disposto no nº 2, alínea a, do artigo 31º do Decreto-Lei nº 230/2009, utilizaram-se neste trabalho resultados já publicados ou vias de publicação que a seguir se discriminam:

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No cumprimento do Decreto-Lei supra mencionado, o autor desta dissertação declara que interveio na concepção e execução do trabalho experimental, na interpretação e discussão dos resultados e na sua redação. Todo o trabalho experimental foi realizado pelo autor desta tese de doutoramento, Carlos Alexandre Lopes Rodrigues Ribeiro.

Aos meus pais e irmã,

por me terem sempre apoiado nos meus sonhos

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“Todas as pessoas grandes foram um dia crianças.

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[Antoine de Saint-Exupéry]

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SUMMARY

Alzheimer's disease (AD) is the most common cause of dementia. The number of patients with AD is increasing every day and it is estimated that, in Portugal, there are more than 90 thousand people with AD. Due to the aging of population, it is expected that the number of AD patients will double in 2040. At present, a definitive AD diagnosis can only be obtained at autopsy through the histological quantification of two AD hallmarks: senile or neuritic plaques and neurofibrillary tangles (NFTs). However, individuals are diagnosed *antemortem* with possible or probable dementia of the Alzheimer type, using clinical criteria, but it is very difficult to diagnose AD at its earliest clinical stages. Thus, the search for biomarkers either genetic, imaging and/or biochemical, either with diagnosis and/or prognosis value is extremely important.

The most important genetic risk factor is the presence of *APOE* ε4 allele and therefore ApoE genotyping is used as a biomarker. Homozygous ε4 allele carriers develop AD up to 10 years earlier than individuals who do not have this allele. On the other hand, the presence of ε2 allele seems to be protective in the development of the disease. Mutations in three genes, APP, presenilin1 and presenilin 2 are associated with familial early-onset forms of AD, although the prevalence of these cases is very low.

β-amyloid 42 peptide (Aβ42), thought to be the causative agent in AD, is decreased in the cerebrospinal fluid (CSF) of AD patients and is used as a biomarker in AD diagnosis. While levels of β-amyloid 40 peptide (Aβ40) seem to be unchanged in AD, the ratio of $A\beta_{42}/A\beta_{40}$, rather than either marker alone, has been used to better distinguish AD subjects from controls or other dementias, and to identify incipient AD in subjects with amnestic mild-cognitive patients (aMCI). Another important molecule for AD diagnosis is tau. Elevated levels of total-Tau (T-tau) in the CSF and several kinases and phosphatases such as cdk5 and Gsk3β have been also associated to the conversion of aMCI to AD. Several other molecules are currently under investigation for their interest as biomarkers, such as transthyretin (TTR).

Several lines of evidence suggest TTR as a protective molecule in AD, including its decrease in the CSF. However, CSF measurements, although attractive in neurodegenerative disorders, imply invasive procedures, specialized personnel to collect the fluid, and the volumes obtained are relatively low. Plasma biomarkers assume an important role in this circumstance, since they are easily accessible to be used as a routine procedure. We thus investigated the behavior of TTR in plasma from AD patients along the development of the disease and searched for factors that could explain the alterations found.

We showed that TTR levels were decreased in plasma not only from AD patients, but also from aMCI patients, suggesting TTR might be used for staging early AD. In both patient groups, women showed significantly lower plasma TTR levels when compared to

MCI and AD men, respectively, and to women control group. In the AD women group, TTR levels correlated with disease stage, reflecting disease severity. Although MCI and AD men groups presented TTR levels lower than men in the control group, the difference was not statistically significant. Since 17β-estradiol induces TTR expression, we also measured plasma estradiol levels in women and showed a reduction in both patient groups as compared to control women. These results indicated that disease modulation by TTR is gender dependent.

With regard to other factors that might influence TTR in plasma, no association was found with ApoE and the presence of the ε4 allele, in both men and women. We also investigated TTR functionality by assessing thyroxine (T_4) binding to TTR in plasma and we found, in both MCI and AD groups, that TTR had reduced capacity to carry the hormone; Because T_4 binds to TTR in the central channel implying the formation of the TTR tetramer, we hypothesized that, in AD, TTR is destabilized and, its clearance accelerated, as a response to a loss of function. These results, together with previous observations that different TTR mutations bind differently to Aβ, correlating inversely with the amyloidogenic potential, underlie the importance of the TTR stabilization concept in AD.

In order to test the "stabilization hypothesis", we compared the ability of WT TTR, T119M TTR (a protective mutation in familial amyloidotic polyneuropathy (FAP)) and L55P TTR (a severe mutation) at inhibiting Aβ fibrillogenesis and toxicity. Using a sensitive conformational assay that relies on the use of the A11 antibody specific for oligomers, we showed that WT TTR and T119M TTR avoided further aggregation of the peptide, arresting the oligomeric state; on the contrary, L55P TTR allowed the peptide to continue to aggregate as deduced by the lack of reactivity with A11 after day 8, indicating that Aβ evolved to fibrils. With regard to the effect of TTR in Aβ toxicity, as demonstrated by caspase 3 activation, only the non-amyloidogenic variants were able to protect against the peptide toxic effects, whereas in the presence of L55P the levels of toxicity were similar to the ones measured for the peptide alone. Next, we postulated that if TTR stability is important for binding to Aβ, it should be possible to improve this interaction through the use of TTR stabilizers, screened and selected in the context of FAP therapies. These compounds, usually non-steroid anti-inflammatory drugs and usually presenting structural similarities with T_4, have high affinity to TTR, displacing the hormone and binding to the T_4 central binding channel. Amongst the compounds tested, iododiflunisal (IDIF), resveratrol (resv), Dinitrophenol (DNP), 2-((3,5-Dichlorophenyl)amino)benzoic acid (DCPA) and 4-(3,5-difluorophenyl) (DFPB) were able to improve TTR binding to Aβ peptide, while diflunisal (DIF), Epigallocatechin gallate (EGCG), flufenamic acid (Fluf), genistein, benzoxazole and Tri-iodophenol (TIP) were not. This indicated that most probably,

although stabilizing TTR, several of the compounds interfered/affected the sites were Aβ binds to TTR.

We also investigated the importance of TTR stabilization in its ability to degrade the peptide, previously suggested as one possible mechanism for TTR protection in AD. We used a fluorogenic peptide encompassing 6 residues of the Aβ peptide sequence containing one of the TTR cleavage sites (Abz-VHHQKL-EDDnp). Only DCPA and DFPB were able to improve TTR proteolytic capacity, indicative that: 1) TTR proteolytic activity against Aβ is separated from simple binding, and thus TTR might exert its protection through different mechanisms, 2) the two events may be related and depend on each other, however, TTR regions involved in binding and proteolytic activity are different. Furthermore, our work demonstrated that RBP, contrarily to T_4, abrogated TTR/Aβ binding and also TTR proteolytic ability to process Aβ peptide. The ability of TTR to disrupt amyloidogenic fibrils was also evaluated. In our work, we suggest that TTR is not a universal disrupter, but rather is specific for Aβ. Amongst the amyloid fibrils tested: amylin, insulin, β2-microglobulin and Aβ, TTR only disrupted the latter, suggesting that TTR recognizes a sequence and not a structural motif, further supporting the proteolytic hypothesis.

To further test the TTR stability hypothesis *in vivo*, we assessed the effects of IDIF, a potent TTR stabilizer and one of the best at improving TTR/Aβ interaction, amongst the ones tested, in an AD transgenic mouse model shown to present gender-associated modulation of brain Aβ levels by TTR. Five month old AD female mice, either with just one (AD/TTR$^{+/-}$) or with two (AD/TTR$^{+/+}$) copies of endogenous TTR were given IDIF, orally administered for 2 months, and assessed for several AD features. We showed that IDIF was able to bind and stabilize TTR in plasma as deduced from its ability to displace T_4 from TTR and to reach the CSF thus making it accessible to the brain.

Evaluation of Aβ levels showed that AD/TTR$^{+/-}$ animals treated with IDIF presented reduced levels of formic acid-soluble Aβ40 and Aβ42 compared to age-matched controls. We also analyzed Aβ plaque burden by immunohistochemistry and observed that AD/TTR$^{+/-}$ treated mice presented a decrease in plaque burden compared to the non-treated animals. CSF and plasma Aβ42 levels were also assessed and we reported a significant reduction in Aβ42 levels in plasma in AD/TTR$^{+/-}$ treated mice and a trend for reduction in CSF, when compared to the non-treated animals. Finally, we evaluated the impact of IDIF treatment on spatial learning and memory through the MWM (Morris water maze) test and we showed that while IDIF-treated AD/TTR $^{+/-}$ mice learned to find the platform, demonstrating a decreased in the latency along the 7-day period, non-treated AD/TTR $^{+/-}$ animals presented impaired ability to learn. Our results demonstrated that TTR stabilization by IDIF, results in decreased Aβ deposition in the brain and amelioration of

the cognitive deficits in the AD mouse model, suggesting TTR promotes Aβ clearance from the brain and periphery.

In conclusion, our work suggests TTR as a biomarker for early diagnosis of AD in particular in women, and proposes that TTR tetrameric stability plays an important role in AD pathogenesis. Moreover, we suggested for the first time the hypothesis that restoring or improving TTR/Aβ binding can be a therapeutic avenue in AD.

SUMÁRIO

A doença de Alzheimer (DA) é uma das causas mais comuns de demência. O número de pacientes com DA está a aumentar dia após dia e é estimado que em Portugal sejam já mais de 90 mil as pessoas com DA. Face ao envelhecimento da população é expectável que o número de pacientes com a doença duplique em 2040. Até ao momento, um diagnóstico definitivo da doença só pode ser obtido pela realização de autópsia e através da quantificação histológica dos dois marcadores da DA: placas senis ou neuríticas e os novelos neurofibrilares. Contudo, os pacientes são diagnosticados *antemortem* como possíveis ou prováveis doentes de Alzheimer, usando-se critérios clínicos. Contudo, é muito difícil diagnosticar a DA nos seus estádios precoces. Por isso, a procura por biomarcadores, genéticos, imagiológicos e/ou bioquímicos com capacidade de diagnóstico e/ou mesmo de prognóstico da doença é extremamente importante.

O mais importante factor de risco genético é a presença do alelo ε4 da ApoE e por isso a genotipagem da ApoE é usada como um biomarcador. Os portadores homozigóticos do alelo ε4 desenvolvem DA até 10 anos mais cedo do que os indivíduos que não possuem esse alelo. Por outro lado, a presença do alelo ε2 parece ser protectora no desenvolvimento da doença. Até à data foram identificadas mutações em três genes, APP, presenilina 1 e 2, que estão associadas a formas familiares precoces de DA, embora a prevalência destes casos seja muito baixa.

O péptido β amilóide 42 (Aβ42) que se pensa ser o agente causador da DA, está diminuído no líquido cefalorraquidiano (LCR) de pacientes com DA e é utilizado como um biomarcador no diagnóstico da doença. Embora os níveis do péptido Aβ40 pareçam estar inalterados nesta patologia, o rácio Aβ42/Aβ40 - ao invés do uso de qualquer um dos marcadores *per si* - tem sido usado para distinguir melhor os pacientes de DA dos controlos ou mesmo dos pacientes com outras demências e até para identificar a DA inicial em pacientes com défice cognitivo ligeiro amnésico (DCLa). Outra molécula importante para o diagnóstico da DA é a tau. Os níveis elevados de tau total (T-tau) no LCR e várias cinases e fosfatases, tais como cdk5 e Gsk3β também têm sido associadas à conversão da DCLa para AD. Várias outras moléculas estão a ser atualmente estudadas pelo seu interesse como possíveis biomarcadores, como é o caso da transtirretina (TTR).

As evidências que sugerem a TTR como uma molécula protectora na DA têm aumentado nos últimos anos, incluindo o fato desta estar reduzida no LCR. No entanto, as quantificações no LCR, embora atraentes em doenças neurodegenerativas, implicam procedimentos invasivos, pessoal especializado para recolher o líquido, e para além disso os volumes obtidos são relativamente baixos. Os biomarcadores no plasma assumem, por isso, um papel importante nesta circunstância, uma vez que são facilmente acessíveis para ser utilizados como um procedimento de rotina. Por isso, investigamos o

comportamento da TTR no plasma de pacientes com DA ao longo do desenvolvimento da doença e procuramos fatores que pudessem explicar as alterações encontradas.

No nosso trabalho, mostrámos que os níveis de TTR estavam diminuídos no plasma não só em pacientes com DA, mas também em pacientes com DCLa, sugerindo que a TTR pode ser utilizada como biomarcador para estádios iniciais da DA. Em ambos os grupos de pacientes, as mulheres mostraram níveis significativamente mais baixos de TTR no plasma, quando comparadas com os homens com DA e DCLa, respectivamente, e com as mulheres do grupo controlo. No grupo de mulheres com DA, os níveis de TTR correlacionaram-se com a fase da doença, refletindo a gravidade da mesma. Embora os homens dos grupos DCLa e DA apresentassem níveis de TTR inferiores aos dos homens do grupo controlo, a diferença não foi estatisticamente significativa. Uma vez que a hormona 17β-estradiol induz a expressão de TTR, também medimos os níveis plasmáticos de estradiol em mulheres e mostrámos uma redução em ambos os grupos de pacientes em relação ao grupo das mulheres controlo. Estes resultados indicam que a modulação da doença pela TTR é dependente do género.

No que diz respeito a outros factores que podem influenciar a TTR no plasma, não foi encontrada qualquer associação com a ApoE e a presença do alelo ε4, em homens e mulheres. Também investigámos a funcionalidade da TTR avaliando a ligação à tiroxina (T_4) no plasma e verificámos que, tanto no grupo DCLa como no grupo DA, a TTR apresentou diminuição na capacidade de transportar a hormona; Uma vez que a T_4 se liga à TTR no canal central, estando subjacente a formação do tetrámero da TTR, formulámos a hipótese de que, na DA, a TTR está desestabilizada e, por isso, a sua eliminação é acelerada, como resposta à sua perda funcional. Estes resultados, juntamente com as observações anteriores de que diferentes mutações de TTR se ligam de forma diferente ao Aβ, correlacionando-se inversamente com o potencial amiloidogénico, fundamentam a importância do conceito de estabilização da TTR na DA.

A fim de testar a "hipótese de estabilização", comparámos a capacidade da TTR WT, T119M (uma mutação protectora na paramiloidose amiloidótica familiar (PAF)) e L55P (uma mutação severa) na inibição da fibrilização e toxicidade do Aβ. Utilizando um ensaio conformacional sensível que depende do uso de um anticorpo específico para oligómeros (A11), mostrámos que a TTR WT e T119M evitaram a agregação do péptido, mantendo o estado oligomérico; pelo contrário, a TTR L55P permitiu que o péptido continuasse a agregar conforme deduzido pela falta de reactividade com o A11 após 8 dias, o que indica que o Aβ evoluiu para uma forma fibrilar. No que diz respeito ao efeito da TTR na toxicidade do Aβ, conforme demonstrado pela activação da caspase 3, apenas as variantes não-amiloidogénicos foram capazes de proteger contra os efeitos tóxicos do péptido, ao passo que na presença de L55P os níveis de toxicidade foram

semelhantes aos medidos com o péptido sozinho. Em seguida, lançámos a hipótese de que, se a estabilidade da TTR é importante para a ligação ao Aβ, deve ser possível melhorar esta interacção através do uso de estabilizadores da TTR, investigados e seleccionados no âmbito da investigação de terapias em PAF. Estes compostos, em geral drogas anti-inflamatórias não-esteróides e apresentando geralmente semelhanças estruturais com a T_4, têm uma elevada afinidade para a TTR e são capazes de substituir a hormona, ligando-se ao canal central de ligação da T_4. Entre os compostos testados, o iodo-diflunisal (IDIF), o resveratrol (resv), o dinitrofenol (DNP), o 2 - ((3,5-diclorofenil) amino) benzóico (DCPA) e o 4 - (3,5-difluorofenil) (DFPB) foram capazes de melhorar a ligação da TTR ao péptido Aβ, enquanto que o diflunisal (DIF) , epigalocatequina galato (EGCG), ácido flufenámico (Fluf), a genisteína, benzoxazole e Tri-iodofenol (TIP) não. Isto indica que, muito provavelmente, embora estabilizando a TTR, vários dos compostos interferiram/afectaram os locais onde o Aβ se liga à TTR.

Também foi investigada a importância da estabilização da TTR na sua capacidade para degradar o péptido, anteriormente sugerida como um mecanismo possível para a protecção da TTR na DA. Para isso, foi utilizado um péptido fluorogénico abrangendo 6 resíduos da sequência de péptido Aβ contendo um dos locais de clivagem pela TTR (Abz-VHHQKL-EDDnp). No entanto, apenas o DCPA e o DFPB foram capazes de melhorar a capacidade proteolítica da TTR, o que pode indicar que: 1) a actividade proteolítica da TTR é distinta da ligação ao Aβ, e, assim, a TTR pode exercer a sua protecção via diferentes mecanismos; 2) os dois eventos podem estar relacionados e dependerem um do outro, no entanto, as regiões da TTR envolvidas quer na ligação ao Aβ quer na sua actividade proteolítica são diferentes. Para além disso, o nosso trabalho demonstrou que o RBP, contrariamente à T_4, impede não só a ligação da TTR ao Aβ como também a sua capacidade de processar proteoliticamente o Aβ. Também foi avaliada a capacidade da TTR para romper fibras amiloidogénicas. No nosso trabalho, sugerimos que a TTR não é um disruptor universal, mas é específico para o Aβ. De entre as fibras amilóide testadas: amilina, insulina, β2-microglobulina e Aβ, a TTR apenas foi capaz de clivar as últimas, sugerindo que a TTR reconheceu uma sequência de aminoácidos e não um motivo estrutural, apoiando a hipótese proteolítica.

Para testar a hipótese da estabilidade da TTR *in vivo*, foram avaliados os efeitos do IDIF, um potente estabilizador da TTR e um dos compostos que melhor aumenta a capacidade de ligação da TTR ao Aβ, num modelo AD transgénico de murganho que demonstra modulação dos níveis do Aβ no cérebro pela TTR de forma dependente do género. Murganhos fêmeas AD com 5 meses de idade e com apenas uma ($AD/TTR^{+/-}$) ou com duas ($AD/TTR^{+/+}$) cópias de TTR endógena foram tratados com IDIF, administrado por via oral durante 2 meses e avaliados para vários marcadores da DA. No nosso

trabalho, mostrámos que o IDIF foi capaz de se ligar e estabilizar a TTR no plasma, conforme deduzido a partir da sua capacidade de substituir a T_4. O IDIF foi ainda capaz de chegar ao LCR tornando-se assim acessível ao cérebro.

A avaliação dos níveis de Aβ mostrou que os animais AD/TTR$^{+/-}$ tratados com IDIF apresentaram níveis reduzidos de Aβ40 e Aβ42 solúvel em ácido fórmico quando em comparação com os respectivos controlos. Foi também analisado o Aβ depositado no cérebro, através de imuno-histoquímica e observou-se que os animais AD/TTR$^{+/-}$ tratados apresentaram uma diminuição na deposição de Aβ comparativamente com os animais não tratados. Os níveis plasmáticos e do LCR de Aβ42 foram também avaliados e verificou-se uma redução significativa nos níveis plasmáticos de Aβ42 nos animais AD/TTR$^{+/-}$ e uma tendência para redução dos níveis no LCR, em comparação com os animais não tratados. Finalmente, foi avaliado o impacto do tratamento do IDIF na aprendizagem e memória espacial com o teste MWM (Morris water maze) e mostrou-se que, enquanto os animais AD/TTR$^{+/-}$ tratados com IDIF aprenderam a encontrar a plataforma, o que foi demonstrado por uma diminuição na latência ao longo dos sete dias de teste, os animais AD/TTR$^{+/-}$ não tratados apresentaram uma diminuição na capacidade de aprendizagem. Os resultados demonstraram que a estabilização da TTR pelo IDIF resultou na redução da deposição do Aβ no cérebro, na melhoria dos défices cognitivos, neste modelo animal, sugerindo ainda que a TTR promove a eliminação do Aβ a partir do cérebro e da periferia.

Em conclusão, o nosso trabalho sugere a TTR como um biomarcador para o diagnóstico precoce da DA, em particular nas mulheres, e propõe que a estabilidade do tetrámero da TTR desempenha um papel importante na patogénese da DA. Além disso, sugere-se pela primeira vez a hipótese de que o restabelecimento da ligação ou a melhoria da ligação da TTR ao Aβ pode ser uma via terapêutica para a DA.

GENERAL INTRODUCTION

In the next section will be discussed the state of the art of several aspects related to one of the major neurodegenerative diseases, Alzheimer's disease (AD) and will be debated the protective role of transthyretin (TTR) in AD, the focus of the experimental work described in section III. In this regard, the importance of the modulation in the binding of TTR to amyloid-beta peptide (Aβ) – a crucial peptide involved in AD development -, the importance of disease biomarkers and the mechanisms involved in the clearance of Aβ peptide are some of the topics that will be discussed in the following pages.

AMYLOIDOSIS

In the nineteenth century Rudolph Virchow popularized the definition of amyloid, characterizing it by a "macroscopic tissue abnormality that presented a positive iodine staining reaction" (Sipe and Cohen, 2000). With the evolution of the light microscopies with polarized optics, amyloid has been described according to the green birefringence produced after staining with Congo Red under polarized light (Sipe et al., 2012). Indeed, this is the main characteristic that allows the definition of amyloid in clinical practice. Amyloidosis include a group of protein misfolding diseases typified by the abnormal deposition of amyloid protein in one or more organs and tissues of animals or humans (Woldemeskel, 2012). Amyloidosis is classified according to the chemical nature of the fibril protein, designated protein A and followed by a suffix, an abbreviated form of the parent or precursor protein name (Sipe et al., 2012).

More than 25 proteins which form amyloid have been recognized and associated with several diseases (Eisenberg and Jucker, 2012). Depending on the distribution of the amyloid deposits, amyloid disorders are divided in two main categories: localized and systemic amyloidosis. Systemic amyloidosis (table 1) are characterized by the deposition of amyloid in many or most tissues and organs, such as Familial amyloid Polyneuropathy (FAP). On the other hand, localized amyloidosis (table 2) occurs in one single tissue or organ, such as AD (Westermark, 2005).

Table 1 - Fibril proteins and their precursors in human systemic amyloidosis. (Westermark, 2005)

Amyloid protein	Precursor	Main disease or involved tissues
AL	Immunoglobulin light chain	Primary
		Myeloma-associated
AH	Immunoglobulin heavy chain	Primary
		Myeloma-associated
$A\beta_2M$	β_2-microglobulin	Hemodialysis
ATTR	Transthyretin	Familial
		Senile systemic
AA	(Apo)serum AA	Secondary, reactive
AApoAI	Apolipoprotein AI	Familial
AApoAII	Apolipoprotein AII	Familial
AApoAIV	Apolipoprotein AIV	Unknown
AGel	Gelsolin	Familial
ALys	Lysozyme	Familial
AFib	Fibrinogen α-chain	Familial
ACys	Cystatin C	Familial
ABri	ABriPP	Familial dementia, British
ADan	ADanPP	Familial dementia, Danish

Table 2 - Fibril proteins and their precursors in human localized amyloidosis (Westermark, 2005)

Amyloid	Precursor protein	Main disease or involved tissues
AL	Immunoglobulin light chain	Local plasma cell clone expansion
AH	Immunoglobulin heavy chain	Primary
Aβ	Aβ protein precursor (AβPP)	Alzheimer's disease, aging
APrP	Prion protein	Spongioform encephalopathies
ACal	(Pro)calcitonin	C-cell thyroid tumors
AIAPP	Islet amyloid polypeptide	Islets of Langerhans
		Insulinomas
AANF	Atrial natriuretic factor	Cardiac atria
APro	Prolactin	Aging pituitary
		Prolactinomas
AIns	Insulin	Iatrogenic
AMed	Lactadherin	Senile aortic, media
AKer	Kerato-epithelin	Cornea; Familial
ALac	Lactoferrin	Cornea; Familial
ASem	Semenogelin I	Seminal vesicles
APin	Protein expressed by tumor	Pindborg tumors

ALZHEIMER'S DISEASE

One of the best known examples of localized amyloidosis is AD. First described by Alois Alzheimer in 1907, AD is currently one of the most devastating neurodegenerative diseases, comprising approximately 70% cases of dementia worldwide, with one of the highest prevalence rates in Western Europe (Reitz et al., 2011). AD is usually associated with aging and clinically characterized by a progressive loss of the cognitive functions leading to death (Alzheimer et al., 1995).

In the beginning of XIX century Alois Alzheimer, a German neuropathologist and psychiatrist, identified the first case of "presenile dementia", which his colleague Kraeplin would later identify as AD. The patient was Auguste Deter, a 50 years-old woman, who presented symptoms that did not fit into any other known diseases at the time. Clinically, Deter showed some symptoms that are now related with AD such as rapid loss of memory and disorientation in time and space (Alzheimer et al., 1995).

After Deter's death, her brain was analyzed, and Alzheimer reported an atrophic brain with the "existence of some fibrils inside the cells", describing the presence of neurofibrillary tangles (NFTs), for the first time (Alzheimer et al., 1995). Indeed, senile plaques – another typical lesion in AD brains – were not firstly described by Alzheimer. In the same year, Fischer had already published a study with the first description of senile plaques (Goedert, 2009). In 1910, Emil Kraepelin included the disease in the Textbook of Psychiatry (Verhey, 2009).

1. Histopathological lesions of AD

In addition to the typical clinical features of AD, the disease is characterized by two cardinal lesions which are senile plaques, constituted of aggregated Aβ peptide and neurofibrillary tangles (NFTs) which are intracellular aggregates of abnormally hyperphosphorylated tau protein (Pimplikar, 2009).

1.1. Neurofibrillary Tangles (NFTs)

Firstly described by Alzheimer as some fibrils inside the cells, NFTs (figure 1) are composed by abnormal fibrils with approximately 10 nm in diameter that occur in pairs wound in a helical fashion with a regular periodicity of 80 nm - these structures are called: paired helical filaments (PHF) (Perl, 2010). The main constituent of the NFTs is the microtubule-associated protein tau which presents an abnormal phosphorylation and also other post-translational modifications such as glycosylation, ubiquitination or glycation. Generally, tau is a cytosolic protein responsible for the stimulation and stabilization of microtubule assembly from tubulin subunits. The protein is the major neuronal microtubule-associated protein and the tau gene is located on the long arm of chromosome 17 (position 17q21) and contains 16 exons (Gong et al., 2005).

It is unclear why tau becomes abnormally phosphorylated, as it can be the consequence of an upregulation of tau kinase or a downregulation of tau phosphatase (Martin et al., 2011). Nevertheless, the phosphorylation process, when at normal levels, is essential to the normal functioning of tau, because it regulates its binding to the microtubules. On the other hand, the hyperphosphorylation leads to the loss of its biological activity and tau is not able to bind to the microtubules causing the increase of soluble tau in the monomeric form. Then tau aggregates and forms dimers and oligomers/protomers resulting in the formation of PHFs and finally in NFTs (figure 1) (Martin et al., 2011).

The distribution of NFTs in AD is generally predictable. NFTs are mainly placed in the layer II neurons of the entorhinal cortex, in the CA1 and subicular regions of the hippocampus, and in the amygdala and the deeper layers (layers III, V and superficial VI) of the neocortex. Furthermore, it was described that the distribution of NFTs in AD is correlated with the stage and duration of the disease (Perl, 2010). However, tau is not just a hallmark lesion of AD but also of other neurodegenerative diseases – taupathies – such as Amyotrophic lateral sclerosis (ALS), Pick’s disease, down’s syndrome or Prion diseases (Gong et al., 2005).

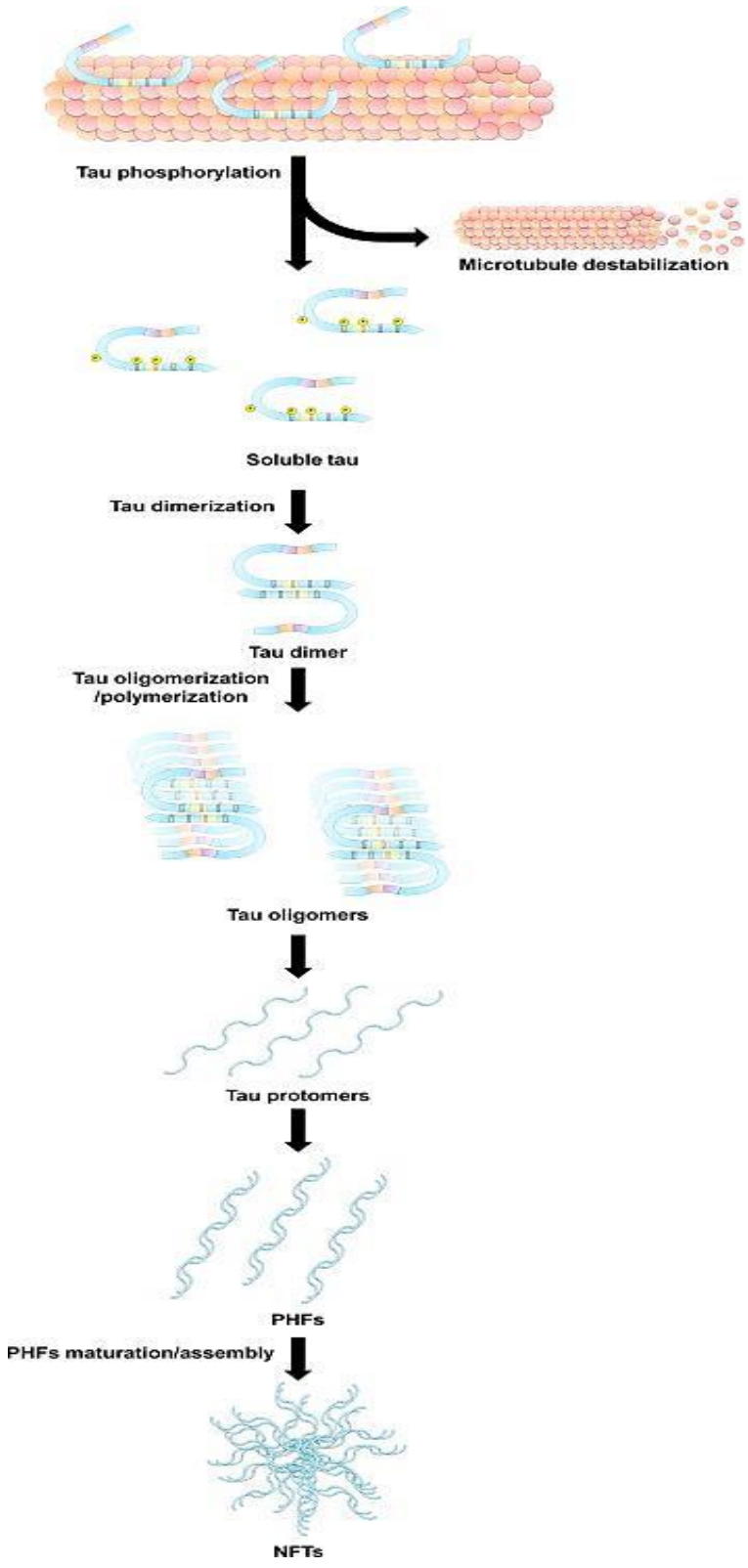

Figure 1 – Representation of tau aggregation. The impairment of tau binding to microtubules results in the increase of soluble tau. Then, monomers aggregate into dimers and oligomers resulting in the formation of PHFs and NFTs (Martin et al., 2011).

1.2. Senile Plaques

Senile plaques, the other hallmark lesion in AD (figure 2), are spherical extracellular lesions mainly located in cerebral cortex and mostly constituted by Aβ peptide.

The lesions observed in AD can be classified in diffuse and neuritic/senile plaques. Although proposed that the diffuse plaques represent the earliest cerebral lesions in AD which then progress to compact plaques, neuritic/seniles plaques do not necessarily correspond to an evolution form of diffuse plaques; in fact, in severe cases of AD, diffuse plaques accounts for the majority of Aβ protein-immunorreactive material (Castellani et al., 2010).

Understanding the origin of senile plaques is another major focus of research in this area. Several hypotheses have been proposed to explain the genesis of senile plaques but it remains unclear if Aβ deposition is a consequence of the evolution of senile plaques or is the first event in its formation. (Castellani et al., 2010). Aβ deposits in the form of senile plaques are not exclusive to AD and are also found in other diseases such as Down syndrome, dementia pugilistica, diffuse Lewy body disease or acute traumatic brain injury (Castellani et al., 2010).

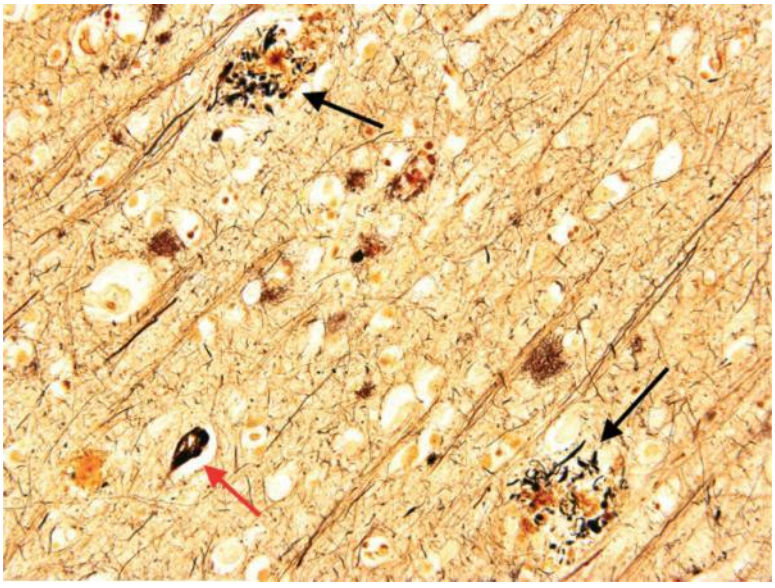

Figure 2 – Representation of the two hallmarks of AD. Neurofibrillary tangles (red row) and senile plaques (black row) (Perl, 2010).

2. Amyloid β Precursor Protein and Aβ formation

2.1. APP processing

Aβ is a small peptide of approximately 4kDa derived from a larger amyloid-β precursor protein (APP). APP is a family of glycosylated transmembrane proteins which are universally expressed, but are most abundant in the brain (Rossner, 2004). The APP gene is located in the chromosome 21 in humans (Korenberg et al., 1989), contains 18 exons and its evolutionary conservation extends to invertebrate species (Zhou et al., 2011). In fact, APP is a member of an evolutionary conserved gene family which includes APPL in Drosophila, appa and appb in zebrafish, APL-1 in *Caenorhabditis elegans* and APLP1 and APLP2 in humans (Muller and Zheng, 2012). The region that corresponds to the Aβ sequence includes the exon 16 and 17 and is composed of 40 to 43 amino acid residues (Zhou et al., 2011). APP processing can involve amyloidogenic or non-amyloidogenic pathway as shown in figure 3. APP processing is initiated by the cleavage by α-secretase within the Aβ region (non-amyloidogenic process) or by β-secretase at the amino terminus of Aβ peptide (amyloidogenic process).

2.2. The Non-amyloidogenic and the amyloidogenic pathways

In the non-amyloidogenic pathway the cleavage by α-secretase within the Aβ region abrogates Aβ formation and generates a soluble fragment (sAPPα) and a carboxyl-terminal fragment (CTF83). The following cleavage by γ-secretase generates a non-amyloidogenic peptide (p3) and an APP intracellular domain (AICD) fragment.

In the amyloidogenic cleavage, APP is cleavage by β-secretase generating a soluble secreted form of APP (sAPPβ) and a CTF99 fragment which contains the Aβ sequence. The CTF99 fragment is then cleavage by γ-secretase generating the APP intracellular domain (AICD) and the amyloidogenic Aβ peptide (Muller and Zheng, 2012).

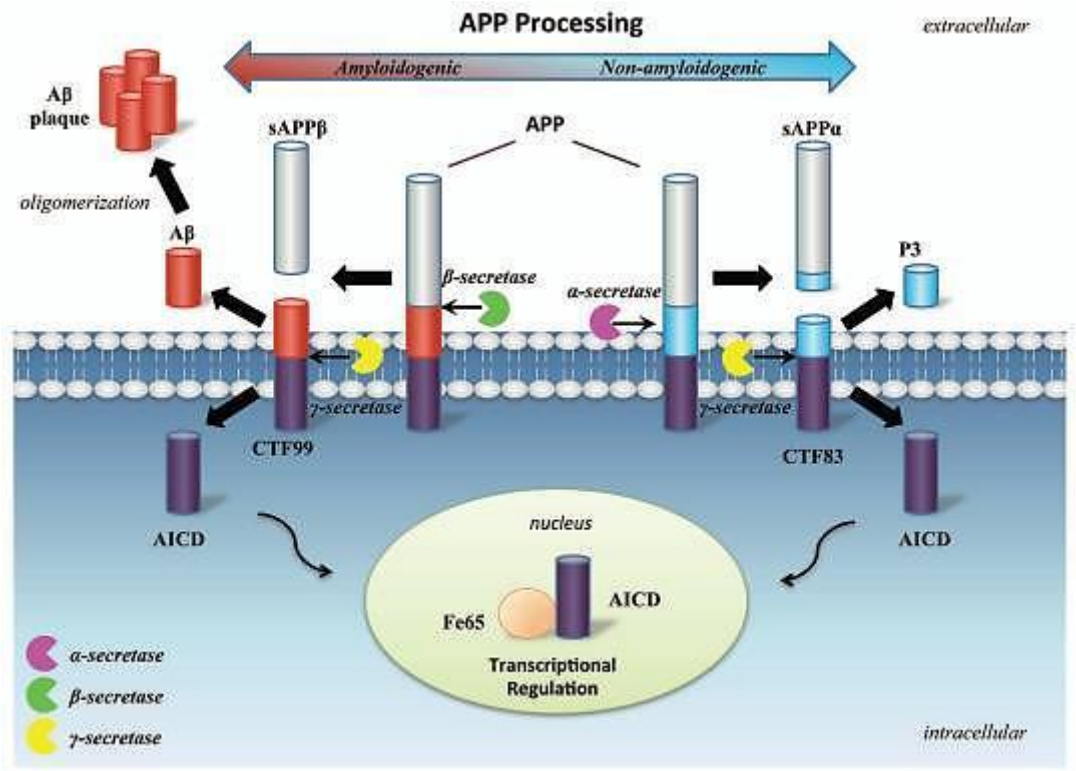

Figure 3 – APP processing and cleavage products. In the right it is represented the non-amyloidogenic pathway and the cleavage products (P3, CTF83 and AICD) and in the left the amyloidogenic pathway which results in the Aβ formation and the other cleavage products (CTF99 and AICD) (Zhou et al., 2011).

The sAPPα generated in the non-amyloidogenic pathway was shown to protect neuron cells and promote neurogenesis. AICD – generated in both pathways – has been associated with multiple genes transcription activation (APP, GSK-3β, KAL1, NEP, BACE1, P53, EGFR and LRP1), after being translocated to the nucleus (Zhang et al., 2011). Furthermore, AICD has also been described as a promotor of the interaction between APP and several cytosolic factors (Zhang et al., 2011). Nevertheless, the function of other generated fragments is still unclear, such as fragment p3 - generated in non-amyloidogenic process - and that is rapidly degraded. For instance, although APP is ubiquitously expressed in mammalian cells, much is still unclear about its physiological roles and also how they can contribute to AD. However, there is increasing evidence that APP is important for neuron generation, differentiation and migration (Zhou et al., 2011).

AD is commonly divided in two forms: familial and sporadic. AD familial cases are usually associated with autosomal dominant mutations and three genes account for these familial types: presenilin 1 (PSEN1) and presenilin 2 (PSEN2) - that forms part of the γ-secretase complex involved in the APP processing (Small et al., 2010) – and APP (van der Flier et al., 2011). PSEN1 mutations are the most common genetic cause for early-onset familial AD (EOAD) (<45 years) and to date, 185 pathogenic PSEN1 mutations in

patients with EOAD & Frontotemporal Dementia have been identified (http://www.molgen.ua.ac.be/ADMutations). Some of these mutations such as L85P, P117L, P117S, insF1 and L166P are also associated with very early onset of cognitive decline (<30 years). Usually, PSEN1 mutations lead to an increase in relative production of more toxic Aβ42 peptides (Duan et al., 2012). Compared with the prevalence described for PSEN1 gene, only 13 pathogenic PSEN2 mutations were found patients with EOAD. Finally, mutations in APP comprise about 9% of familial cases (Castellani et al., 2010) and to date 33 pathogenic APP mutations were reported http://www.molgen.ua.ac.be/ADMutations. These mutations, located inside or close to the Aβ domain, can cause the increase of Aβ produced and/or increase in the ratio of Aβ42/40, promoting the production of Aβ variants with higher tendency to aggregate (Castellani et al., 2010, Kumar and Walter, 2011).

Nonetheless, these mutations are rare and the majority of AD cases is classified as sporadic and has a later onset age (LOAD) (Castellani et al., 2010).

2.3. Soluble amyloid precursor protein α and β

Although the physiological functions of sAPPα are still unclear, several roles have been attributed and generally considered beneficial to neurons. These functions include neuroprotection, regulation of cell proliferation and early development of the central nervous system (CNS) (Furukawa et al., 1996, Ohsawa et al., 1999, Caille et al., 2004). sAPPα also promotes neurite outgrowth and cell adhesion (Mattson, 1997, Gakhar-Koppole et al., 2008) and when administered intracerebroventricularly, improves learning and memory in mice and rats (Meziane et al., 1998, Taylor et al., 2008). The sAPPα concentrations in cerebrospinal fluid (CSF) are decreased in carriers of the Swedish APP mutation (APP 670/671) (Lannfelt et al., 1995), however different and contradictory data were reported in sporadic AD cases: while some authors showed an increase of sAPPα concentrations in CSF (Lewczuk et al., 2010), others reported unchanged levels of this peptide (Olsson et al., 2003, Brinkmalm et al., 2013).

sAPPβ differs from sAPPα by lacking a region of 16 amino acids at the C-terminus (Brinkmalm et al., 2013). Contrarily to sAPPα, sAPPβ is not associated with neuroprotection and is thought to be involved in pruning of synapses during development of both central and peripheral neurons (Nikolaev et al., 2009).

Although several studies showed sAPPα, sAPPβ, or total sAPP as potential biomarkers for AD the results are still inconclusive.

2.4. Aβ peptide

Aβ is a ±4kDa peptide with a common core sequence but heterogeneous N- and C-termini (Tomidokoro et al., 2010). It has been suggested that the hydrophobic C-terminal region of Aβ, which is derived from the transmembrane domain of APP, is mainly responsible for Aβ aggregation (Jarrett et al., 1993) and that the hydrophilic N-terminal domain participates in amyloid formation (Fraser et al., 1991). Aβ peptide occurs in different lengths variants (39-43 amino acids) depending on the site where γ-secretase cleaves APP. The two major forms of Aβ that have been observed are composed by 40 and 42 residues (Aβ40 and Aβ42, respectively) and the later has a much higher tendency to form aggregates (Kumar and Walter, 2011). However, in a normal individual, the majority of Aβ produced is Aβ40, and 5-15% of the total Aβ is the 42 residues form (Findeis, 2007).

Aβ is produced during normal cellular metabolism and the peptide is secreted to the extracellular milieu of the human brain, but is also found in CSF (Kumar and Walter, 2011). The effective role of Aβ peptide is another unrevealed mystery, however some physiological functions have been attributed to the peptide, such as: ion channel modulation, kinase activation, cholesterol transport regulation, learning and memory or regulation of AD-associated genes (Kumar and Walter, 2011). Recently, it was showed that Aβ is associated with proliferation and differentiation in the choroid plexus epithelial cells. Bolos and colleagues described that treatment of cultured plexus epithelial cells of APP/PS1 mice with oligomeric Aβ in, increased proliferation and differentiation of neuronal progenitor cells. (Bolos et al., 2013).

The formation of amyloid in AD is, as described before, one of the key factors in AD pathogenesis. A commonly accepted concept for the formation of amyloid fibrils is the nucleation-dependent polymerization model that divides the fibrilization process into a nucleation phase and an elongation phase (Kumar and Walter, 2011). As depicted in figure 4, in the nucleation phase, monomeric Aβ (composed of α-helical and/or unordered structure) can suffer conformational changes to form oligomeric species (composed of β-sheets). After the formation of oligomers, the species evolve to larger aggregates and fibrils (elongation phase) (Kumar and Walter, 2011). Apparently, the monomers are harmless species which become neurotoxic when evolve to oligomers and aggregates. Additionally, it was described that there is a positive correlation between soluble oligomeric Aβ levels and the stage of the disease (Kumar and Walter, 2011).

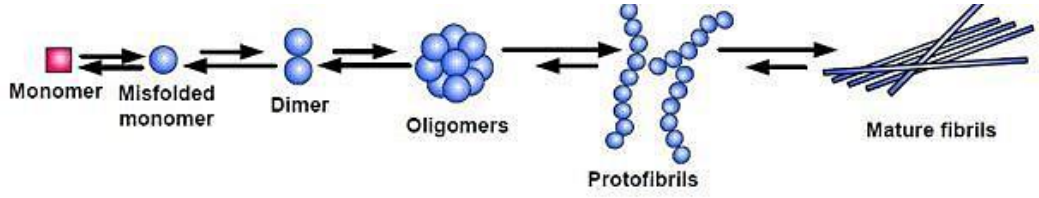

Figure 4 – Representation of Aβ assembly. Adapted from (Kumar and Walter, 2011)

3. Alzheimer's disease - The Hypotheses

Different hypotheses try to explain the AD cascade, but in all cases the theories are not absolutely consistent. Besides that, and as Hardy, J. (Hardy, 1997a) said, it is not correct to present AD as a "dementia of unknown cause and inexorable progression" because are known several causes of the disease and in all of them APP metabolism and Aβ production and deposition have been the key early event in disease pathogenesis. However and unfortunately it seems to be correct that we can continue to consider AD as a dementia of "inexorable progression" (Hardy, 1997a).

The "Amyloid cascade hypotheses" is the dominant hypothesis concerning the etiology and pathogenesis of AD. It considers that Aβ peptide is the initiator of a pathological cascade that leads to amyloid plaques, neurofibrillary tangle formation, neuronal dysfunction, possibly inflammatory responses and finally dementia (Sorrentino and Bonavita, 2007). This hypothesis is supported by studies of rare cases of genetically inherited forms of AD. In affected families, a particular aggressive and early-onset form of the disease is observed. The proteins aggregated in the brain are in general very similar to the ones observed in the much larger group of sporadic AD.

In spite of the dominance of this hypothesis nowadays, there are some evidences that are usually used to reject it. Pathological studies are one of these examples, because the relationship between deposition and the degree of dementia is not directly correlated. The argument of poor correlation between amyloid deposition and clinical features was refused by Hardy and co-workers, because the analyses depend directly on the presence of amyloid deposits around for neuropathologists to count them. The fact is that all known AD-causing mutations lead to an increase in Aβ formation and its deposition in the disease process (Hardy, 1997b). Other arguments against the "Amyloid Cascade hypothesis" include studies performed *in vivo* and *in vitro* using different models showing that although Aβ appears to be toxic, the concentrations required to induce the neuronal death were excessive (Sorrentino and Bonavita, 2007). Other example is associated with

transgenic animal studies, which showed that behavioral deficits appear before amyloid deposition in mice that overexpress APP (Hardy, 1997b).

Another hypothesis pretends to justify the correlation between synaptic loss and the levels of Aβ immunoreactivity in soluble extracts. Aβ derived diffusible ligands (ADDL) hypothesis uses, basically, the same background of Aβ amyloid cascade hypothesis. ADLLs are non-fibrillary neurotoxins, mainly constituted by small globular Aβ oligomers. This theory states that peptide oligomers play a central role in AD. ADLLs altered synaptic signaling, which first cause early-stage memory loss (Klein, 2002). The peptide forms non-fibrillary plaques that culminate in the formation of fibrils. The organism has to defend against the fibrils and does so by causing a local inflammatory response that leads to the activation and accumulation of microglia and astrocytes. It results in synaptic spine loss, neuritic dystrophy, oxidative stress, changes in ionic homeostasis and other biochemical changes (Walsh and Selkoe, 2007).

4. How Aβ is removed?

AD patients that present an abnormal increase in Aβ production or APP overexpression in brain are rare. The larger part of AD patients do not present this type of dysfunctions and the accumulation of Aβ peptide is associated with the unbalance between its production and clearance (Wang et al., 2006b). The cause of this impairment is not known but several factors are involved such as: peptidases, anti-β autoantibodies, Aβ-bindable substances and receptors.

Aβ is degraded by several peptidases, mainly two zinc metaloendopeptidases: Neprilysin (NEP) and insulin-degrading enzyme (IDE). NEP is a 97kDa type II membrane-associated protein enzyme - primarily localized at the presynaptic terminals and on axons with its ectodomain facing the extracellular space - and responsible for the cleavage of various peptides (Hellstrom-Lindahl et al., 2008). NEP is usually expressed in many normal tissues including the brush-border of intestinal and kidney epithelial cells, neutrophils, tyhomcytes, lung, prostate and brain, in this last case preferentially localized in the nigrostriatal and in the brain areas susceptible to amyloid plaque deposition, like the hippocampus (Wang et al., 2006a). IDE is a zinc metalloendopeptidase that hydrolyzes several peptides, such as insulin, glucagon, amylin and the APP intracellular domain (AICD) in addition to Aβ. Some studies described that although IDE is expressed in neurons it is also located in senile plaques in AD brain (Wang et al., 2006a). Caccamo and co-workers correlated NEP and IDE levels, reporting that their levels decrease in hippocampus with aging and raise in the cerebellum of older mice and moreover, that

NEP and IDE levels are higher in the cerebellum compared to AD vulnerable regions such as hippocampus and cortex in mice and humans. Authors also showed that IDE is more oxidized in the hippocampus compared to the cerebellum of AD patients (Caccamo et al., 2005). Taken together, these results underline the vulnerability of particular regions of the brain for Aβ accumulation with NEP and IDE levels and also the importance of aging in the levels of these Aβ degrading proteases.

Autoantibodies also assume an important role in Aβ clearance. They have been found in AD patients and healthy individuals, but tend to be reduced in the former. Basically, autoantibodies which exist in low levels prevent the Aβ neurotoxicity, blocking Aβ fibrils formation and disrupting formation of fibrilar structures (Brettschneider et al., 2005, Geylis et al., 2005).

Such as peptidases and autoantibodies, other substances can participate in Aβ clearance. Peripheral anti-Aβ antibodies and Aβ-binding substances are able to enter the brain, preventing Aβ aggregation and promoting its elimination. Furthermore, and by binding to peripheral Aβ, they can also promote the efflux of Aβ from the brain, contributing to Aβ clearance (Wang et al., 2006b). However, very recently, Walker and co-workers reported that the peripheral reduction of Aβ does not result in reduced levels of Aβ brain levels in mice. NEP was intravenously administered and resulted in a dose-dependent clearance of plasma Aβ. Nonetheless, this result did not correspond to reduced levels of soluble Aβ in the brain (Walker et al., 2013).

Soluble Aβ can also be removed via interstitial fluid bulk flow, into the bloodstream, but this type of clearance comprises only 10-15% of the total Aβ in the brain and circulating Aβ also can influx into the brain from the plasma (Wang et al., 2006b). So, the receptor-mediated transport of Aβ is the main responsible for the transport of Aβ across the blood-brain barrier (BBB) (Wang et al., 2006b). Thus, Aβ peptide burden is also a result of its flux across the brain through the action of receptors, namely: the lipoprotein receptor-related protein (LRP) and the receptor for advanced glycation end products (RAGE). LRP mediates the efflux of Aβ peptide from the brain into blood, through the endocytosis and transcytosis mechanisms. Obviously, failure in this mediation leads to an increase of Aβ deposition in the brain. Moreover literature indicated that expression of LRP is negatively regulated by Aβ levels (Wang et al., 2006b). If LRP mediates the efflux of Aβ peptide, the other receptor – RAGE – is the responsible for the influx of Aβ from blood to brain and is upregulated by excess amounts of Aβ in brain (Wang et al., 2006b). Thus, LPR and RAGE play opposite roles in Aβ transport across the BBB and, for instance, in AD patients LPR is downregulated and RAGE upregulated, at the BBB (Shibata et al., 2000).

5. The search for AD biomarkers

A definitive AD diagnosis can only be made by postmortem neurophatological examination; however it is consensual that a treatment for the disease can only be effective if we could early detect AD before the most aggressive symptoms. It is probable that the pathogenic process of AD starts decades before the first symptoms. The preclinical period characterized by a progressive cognitive impairment is designated as mild cognitive impairment (MCI) and this period may be determinant to the application of effective treatments. The annual conversion rate from MCI to AD is estimated to be around 15% (Petersen et al., 2001a). Due to the importance of an early AD diagnosis, the goal for the search of accurate and useful biomarkers for AD has evolved in last years.

5.1. CSF biomarkers

The direct contact with the brain makes CSF an important source of potential biomarkers in neurological diseases, and the crucial involvement of Aβ peptide and tau make them primary targets in AD.

Analysis of CSF Aβ42 levels shows a significant reduction in AD patients (<500 pg/ml) compared to controls (±700 pg/ml) (Humpel, 2011). It has been suggested that Aβ42 reduction in CSF is related to the reduced clearance of Aβ from brain to the blood/CSF and also to the enhanced aggregation and plaque deposition in the brain (Humpel, 2011). It has been also suggested that Aβ could be useful to an early diagnosis of AD, since reduced levels in CSF have been described in asymptomatic healthy elderly patients who develop AD in around 2 years (Chintamaneni and Bhaskar, 2012). However, and regarding to Aβ levels, some controversial data was found in the Aβ40 levels analysis in the CSF (unchanged or increased?). Therefore, rather than Aβ42 and Aβ40 levels alone, it has been suggested that the Aβ42/Aβ40 ratio can improve the accuracy of AD diagnosis. A decrease in this ratio in the CSF has been found in AD and this reduction seems more pronounced than the reduction of CSF Aβ42 alone (Fukuyama et al., 2000, Mehta et al., 2000, Hansson et al., 2007). Levels of total tau (T-tau) are also used in AD diagnosis, because it was reported that CSF T-tau levels are increased in AD patients (>600 pg/ml) as compared with healthy controls. Higher levels of T-tau in CSF (>3000 pg/ml) are also associated with another neurodegenerative disorder (Creutzfeldt–Jacob disease (CJD)) (Humpel, 2011). Similar to Aβ42 levels, T-tau levels might also be used as an early AD marker because increased CSF T-tau levels were reported in 90% of MCI

cases that evolved to AD (Blennow, 2004). Phosphorylated tau (P-tau) levels are also used in the diagnosis of AD. Several forms of P-tau have been studied and it has been found that P-tau levels are increased in AD patients compared to age-matched controls. Moreover, it has also been described that cognitive decline in patients with MCI correlates with CSF P-tau in threonine-231 levels and that MCI patients who convert to AD have higher P-tau levels compared to other MCI patients (Brys et al., 2009). The combination of these three CSF biomarkers (Aβ42,T-tau and P-tau) increases significantly the accuracy of AD diagnosis, with a sensitivity of >95% and a specificity of >85% (Humpel, 2011).

Great efforts to find other potential biomarkers in CSF have been made and as a result, other molecules appear as candidate markers to AD. Higher levels of cytochrome c in CSF were found in MCI patients who evolved to AD compared to MCI stable patients, discriminating these groups with a sensitivity and specificity of >75% (Papaliagkas et al., 2009). It was suggested that these increased levels of cytochrome c and its release from the mitochondria can result in the mitochondrial dysfunction and destruction that are correlated with oxidative stress in AD (Papaliagkas, 2013). β-secretase has been also reported as a candidate biomarker for AD because it was described that β-secretase levels in CSF were increased in AD patients and in MCI patients compared to controls (Zetterberg et al., 2008). Other study also reported that sAPP levels are increased in CSF in MCI patients who progressed to AD compared to MCI stable patients (Perneczky et al., 2011). Transthyretin (TTR) is another molecule that has emerged in recent years as a potential biomarker in AD, as several studies reported its reduction in CSF of AD patients (Riisoen, 1988, Merched et al., 1998, Puchades et al., 2003, Castano et al., 2006, Gloeckner et al., 2008) and also that this decrease seems to be specific for AD (Hansson et al., 2009)..

5.2. Plasma Biomarkers

Despite promising biomarkers in CSF, its collection is invasive and thus difficult to be a regular procedure in AD diagnosis. Plasma biomarkers are easily accessible and thus more attractive to be used as a routine procedure. However, nowadays it is unclear if its levels reflect the state of the disease, and moreover tau and Aβ levels in plasma are low and thus difficult to analyze. It was described that plasma Aβ42 levels are correlated with an increase in the risk of developing AD and that these levels decrease over time in newly diagnosed patients according to the concept of accelerated accumulation of Aβ42 in neuronal deposits as disease developments (Mayeux et al., 2003). However, studies on plasma Aβ levels, revealed inconsistence (Irizarry, 2004, Borroni et al., 2006, Freeman et

al., 2007) and one of the major difficulties in the detection and measurement of plasma Aβ is the nature of the species that are measured by the antibodies (Chintamaneni and Bhaskar, 2012). Pesaresi and co-workers described that a reduction of Aβ42 in plasma could be a marker for AD status in the transition from MCI to AD (Pesaresi et al., 2006). Nevertheless, another report showed that plasma Aβ42 is increased in early AD and that alteration in Aβ levels could indicate a transition from MCI to AD; however other authors argued that Aβ42 alone it is not a good biomarker (Blasko et al., 2008). Due to the large variability of person-to-person absolute Aβ levels, recent data show that the ratio of Aβ42 to Aβ40 is more suitable to confirm the diagnosis of probable AD and that the plasma Aβ42/Aβ40 ratio, similarly to what is observed in the CSF, has been shown to be a good biomarker to predict the conversion of MCI to AD (Graff-Radford et al., 2007). Additionally, a high concentration of Aβ40 in plasma, especially when combined with low concentrations of Aβ42 may indicate an increased risk of dementia (van Oijen et al., 2006). With little consistency, GSK-3 was also reported as a potential biomarker since it was shown that GSK-3 levels are increased in white blood cells in AD and MCI patients compared to controls (Hye et al., 2005). Recently, TTR levels in plasma were also analyzed and, supporting the data reported from CSF, decreased TTR levels were found in plasma from AD patients compared to age-matched controls (Han et al., 2011, Velayudhan et al., 2011).

5.3. Genetic Biomarkers

At present, the main biomarker for AD remains the presence of APOE ε4 allele. This is the most important genetic risk factor for sporadic AD. Epidemiologic studies showed that only about 20–25% of the general population carries one or more ε4 alleles, whereas 50–65% of people with AD are ε4 carriers. Furthermore, it was reported that the risk of developing disease is increased in carriers of ε4 allele and that homozygous ε4 allele carriers develop AD up to 10 years earlier than individuals who do not have this allele. However, the presence of this allele is not vital for the disease's manifestation. In contrast, the presence of ε2 allele seems to be protective in the development of AD (van der Flier et al., 2011).

6. Being women - A risk factor?

AD affects about twice as many women as men. It has been suggested that this fact is only related with the higher life expectancy of women than men (Hebert et al., 2001) but some studies also suggested that AD incidence is increased for women compared to men among the very old (Henderson, 1997). Sex steroid hormones, have been associated with the greater susceptibility for the disease of women and is known that these gradually decrease with age and that women in menopause suffer rapid declines in hormone production.

Ovariectomy was associated with increased brain Aβ levels in guinea pigs when compared to control animals. Moreover, the administration of estradiol results in decreased brain Aβ levels in these animals (Petanceska et al., 2000). Curiously, it was also described that mitochondria from young women are protected against Aβ toxicity compared to men, but this advantage is lost in mitochondria from old women, suggesting the importance of estrogens due to its ability to protect mitochondria against the toxicity of Aβ peptide (Vina and Lloret, 2010).

Data from a clinical trial involving 120 women described that estrogen replacement therapy (ERT) administered for one year did not prevent the progression of the disease nor improved cognitive functions in women with mild to moderate AD (Mulnard et al., 2000). On the other hand, another study investigated the effect of HRT on the association between the presence of APOE ε4 allele and telomere attrition and revealed that APOE ε4 carriers presented decreased signs of cell aging compared to the respective controls, that exhibited telomere shortening as predicted for the at-risk population (Jacobs et al., 2013). Other study evaluated the impact of 17β-estradiol in AD transgenic mouse model and described that mice treated with 17β-estradiol presented a reduction of Aβ42 levels and plaque burden (Amtul et al., 2010). 17β-estradiol was also associated with the expression of TTR by Quintela and colleagues. It was reported that TTR is up-regulated by 17β-estradiol and 5alpha-dihydrotestosterone in liver (Goncalves et al., 2008). The same authors also showed that 17β-estradiol and progesterone induce TTR expression in rodents' choroid plexus (Quintela et al., 2009, Quintela et al., 2011). Oliveira and co-workers also evaluated the impact of TTR in brain levels of sex hormones and concluded that TTR reduction/abolition influences brain levels of 17β-estradiol and testosterone in female mice (Oliveira et al., 2011).

TRANSTHYRETIN (TTR)

1. TTR – Structure

TTR is a plasma protein originally discovered in 1942.The first reference to the protein was made by Seibert and co-workers when they described a protein “X” with slightly mobility greater than albumin in plasma (Seibert and Nelson, 1942) and for this reason named pre-albumin. In the same year, Kabat et al. described the presence of a similar protein in the human cerebrospinal fluid (CSF) (Kabat et al., 1942). The name “transthyretin” reflects its principal function, which is the transport of thyroid hormone, thyroxine (T_4) (Woeber and Ingbar, 1968) and retinol, through binding to retinol-binding protein (RBP).

The TTR mRNA codifies for the TTR-monomer originating a polypeptide of 147 amino acids residues whose N-terminal region is a hydrophobic signal of 20 amino acids residues. The monomer is subjected to a cleaving process during its migration through the endoplasmatic reticulum giving rise to the native TTR monomer after breaking of the signal peptide (Soprano et al., 1985). Assembly of four identical subunits (13745 Da) occurs yielding the mature tetrameric protein with a molecular mass of 54980 Da (Kanda et al., 1974). Each monomer consists of 8 antiparallel β-strands (A through H) which are organized into two four-stranded β-sheets (DAGH-CBEF) and only a short α-helix located on β-strand E (Blake et al., 1978). As we can see in figure 5, a dimer is formed by the interaction between β-strands F and H of each subunit with hydrogen bonds. The tetramer formation results from the interaction between two dimers involving β-strands G to H and A to B (Blake et al., 1978, Hamilton and Benson, 2001).

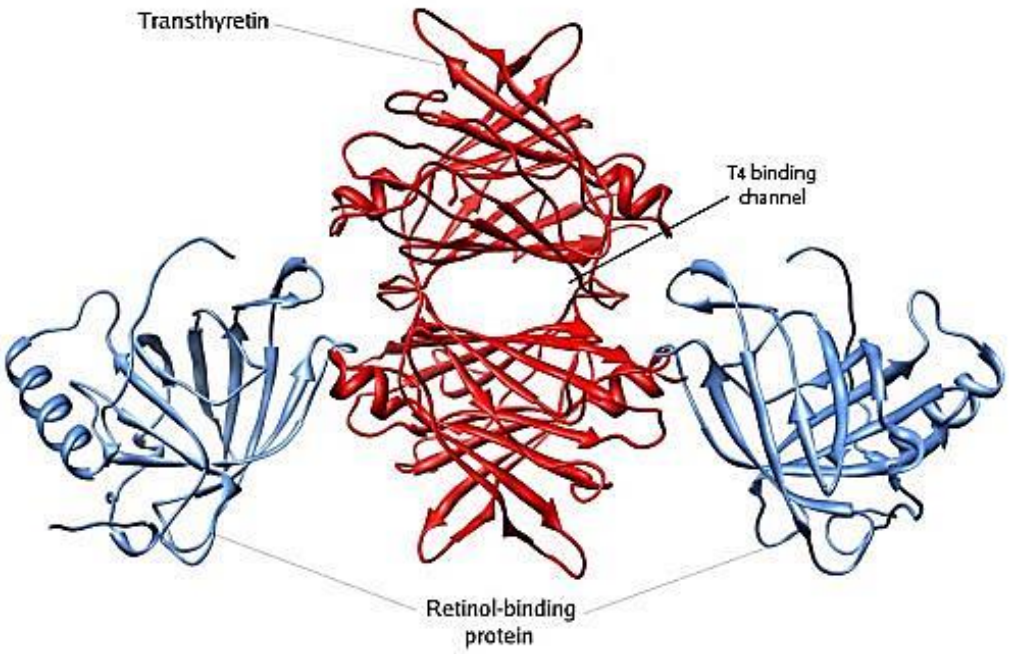

Figure 5 – The native state of human transthyretin (TTR) – tetramer form – with the representation of central channel where thyroxine (T4) binds and the representation of TTR/retinol-binding protein (RBP) complex (Monaco et al., 1995) (adapted).

TTR is an evolutionarily conserved protein that is found in many vertebrates' species including mammals, marsupials, birds, reptiles, amphibians and teleost fish (Schreiber and Richardson, 1997, Power et al., 2000). Additionally, sequences homologous to TTR were found in bacteria, nematodes and plants – transthyretin-like proteins (TLPs) and these TLPs were also able to form homotetramers in *Escherichia coli* and *Caenorhabditis elegans* but without the capacity to bind T_4 (Eneqvist et al., 2003).

TTR is mainly synthesized in the liver, responsible for the production of more than 90% of the protein. The remaining is produced in the choroid plexus and the retinal pigment of the eye (Soprano et al., 1985, Dickson and Schreiber, 1986). Animal experiments have shown considerable amounts of TTR mRNA in specific Central Nervous System (CNS) regions, varying from 11 to 30% of the hepatic levels (Soprano et al., 1985). A different study revealed that the highest TTR mRNA concentration occurs in the epithelial cells lining the ventricular surface of the choroid plexus. In spite of the low TTR levels in CSF (2 mg/dL), the choroid plexus is presented as the major site of TTR expression, expressed as a ratio of tissue/mass, corresponding to a 30-fold higher than that found in plasma. TTR represents 20% of the total CSF proteins (Weisner and Roethig, 1983).

2. The association between TTR and FAP

FAP is one of the examples of systemic amyloidosis and is associated with mutations in the TTR gene. It was first described by the portuguese Corino de Andrade in 1952 (Andrade, 1952). The disease was detected in the Portuguese population localized in the north of the country. FAP is a neurodegenerative disorder with onset between 20-35 years of age and histologically characterized by the presence of amyloid fibrils, especially through the peripheral nervous system (PNS) leading to organ dysfunction and ultimately, death. FAP is a hereditary autosomal dominant polyneuropathy, characterized by early impairment of temperature and pain sensation in the feet, and autonomic dysfunction leading to the paresis, malabsorption and emanciation. Motor involvement occurs as the disease develops causing wasting and weakness and there is a progressive loss of reflexes (Reilly and King, 1993). Symptoms usually start between the ages of 20-35 years and leads to a progressive and severe sensory, motor and autosomic polyneuropathy fatal in about 10 to 20 years (Reilly and King, 1993).

The association between TTR and FAP was established in 1978, when Costa et al., described TTR as the main protein in FAP fibrils (Costa et al., 1978). After this important discovery, Saraiva et al. identified a valine substituted by a methionine at position 30 (V30M TTR) in the protein isolated from Portuguese FAP patients (Saraiva et al., 1985). This mutation is the most frequent in Portugal and results from a point mutation in the exon of the TTR gene. It has also been identified in patients from different origins (Costa et al., 1978, Dwulet and Benson, 1984, Nakazato et al., 1984, Saraiva et al., 1985, Holt et al., 1989, Bhatia et al., 1993). After the identification of this mutant, others were identified in the TTR protein, and all are associated with different clinical phenotypes; For example, L55P TTR which is associated with one of the most severe forms of TTR-related amyloidosis (Jacobson et al., 1992, Yamamoto et al., 1994) and Y78F TTR, another aggressive mutation. Curiously, Almeida et al. described T119M TTR as an example of a non-aggressive mutation that inclusively has a protective role against the disease (Almeida et al., 2000). More than 100 TTR mutations were identified so far, most of them associated with amyloid deposition (Saraiva, 2001).

3. TTR Functions

3.1. Transport of thyroxine (T4)

Thyroid hormones (THs) are iodinated compounds essential for development, tissue differentiation and maintenance of metabolic balance in mammals. The thyroid gland is responsible by the synthesis of three THs: tetraiodothyronine (T_4), triiodothyronine (T_3) and a biologically inactive reverse T_3 (rT_3) (Oliveira et al., 2012). T_4 is the most abundant TH secreted by the thyroid gland and the majority of plasma T_4 circulates bound to TH binding proteins such as thyroxine-binding globulin (TBG), TTR and albumin, and only a small part of T_4 (0.03-0.05%) circulates in a free form (Bartalena, 1990). In humans it is reported that 65% of plasma T_4 is bound to TBG, 20% to albumin and 15% to TTR; in rodents 50% of total T_4 is carried by TTR. On the other hand, in CSF – in rodents and humans – TTR is the principal carrier of T_4 and transports about 80% of the total CSF T_4 (Oliveira et al., 2012).

TTR, in its homotetrameric structure, has two binding sites for T_4, located in the binding pocket formed by the central hydrophobic channel (Blake et al., 1974) (see figure 6). Despite presenting two T_4 binding sites, these channels have negative cooperativity and therefore only one molecule is transported by TTR. TTR also binds T_3, but with lower affinity (Andrea et al., 1980).

There is no consensus regarding the delivery of T_4 to cells. Firstly, it was proposed that TTR mediated T_4 transport into and within the brain, but Dickson and colleagues suggested that T_4 is transported from blood into the choroid-plexus where, by itself or bound to TTR, is secreted to CSF (Dickson et al., 1987). It has been also suggested that the ability of T_4 to passively cross the double lipid layer membranes is independent of the CSF TTR transport (Chanoine et al., 1992). Studies in mice (Episkopou et al., 1993) support the free T_4 tissue uptake hypothesis since a 50% decrease in total T_4 and normal free plasma hormone levels was reported in TTR null mice ($TTR^{-/-}$), when compared to wild-type ($TTR^{+/+}$) animals. Moreover, an increase in T_4 binding to TBG was observed although TBG levels were the same, suggesting a competition between TBG and TTR for T_4 binding. Authors concluded that $TTR^{-/-}$ mice are euthyroid in the absence of the major plasma T_4 carrier in rodents, suggesting that TTR is not essential to T_4 metabolism (Palha et al., 1994). Furthermore, in other reports authors showed that CSF of $TTR^{-/-}$ mice presented 30% lower levels of T_4 when compared to $TTR^{+/+}$ animals, but no differences were found in T_4 content in cortex, cerebellum or hippocampus, proposing that TTR is not

crucial for T_4 to reach brain and other tissues in this mutant (Palha et al., 1997). However, a more recent study described that the transport of T_4 from the CSF into the brain was TTR dependent (Kassem et al., 2006). Furthermore, Landers and co-workers also showed another important role for TTR in the transport of T_4 across the placenta and delivery to the fetus (Landers et al., 2009).

3.2. Transport of retinol

TTR is also responsible for the transport of retinol (vitamin A) through the binding to retinol-binding protein (RBP). In vertebrates, retinol is obtained from the diet, and retinol and its metabolites are associated with important physiological roles (Gudas, 2012). Retinol is transported by RBP, a 21.000 Da protein described firstly by Kanai and co-workers (Kanai et al., 1968). RBP is mainly synthetized in the liver and its binding to retinol promotes RBP secretion in plasma.

TTR-RBP complex is a very stable retinol transporting system to cells (Noy et al., 1992). Studies *in vitro* revealed that TTR tetramer has four RBP-binding sites, two in each dimer at surface of the protein. Due to the steric hindrance, only two RBP molecules are carried by each TTR molecule (see figure 6) (Monaco et al., 1995). Raz and co-workers described that the binding capacity and also affinity of TTR for T_4 is not affected by the presence or absence of RBP (Raz and Goodman, 1969).

As in the case of T_4 transport by TTR, studies in $TTR^{-/-}$ mice were important to better understand the importance of TTR in the transport of retinol. $TTR^{-/-}$ mice showed a reduction of 95% in retinol and RBP plasma levels when compared to $TTR^{+/+}$ animals (Episkopou et al., 1993). Van Bennekum and colleagues justified this reduction by the increase of renal filtration of the retinol-RBP complex (van Bennekum et al., 2001). Increased hepatic RBP levels were also found in $TTR^{-/-}$ mice (Wei et al., 1995). Nonetheless, van Bennekum et al. showed that RBP liver secretion to plasma was unaltered, suggesting that decreases of RBP levels and retinol in plasma are not related with a failure of secretion (van Bennekum et al., 2001). Furthermore, the symptoms normally associated with the absence of vitamin A were not observed in $TTR^{-/-}$ mice, such as infections and eye abnormalities or/and loss of weight suggesting that TTR does not have an essential role in retinol metabolism, in mice (van Bennekum et al., 2001).

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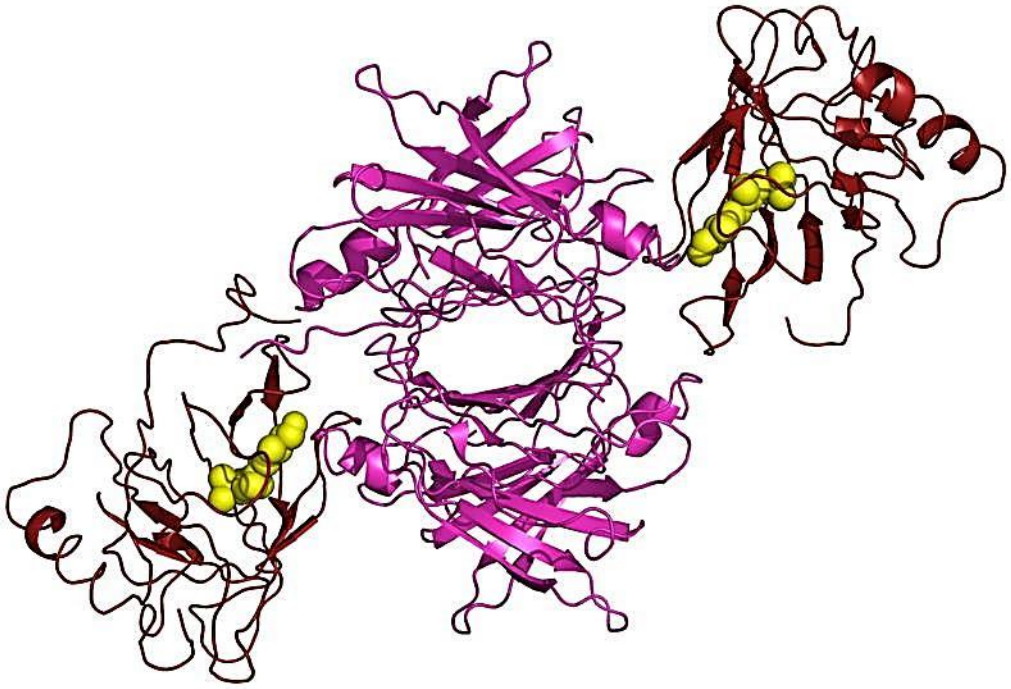

Figure 6 - The three dimensional crystal structure of the retinol–RBP–TTR complex. In pink it is plot the TTR tetramer, linked with two RBP molecules (red) each one with a retinol molecule (yellow) inside. The RBP binding sites are independent of TH-binding sites, located in the central of TTR channel (Berry and Noy, 2012).

4. TTR as a protease

Besides the function as a carrier for T_4 and T_3 and the retinol transport through RBP, another TTR function was described. Liz et al. showed that TTR is a cryptic protease: authors showed that, when TTR was incubated with apoA-I, a major apoA-I fragment of 26kDa was observed, suggesting for the first time that TTR might be a protease able to use apoA-1 as a substrate, concluding by Maldi-MS that the cleavage occurred in the C-terminus of protein (Liz et al., 2004, Liz et al., 2007). TTR is also able to cleave lipidated apoA-I (mainly in the lipid poor pre β-HDL subpopulation) which can be relevant in the lipoprotein metabolism. Moreover, apoA-I cleaved by TTR showed less ability to promote cholesterol efflux. Additionally, this study showed that TTR increased apoA-I amyloidogenicity *in vitro*, because the apoA-I cleaved by TTR had a higher propensity to form aggregates (Liz et al., 2007). More recently, Costa et al. showed for the first time that TTR can cleave Aβ peptide *in vitro*, suggesting TTR proteolytic activity as a mechanism for Aβ clearance. Moreover, Costa and co-workers demonstrated that newly generated Aβ peptides presented reduction of amyloidogenic potential, when compared to

the full counterpart (Costa et al., 2008, Sciarrone et al., 2008). Liz et al. also described that TTR was able to cleave amidated neuropeptide Y (NPY) and that its proteolytic activity affects axonal growth, leading to the conclusion that TTR has natural substrates in the nervous system (Fleming et al., 2009, Liz et al., 2009). Newly, the same authors described TTR as a metallopeptidase (Liz et al., 2012) and this result was supported by another study that showed the involvement of a carboxylate and an ammonium group, possibly from a lysine side chain, in the TTR hydrolytic activity (Gouvea et al., 2013).

5. TTR in the nervous system

$TTR^{-/-}$ mice are viable, fertile and phenotypically similar to $TTR^{+/+}$ animals (Episkopou et al., 1993). However, the absence of TTR was associated with increased exploratory activity and reduced signs of depressive-like behavior, probably due to the modulation of noradrenergic system by the increase of noradrenaline in the limbic forebrain (Sousa et al., 2004). Increased levels of NPY – a antidepressant neurotransmitter - (Heilig, 2004) in dorsal root ganglia (DRG), sciatic nerve, spinal cord, hippocampus, cortex and CSF were also found in $TTR^{-/-}$ mice (Nunes et al., 2006). These alterations may be a consequence of up-regulation of Peptidylglycine alpha-amidating monooxygenase (PAM) (Nunes et al., 2006). Taken together, these results underlie the importance of TTR in the modulation of depressive behavior. Furthermore, Sousa et al. also described that $TTR^{-/-}$ mice presented memory impairment when compared with $TTR^{+/+}$ animals. Authors described that the absence of TTR accelerate cognitive deficits usually associated with aging (Sousa et al., 2007).

Additionally, TTR was associated to nerve regeneration. Fleming and co-workers showed, for the first time, that TTR can act as a nerve regeneration enhancer. They observed that $TTR^{-/-}$ mice presented a decrease in regeneration of a sciatic crushed nerve. On the other hand, in animals with the same $TTR^{-/-}$ background but with expression of TTR in neurons, this phenotype was rescued, emphasizing the importance of TTR in nerve regeneration (Fleming et al., 2007).

Recently, TTR was also associated with ischemia, one of the major causes of brain injuries worldwide. Santos and co-workers described that in a compromised heat-shock response, CSF TTR contributes to control neuronal cell death, edema and inflammation, influencing the survival endangered neurons (Santos et al., 2010). Other authors reported that, in young rats subjected to focal cerebral ischemia, TTR was one of

differentially expressed proteins in plasma (Chen et al., 2011). Moreover, in a rat model of transient middle cerebral ischemia artery occlusion, monomeric form of TTR was increased, suggesting that monomeric TTR may represent an ischemia-specific CSF marker to specify the sequential changes according to ischemia insults of the brain (Suzuyama et al., 2004).

ALZHEIMER'S DISEASE AND TRANSTHYRETIN

1. Aβ sequestration

There is growing evidence of a protective role of TTR in AD, and also the importance of the modulation of the interaction between Aβ and TTR.

The report of Schwarzman and colleagues became determinant for the discussion of the relationship between Aβ and TTR. For the first time, they showed the importance of TTR in the Aβ sequestration, demonstrating that TTR is the major Aβ sequestering protein in CSF and that TTR binding to Aβ prevents amyloid formation. Thus, authors suggested that a failure in Aβ sequestration may result in Aβ aggregation and that this failure could be explained by: the abnormal production of Aβ, the reduction of sequestering proteins or the incapacity of those proteins to bind Aβ peptide (Schwarzman et al., 1994, Schwarzman and Goldgaber, 1996). In addition to TTR, other proteins were already associated with Aβ sequestration such as Apoliprotein E (ApoE), Apoliprotein J (ApoJ) and Gelsolin (Ghiso et al., 1993, Goldgaber et al., 1993, Schwarzman et al., 1994, Chauhan et al., 1999).

2. TTR/Aβ interaction

The discussion on the TTR interaction with Aβ and consequent inhibition of aggregation and toxicity raised the hypothesis that mutations in the TTR gene or conformational changes in the protein induced by aging, could affect the sequestration properties (Palha et al., 1996). Experiments performed allowed to conclude that there is no correlation between the presence of mutations in TTR and AD pathology (Palha et al., 1996). Regarding the structural nature of the TTR/Aβ interaction some information was

obtained from computer-assisted modeling (Schwarzman et al., 2004). This model predicts the existence of an Aβ binding domain on the surface of each TTR monomer. Other studies by the same group reported that residues 30-60, especially the 38-42 region of TTR, are the key structure of the binding domain to Aβ (Schwarzman and Goldgaber, 1996, Schwarzman et al., 2005). Moreover, and more recently, Du and Murphy identified the A strand, in the inner β-sheet of TTR, as well as the EF helix, as regions of TTR involved in the Aβ interaction (Du and Murphy, 2010).

Additionally, several authors confirmed Aβ as a TTR ligand with different Aβ species such as soluble, oligomeric and fibrillar forms binding to TTR (Liu and Murphy, 2006, Sciarrone et al., 2008). Costa and co-workers also described that TTR was able to inhibit and to disrupt Aβ fibrils (Sciarrone et al., 2008). Understanding how TTR binds to Aβ is now an important discussion on the field. Some authors argued that TTR bind preferably to Aβ aggregates rather than Aβ monomers (Du and Murphy, 2010).

Other studies reported that Aβ binds to different TTR variants correlating negatively with the amyloidogenic potential of the TTR mutant , showing that T119M TTR presented the highest affinity to Aβ (Sciarrone et al., 2008, Du and Murphy, 2010). Additionally, Schwarzman and colleagues also pointed to the relationship between TTR variants/amyloidogenic potential and binding to the peptide. *In vitro* studies, using different TTR mutations, concluded on the differential binding to Aβ and inhibition of aggregation of the peptide by those variants (Schwarzman et al., 2004). Recently, a novel mechanism of action for TTR was suggested. Yang and co-workers, reported that the EF helix/loop described above, “senses” the presence of soluble toxic Aβ oligomers causing the TTR tetramer destabilization and the consequent exposure of the hydrophobic inner sheet which permits the scavenging of toxic oligomers (Yang et al., 2013).

3. TTR role in AD – *in vivo* evidences

In vivo experiments on the importance of TTR in AD started with the study of Link (Link, 1995). For the first time researchers used a transgenic model (Caenorhabditis elegans) expressing human Aβ42 demonstrating neurodegeneration; Double transgenic strains for Aβ and TTR were generated to evaluate the role of TTR expression in Aβ aggregation and the results suggested a reduction in the number of positive Th S (thioflavine S) deposits (Link, 1995).

Studies with transgenic mice overexpressing mutant APP showed an increase in TTR expression in the hippocampus, a slow disease progression and lack of

neurodegeneration (Stein and Johnson, 2002). The statement of “TTR increase in the hippocampus” is controversial because, until this work, the literature only reported TTR expression in choroid plexus and meninges of the brain (Sousa and Saraiva, 2008). Nevertheless, authors reported that when a chronic infusion of an antibody against TTR was administered into the hippocampus an increase of Aβ, tau phosphorylation, neuronal loss and apoptosis was observed (Stein et al., 2004). The hypothesis is that, the α-secretase cleaves APP and its product (sAPPα) leads to an increase in the expression of protective genes, like TTR, in order to confer neuroprotection (Stein et al., 2004). Lazarov and colleagues reported that transgenic mice exposed to an "enriched environment" presented a reduction in brain Aβ levels and amyloid deposits compared to animals under “standard housing” conditions, this being associated with altered expression of several genes including upregulation of TTR (Lazarov et al., 2005). Furthermore, Choi and co-workers showed that Aβ deposition is significantly accelerated in the hippocampus and cortex in an ADTTR$^{+/-}$ transgenic mice (Choi et al., 2007).

TTR protective role *in vivo* was also demonstrated in APP transgenic mice overexpressing human TTR (Buxbaum et al., 2008). More recently, Oliveira and co-workers reported a gender-associated modulation of brain Aβ levels and brain sex steroid hormones by TTR (Oliveira et al., 2011). On the other hand, data by Waigi and Doggui from different AD transgenic mice strains led to conclusions that do not support a neuroprotective role of TTR in AD (Wati et al., 2009, Doggui et al., 2010).

Interestingly, in humans, TTR levels appear to be in agreement with the idea of TTR protection in AD, as it is described that CSF TTR is reduced in AD (Serot et al., 1997, Castano et al., 2006, Gloeckner et al., 2008). Furthermore, and more recently evaluation of serum/plasma TTR levels in AD also showed that the protein is reduced in AD patients as compared to non-demented controls (Han et al., 2011, Velayudhan et al., 2011), although the reason underlying this alteration and if TTR decrease is a cause or a consequence of the disease, are not known.

4. TTR Stability – A key factor

In 1993, the discussion involving TTR stability and its importance in neurological diseases was launched by McCutchen and colleagues, when they suggested that mutated L55P TTR significantly alters tetramer stability and increases amyloidogenicity. The observation that L55P TTR tetramer was more unstable than the WT TTR and that the ability of L55P TTR to denature to the amyloidogenic intermediate at pHs where the WT protein was stable, could be an explanation to understand why the TTR variant forms, easily, amyloid fibrils *in vitro* and *in vivo*, while the WT TTR remained stable and non-amyloidogenic (McCutchen et al., 1993). Later studies reinforced the importance of TTR mutations in their tendency to form aggregates. Quintas and co-workers showed that the tendency to TTR form aggregates correlates with their known amyloidogenic potential, also describing that the non-amyloidogenic mutant T119M TTR presents the highest stability to dissociation (Quintas et al., 1997). These results, supports the work performed by Longo Alves and co-workers which firstly concluded that T119M TTR presented higher stability to dissociation than V30M TTR mutant (Longo Alves et al., 1997). In the same year, Almeida and colleagues showed that the presence of T119M TTR significantly increased TTR-T_4 binding, while V30M TTR presented a very low affinity to the hormone, highlighting the stability concept through T_4 binding (Almeida et al., 1997). In fact, the T119M TTR crystal structure, later known, showed new H-bonds within each monomer and monomer-monomer inter-subunit contacts (S117-S117 and M119-Y114) that results in the improved of protein stability, possibly leading to the protective impact of the V30M/T119M TTR (Sebastiao et al., 2001). Moreover, this mutation is located in the H strand and participates in the binding to T_4, resulting in increased affinity for the hormone.

Based on these results, several authors showed that small compounds sharing molecular structural similarities with T_4 are candidate ligands for the T_4 binding sites, suggesting these compounds as inhibitors of fibril formation *in vitro* (Baures et al., 1998, Oza et al., 2002). These compounds mostly belong to the class of nonsteroidal and anti-inflamatory drugs and include molecules such as the derivative of diflunisal (iodo-diflunisal - IDIF). IDIF, contrarily to other proposed TTR stabilizers (diclofenac, fluofenamic acid and diflunisal) efficiently inhibited TTR aggregation. Moreover, IDIF was also described as the most potent inhibitor of T_4 binding to TTR and also as the most potent stabilizer of TTR tetramer (Almeida et al., 2004). One year later, Gales and co-workers, presented the crystal structure of TTR complexed with the diflunisal derivate and described that IDIF binds very deep in the hormone-binding channel. They showed that the iodine substituent is tightly anchored into a pocket of the binding site and the fluorine atoms provide extra hydrophobic contacts with the protein. Their analysis proposed that the carboxylate

substituent is involved in an electrostatic interaction with the N of a lysine residue. The author suggested that the new interactions induced by IDIF increase the stability of the tetramer impairing the formation of amyloid fibrils (Gales et al., 2005). In 2007, Cardoso et al. described that [2-(3,5-dichlorophenyl) amino] benzoic acid (DCPA) and tri-iodophenol (TIP) were the best stabilizers of V30M tetramers in plasma from carriers of this mutant, and clearly inhibit aggregation in a cellular system (Cardoso et al., 2007). According to the importance of TTR stabilization, other *in vitro* and *in vivo* reports showed that small compounds that can stabilize TTR tetrameric fold could be used in the treatment of other amyloidosis such as FAP (Ferreira et al., 2011, Coelho et al., 2012, Ferreira et al., 2012b, a)

With regard to the influence of TTR mutations in the TTR/Aβ interaction, Costa et al. described that TTR variants such T119M, Y78F, V30M and L55P that bound differently to Aβ peptide, in the following order: T119M>WT>V30M≥Y78F>L55P. Consequently, they suggested that the ability to rescue Aβ is different between them, with obvious implications in the deposition in the brain. The observation of an inverse relationship between TTR amyloidogenic potential and binding to Aβ, lead the authors to suggest the TTR stability as a factor affecting this interaction. If so, stabilization of TTR should result in increased relation affinity to the peptide (Sciarrone et al., 2008).

Very recently, Hornstrup and co-workers reported an association between genetic stabilization of TTR and a reduction in the risk of developing cerebrovascular disease and an increase in life expectancy. Researchers concluded that plasma TTR and T_4 levels were increased in heterozygotes for T119M TTR versus noncarriers patients. Importantly, the incidence of cerebrovascular disease was decreased and live expectancy increased (Hornstrup et al., 2013).

Together, these results demonstrate the importance of TTR stability in the design of new therapeutic strategies and also that it is essential to further explorer the stability concept associated with TTR/Aβ binding in AD.

CONCLUDING REMARKS

Fundamental research is essential to unravel the mysteries of science and in AD the efforts are quite large. At the same time, AD is a complex disease and the particular interests of different researchers can be quite different; we focused our attention in the role of TTR in AD.

Literature shows us that it is very likely that TTR plays an important role in AD as suggested by its decrease in CSF. It has also been reported that TTR is lowered in plasma however, there is no information about what happens in the early stages of AD and also if there are differences in plasma TTR levels regarding gender. The behavior of TTR in plasma and the possibility to be used as a biomarker is obviously of particular interest. Further efforts, are necessary to better characterize TTR alterations in this fluid, in the AD context.

The involvement of TTR in AD as a protective molecule is also supported by *in vitro* and other *in vivo* data which indicates that TTR modulates Aβ aggregation and toxicity. However, the mechanism underlying TTR protection is not known and can go from simple binding and arresting of the peptide, to proteolysis, hormone involvement and others. The factors affecting TTR/Aβ interaction are not identified yet, and those might be determinant for the design of TTR-based therapies. The work that will be described in the next sections addressed aimed at answering some of these questions related to the role of TTR in AD.

RESEARCH PROJECT

OBJECTIVES

The main aims of this project were to study how TTR exerts its protective role in AD, to identify factor(s) involved in the modulation of the TTR/Aβ interaction, and also to find ways which can improve this interaction, while designing therapeutic strategies to prevent or slow down AD.

The designed experiments had the following goals:

1. To investigate mechanisms for AD modulation by TTR:

    a) ascertain TTR plasma levels in aMCI and in AD patients and correlation with disease state, gender and age;

    b) assess alterations in TTR activity/functionality in aMCI and AD patients.

2. To investigate TTR stability as a main factor in the TTR/Aβ interaction:

    a) probe for differences in binding to Aβ peptide by different TTR variants, using sensitive assays;

    b) investigate the effect of TTR stability in its interaction with Aβ peptide, by screening for compounds known as TTR tetrameric stabilizers;

    c) confirm *in vivo* the importance of TTR stability in TTR/Aβ interaction and in its protective role in AD, using an AD mouse model.

CHAPTER I

Transthyretin decrease in plasma of MCI and AD patients: investigation of mechanisms for disease modulation

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Running Title: Plasma TTR is decreased in MCI and AD patients

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Abstract

Different authors described that transthyretin (TTR) is decreased in the cerebrospinal fluid (CSF) of Alzheimer's disease (AD) patients and thus TTR is a potential CSF biomarker in AD. However, descriptions of what happens to TTR in plasma of these patients are lacking in the literature. We investigated TTR levels in plasma samples from 55 patients with mild-cognitive impairment (MCI), 56 patients with AD and 41 non-demented controls, and found that TTR is decreased in both MCI and AD groups, suggesting that TTR might be used for staging early AD. In MCI and AD groups, women showed significantly lower plasma TTR levels when compared to MCI and AD men, respectively, and to women control group. In the AD women group, TTR levels correlated with disease stage, reflecting disease severity. Although MCI and AD men groups presented TTR levels lower than men in the control group, the difference was not statistically significant. Genetic analysis for ApoE revealed no relationship between TTR levels and the presence of the ε4 allele, for both men and women, in both patient groups. Importantly, we assessed thyroxine binding to TTR in plasma and found, in both MCI and AD groups, that TTR had reduced capacity to carry the hormone. Finally, we measured plasma estradiol levels in women and showed a reduction in both groups. Thus, this study prompts TTR as an early plasma biomarker in AD indicating that disease modulation by TTR is gender dependent; this study provides hypotheses into the mechanisms involved.

Keywords: Alzheimer's disease, ApoE, biomarker, disease severity, estradiol, plasma, transthyretin, thyroxine.

Introduction

Subtle losses of cognitive function can result from normal aging but can also be part of a diagnosis, as in mild cognitive impairment (MCI), in which subtle loss of memory occurs. These alterations are not part of normal aging and even though MCI patients are more forgetful than others in the same age group, their ability to participate in daily activities is not significantly affected. Alterations characteristic of MCI may also represent a transitional state to early Alzheimer's Disease (AD) and are frequently referred to as an amnesic mild cognitive impairment (a-MCI), and therefore, this condition is now recognized as a risk factor for AD (El Sankari et al., 2011). The annual conversion rate from a-MCI to AD is estimated to be 10-15%, compared with 1-2% seen in healthy, non-impaired control subjects (Petersen et al., 2001a).

Aβ peptide, formed through the sequential cleavage of the amyloid precursor protein (APP) by β and γ-secretases, is viewed as the causative agent in AD. Its accumulation has been explained by an imbalance between its production and degradation, although much less is known regarding the latter. ApoE plays an important role in AD and the risk of developing AD is increased in carriers of the ApoE ε4 allele: individuals with one and two copies of the ε4 allele have a 45% and 50–90% probability of developing AD, respectively (Corder et al., 1993). ApoE acts normally to scaffold the formation of high density lipoprotein (HDL) particles which promote the proteolytic degradation of soluble forms of Aβ (Sparks, 2007). Aβ and its precursor protein APP play important roles in neuronal functions. Therefore, control of physiological Aβ levels, rather than complete inhibition seems to be an important strategy to reduce the accumulation of neuritic plaques and thus slowing down the progression of AD (Malito et al., 2008).

Several molecules have been suggested as Aβ carriers (Schwarzman et al., 1994, Chauhan et al., 1999). Among them transthyretin (TTR) which has recently received large attention has been suggested to bind the Aβ peptide, hence avoiding its accumulation, aggregation and toxicity (Schwarzman and Goldgaber, 1996, Sciarrone et al., 2008). TTR is a 55kDa homotetrameric protein produced mainly in the liver and choroid plexus of the brain, being secreted into the plasma and cerebrospinal fluid (CSF), respectively (Soprano et al., 1985). In human plasma, TTR, thyroxine-binding globulin (TBG) and albumin are responsible for the delivery of thyroxine (T_4) into target tissues (Robbins, 1991). Although TBG is much less concentrated in the plasma than TTR, it presents the highest affinity constant for T_4 and transports about 70% of plasma T_4. TTR has an intermediate affinity for T_4 transporting about 15% of the hormone, whereas albumin presents the lowest binding affinity (Robbins, 1991, Loun and Hage, 1992). TTR is also responsible for retinol transport through binding to retinol binding protein (RBP). TTR mutations are associated

with Familial Amyloid Polyneuropathy (FAP), a systemic amyloidosis with a special involvement of the peripheral nerve (Benson and Kincaid, 2007) and, more recently, TTR was shown to have proteolytic activity (Liz et al., 2004, Costa et al., 2008).

The observation that TTR is reduced in the CSF of AD patients, supporting the notion of a TTR protective role in AD, has been done by several reports along time (Serot et al., 1997, Gloeckner et al., 2008, Hansson et al., 2009). Nonetheless, it remains unclear whether this reduction is restricted to AD or, on the contrary, is common to other neurodegenerative disorders as described for normal pressure hydrocephalus (Gloeckner et al., 2008). Furthermore, this issue is still controversial as some authors did not detect differences in TTR levels (Abdi et al., 2006) while others observed increased levels of the protein (Davidsson et al., 2002, Chiang et al., 2009), in the CSF of AD patients.

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. Due to the characteristics of AD, CSF has been obviously used as the primarily source for TTR measurements. Nevertheless, a plasma biomarker is highly recommended and attractive, with obvious advantages. TTR behavior in sera, in the AD context, has not yet been fully explored and, although some attempts have been made, new studies are necessary. Recently, Han and collaborators described decreased sera TTR levels in AD patients but they observed no differences regarding gender, in the patient groups, while significant differences were found between men and women in the control group (Han et al., 2011). On the other hand, in a different study Velayudhan *et al.* (Velayudhan et al., 2011) also described that plasma TTR is diminished in AD patients, but no association with gender was attempted. Most importantly, none of the reports found in the literature ever explored factors that modulate TTR binding to Aβ nor the mechanism(s) responsible for TTR decrease. For instance, it was reported that the age-adjusted AD incidence is higher in women compared with men, mainly in advanced ages (Kaplan, 2006) and it has been suggested that estrogens may play a relevant role in this process (Candore et al., 2006). It has also been established that TTR expression, in the choroid plexus, is regulated by 17β-estradiol (Quintela et al., 2009) and, hence, this relationship in AD needs to be explored. Furthermore, whether TTR decline occurs early, before disease development, or after the pathology is established and advanced, is not known; this information may be relevant for diagnosis and even for treatment purposes, and thus must be addressed.

In this work, we investigated plasma TTR levels in aMCI and AD patients at different disease stages and evaluated factors that may affect TTR levels, such as gender, age, hormones and TTR ligands.

Material and Methods

Patient and control subjects

The patient population was recruited at the Dementia Clinic, Neurology Department of Coimbra University Hospital: 55 MCI patients and 56 AD patients matched for age and gender. All patients underwent a thorough biochemical, neurological and imaging (CT or MRI and SPECT) evaluation. PET, CSF analysis, and genetic studies were more restricted, although considered in younger patients. All patients were in a stable condition, without acute co-morbidities. Cognitive and functional status was assessed by the Mini Mental State Examination (MMSE) (Folstein et al., 1975), Portuguese version (Guerreiro, 2003a), Alzheimer's Disease Assessment Scale – Cognitive (ADAS-Cog)(Mohs et al., 1983, Rosen et al., 1984), Portuguese version (Guerreiro, 2003b), the Clinical Dementia Rating Sale (CDR) (Berg, 1988) and a comprehensive neuropsychological battery with normative data for the Portuguese population (BLAD) (Guerreiro, 1988) exploring memory (Wechsler Memory Scale sub-tests) and other cognitive domains (including language, praxis, executive functions and visuo-construtive tests). Based on these instruments, patients were classified as probable AD or amnestic MCI (aMCI). The diagnosis of AD was based on the guidelines of the Diagnostic and Statistical Manual of Mental Disorders – fourth edition (DSM-IV-TR) (Association, 1994) and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) (McKhann et al., 1984). These patients were further classified into 3 stages in accordance with the CDR score: 32 AD patients were classified as mild AD (class 1.0 of the CDR scale), 15 as moderate AD (CDR=2.0) and 12 as severe AD (CDR=3.0). aMCI diagnosis was made in accordance with the criteria defined by Petersen (Petersen et al., 2001b), and additionally all these patients were classified as class 0.5 of the CDR scale. This study was approved by the Ethics Board of Coimbra University Hospital and all subjects or responsible caregivers, whichever appropriate, gave their informed consent. The control group consisted of 41 healthy subjects, recruited at LabMed Center, matched for age and gender, with no neurological or psychiatric history and without cognitive impairment. All subjects were informed on the purpose of the study and gave their written consent. All women analysed in this study, with the exception of a single AD women, were not subjected to any treatment involving hormone replacement therapy (HRT).

Blood collection and plasma separation

Blood samples were collected into EDTA tubes. Blood was immediately centrifuged at 2000 rpm for 15min, at 4^{o}C, and the supernatant was collected and stored frozen at –80^{o}C until analysis.

Radial immunodiffusion method

A human prealbumin bindarid, radial immunodifusion (RID) kit (Binding Site), was used to determine the concentration of TTR in human plasma. A calibrator plus two dilutions were used to produce a linear calibration curve, and 5 µl of each sample were added into each well. The precipitation rings were allowed to develop for 72 hours at room temperature. After that, the diameter of the rings was measured and the concentration of TTR was determined using the calibration curve. Data were expressed in mg/L.

ApoE genotyping

Patients were genotyped for ApoE status. Briefly, DNA was isolated from whole blood using a commercial kit (Roche Diagnostics GmbH, Manheim, Germany), as described by the manufacturer. ApoE genotype was determined by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) assay, as previously described (Crook et al., 1994).

TTR-T_4 binding

Qualitative studies of the displacement of T_4 from plasma TTR were carried out by incubation of 5 µl samples of whole plasma (control and AD patients) with [^{125}I]T_4 (specific radioactivity 1250 µCi/µg; concentration 320 µCi/ml; Perkin Elmer, Boston, MA, U.S.A.), followed by protein separation by a Native PAGE gel in a glycine/acetate buffer system. Each gel lane was cut in 1mm slices and counted using a gamma counter (Wallac 1470 wizard™). Results were shown as the ratio of ^{125}I-T_4-TTR/^{125}I-T_4 total bound.

Plasma estradiol levels

Quantification of female plasma estradiol levels (18 MCI patients, 22 AD patients and 20 control subjects) was performed by using Cayman's Estradiol EIA Kit (Cayman Chemical Company, USA), according to manufacturer's instructions. This assay is based on the competition between estradiol and an estradiol-acetylcholinesterase (AChE) conjugate (Estradiol Tracer) for a limited amount of Estradiol Antiplasma. Data were expressed in pg/ml.

Statistical analysis

All data were expressed as mean±SEM. D'Agostino and Pearson test were used to evaluate normal distributions. The differences in plasma TTR levels, T_4 binding to TTR and estradiol levels were analyzed using one way analysis of variance (ANOVA) with Tukey's and Bonferroni's post-hoc tests. The relationship between plasma TTR levels and the corresponding ApoE genotyping was analyzed using a T-Test analysis. The effect of age, and the correlation between TTR and T_4 binding capacity, or TTR and estradiol levels was analyzed using Pearson's correlation coefficient. A $p<0.05$ was considered significant for all analyses. GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com, was the statistical software used.

Results

Plasma TTR levels are decreased in aMCI and AD

In this study we used 55 aMCI and 56 AD patients and 41 control subjects. The mean age of aMCI patients, AD patients and control subjects was 71.6 ± 1.2, 73.6 ± 1.4 and 74.2±0.8 years, respectively (see table 1 for a complete characterization). The values presented for TTR were obtained with the RID method (table 2). Our study indicated that TTR is decreased in plasma of aMCI and AD patients when compared to age-matched controls (figure 1A), suggesting that alterations in TTR concentration precede the diagnosis of probable AD, hence prompting TTR as an early biomarker in AD. No significant differences were detected between the two patient groups.

In the control group, no differences were found regarding gender (figure 1B) and age (table 3); although plasma TTR levels in control men were higher than in control women, the difference was not statistically significant. However, in the patient groups, analysis by gender indicated that in women, both in aMCI and AD groups, plasma TTR was significantly decreased when compared to the aMCI and AD men, respectively (figure 1B). Moreover, TTR levels were significantly lower in aMCI and AD women than in those from the control group (figure 1B). Additionally, analysis of TTR amongst AD women according to disease stage, indicated that TTR levels were decreased in the moderate/severe cases, when compared to the mild ones (figure 1C), suggesting that TTR reflects disease severity; however, no correlation was found between TTR and the MMSE score (AD, p=0.86; MCI, p=0.29) (not shown). Moreover, there were no age-related changes in plasma TTR levels in women groups (table 3). In contrast, the aMCI and AD men groups revealed no significant changes in TTR levels relative to control groups, although a trend was observed (figure 1B). Also, no association between TTR levels and disease severity or MMSE score was observed (figure 1C). Curiously, a negative correlation was observed between TTR levels and age in the AD men group (table 3), suggesting that although TTR modulates the disease, the modulation is different for the two genders. Taken together, the female gender is the main contributor to the observed TTR decrease in the aMCI and AD samples.

Genetic analysis for ApoE showed that the ε4 allele was present in approximately 50% of the aMCI and AD cases (table 1), but we did not find any correlation between this allele and TTR levels (p=0.37) (not shown). This result suggests that, although the presence of ApoE ε4 is an important risk factor in late onset AD and even though TTR participates in the mechanisms affected in this disorder, the two molecules do not relate together in AD and are probably involved in different pathological pathways.

Table 1- Demographic and clinical characterization of control and patients subjects.

	Control			aMCI			AD		
	All	**Male**	**Female**	**All**	**Male**	**Female**	**All**	**Male**	**Female**
Age (years)	74.2±0.8 (n=41)	74.2±1.1 (n=18)	74.1±1.2 (n=23)	71.6±1.2 (n=55)	72.5±1.9 (n=24)	71.0±1.8 (n=31)	73.6±1.4 (n=56)	70.3±1.9 (n=26)	76.0±1.9 (n=30)
ApoE genotyping	NA	NA	NA	50.9% ε4	58.3% ε4	44.8% ε4	52.9% ε4	50.0% ε4	55.6% ε4
MMSE score	NA	NA	NA	26.6±0.4	27.3±0.6	26.1±0.6	17.7±0.8	17.2±1.4	18.1±1.0

NA- non-applicable

Table 2 – TTR concentration, TTR-T_4 binding and estradiol levels in control, aMCI and AD and samples.

	Control			aMCI			AD		
	All	**Male**	**Female**	**All**	**Male**	**Female**	**All**	**Male**	**Female**
TTR (mg/L)	237.3±7.5 (n=41)	251.2±10.5 (n=18)	226.5±10.2 (n=23)	201.9±7.6 (n=55)	226.6±11.9 (n=24)	182.8±8.5 (n=31)	207.1±8.4 (n=56)	228.1±10.4 (n=26)	188.9±12.0 (n=30)
TTR-T_4	0.118±0.005 (n=41)	0.124±0.008 (n=19)	0.112±0.006 (n=22)	0.101±0.004 (n=54)	0.098±0.006 (n=24)	0.1037±0.006 (n=30)	0.104±0.005 (n=53)	0.1074±0.007 (n=25)	0.101±0.007 (n=28)
Estradiol (pg/ml)	NA	NA	71.88±7.4 (n=20)	NA	NA	38.96±4.3 (n=18)	NA	NA	44.73±6.1 (n=22)

NA- non-applicable

Table 3 – Correlation of age in aMCI, AD and Control groups, in plasma TTR levels ($p<0.05$).

	Control		aMCI		AD	
	Male (r_p/P)	**Female (r_p/P)**	**Male (r_p/P)**	**Female (r_p/P)**	**Male (r_p/P)**	**Female (r_p/P)**
TTR * Age	r_p=-0.19/ P=0.4	r_p=-0.19/ P=0.4	r_p=-0.24/ P=0.25	r_p=-0.23/ P=0.20	r_p=-0.46/ P=0.02*	r_p=-0.01/ P=0.96

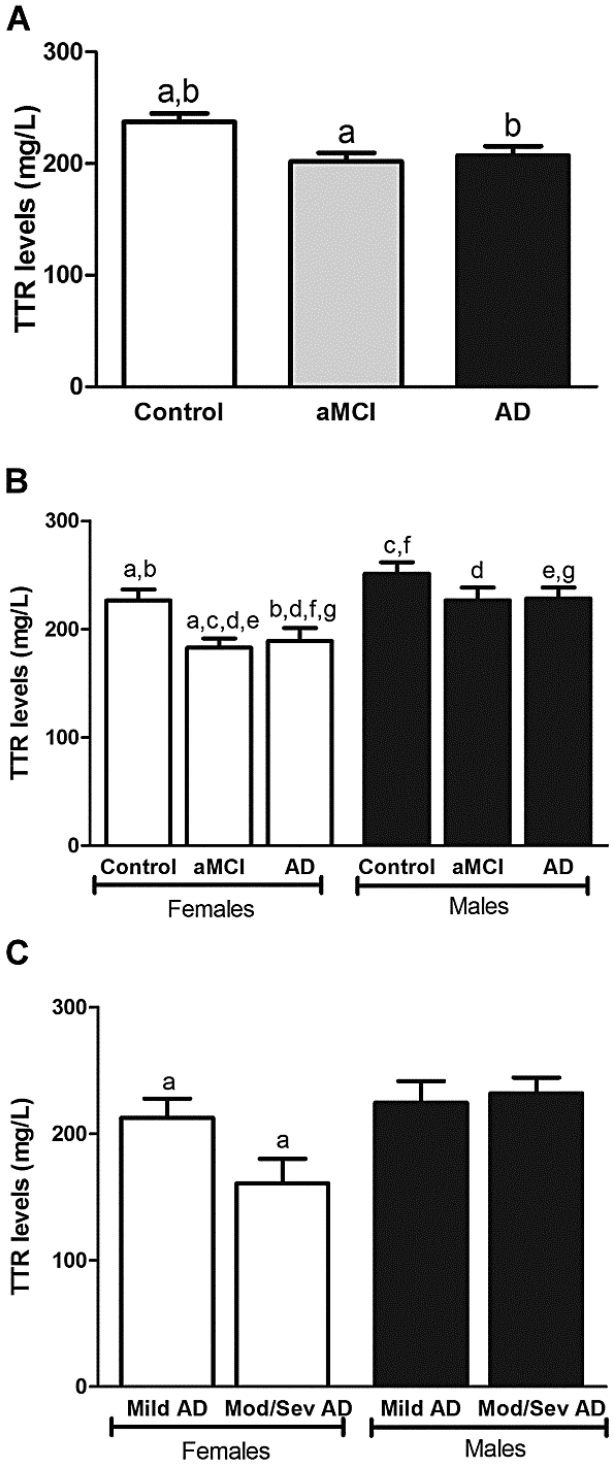

Figure 1- Analysis of plasma TTR levels by RID.

A – Plasma TTR levels in aMCI, AD and Control groups. a=P<0.01 and b=P<0.05;

B – Gender differences in plasma TTR levels in aMCI, AD and Control groups. a, d, g = P<0.01; c=P<0.0001;b,e = P<0.05; f=P<0.001;

C – Differences in plasma TTR levels in mild and moderate/severe AD, in females and in males. a=P<0.05

Plasma TTR ability to bind T_4 is decreased in aMCI and AD

Over 100 mutations have been identified in TTR and associated with amyloid deposition. This is thought to happen because the mutations destabilize the TTR tetrameric fold leading to tetramer dissociation. As a consequence, in the amyloidogenic variants, TTR ability to bind its ligands (T_4 and RBP) is affected or even impaired. These changes can also impact in protein clearance rates (Longo Alves et al., 1997).

To investigate if the lower TTR levels in the aMCI and AD groups were also reflected in TTR affinity for T_4, we evaluated the ability of plasma TTR to bind ^{125}I-T_4, using 54 aMCI, 53 AD and 41 control samples (table 2). Displacement analysis of T_4 from plasma TTR by [^{125}I]T_4 showed that TTR ability to bind T_4 was decreased approximately 14% in aMCI and 12% in AD patients when compared to the control group (Figure 2).

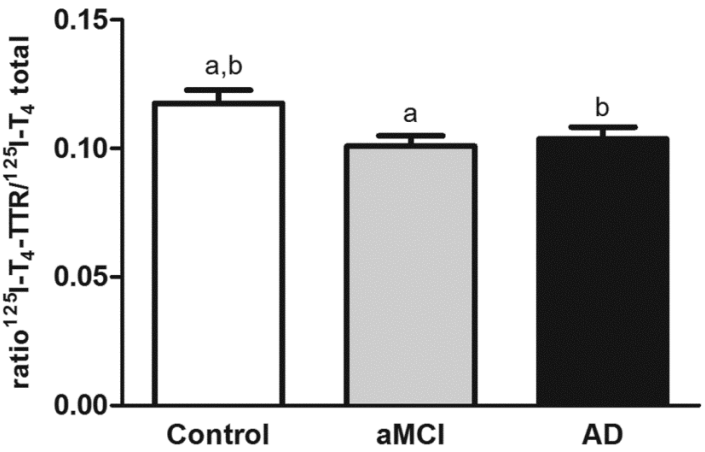

Figure 2 –Analysis of T_4 displacement from TTR. Displacement of T_4 from TTR by [^{125}I]T_4 in Control, aMCI and AD groups. a and b=P<0.05.

Estradiol levels are reduced in women in aMCI and AD

TTR expression is known to be regulated by estradiol (Quintela et al., 2009). To further explore the observed plasma TTR levels in AD, in particular in women, we measured estradiol levels in 18, 22 and 20 women from the aMCI, AD and control groups, respectively (table 2). Here we showed that estradiol was significantly reduced in aMCI and AD women when compared to controls (figure 3A). Since neither the patients nor controls were under HRT (with the exception of one single AD woman), we suggest that the observed decrease of estradiol is related to the disease. No correlation between TTR and estradiol levels was found (AD, *p*=0.96; MCI, *p*=0.21).

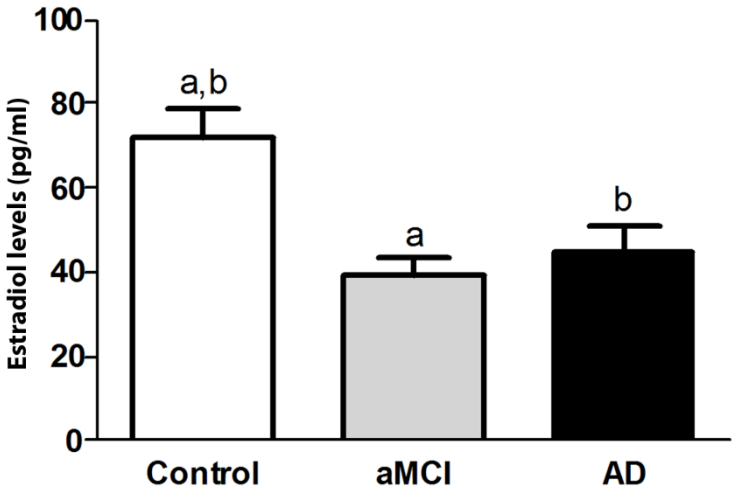

Figure 3 – Analysis of estradiol levels in Control, aMCI and AD women groups. Estradiol levels in women from Control, aMCI and AD groups. a,b=P<0.01.

Discussion

It has been reported by different authors that TTR is decreased in the CSF of AD patients when compared to healthy age-matched controls and even when compared to other neurological pathologies (Puchades et al., 2003, Castano et al., 2006, Gloeckner et al., 2008, Hansson et al., 2009). Although the mechanisms underlying this alteration are not yet known, and no correlation was found between mutations in the TTR gene and AD (Palha et al., 1996), different factors such as mutations and metal ions concentrations might cause this impairment. More recently, polymorphisms in the TTR gene were associated with hippocampal atrophy, although the study could not associate this alteration to AD (Cuenco et al., 2011). Thus, other factors must modulate TTR levels in the AD context, and further studies are necessary.

Several researchers aimed at finding plasma markers in AD as this fluid may also reflect disease disturbances. It has been reported that Aβ generated in the brain binds to cholesterol/ApoE and, in this way, crosses the blood brain barrier (BBB) to be further incorporated in HDL for delivery in the liver (Sparks, 2007). Because plasma TTR binds Aβ it might interfere in its transport and elimination. Similarly to what is observed in CSF, plasma TTR levels can be altered in AD. In fact, very recently Han *et al.* (Han et al., 2011) described that serum TTR is decreased in AD patients using an ELISA approach and Velayudhan *et al.* (Velayudhan et al., 2011) also reported, using a western-blot analysis, that plasma TTR is diminished in AD patients, but no insights on the cause(s) for such decrease are discussed.

In the present work, we used a RID approach to determine plasma TTR concentration from patient and control subjects; the RID method does not require plasma dilutions, contrarily to the ELISA and western-blot techniques. Furthermore, we included in our study not just AD but also aMCI patients to further highlight the behavior pattern of TTR before AD symptoms are evident, which was not addressed by these other two reports. We included 55, 56 and 41 aMCI, AD and control subjects, respectively, and showed that TTR is decreased in plasma of aMCI and AD patients when compared to age-matched controls, further supporting a role for TTR in this pathology, and pointing out the possibility of using plasma levels of this protein as a biomarker.

Data on the relationship between gender/age and TTR levels are controversial. For example, Serot *et al.* reported increasing levels of TTR with age in CSF samples (2-18 < 20-53 < 61-90 years); however, within the age range of AD development, no significant differences were found by the authors (Serot et al., 1997). On the other hand, Kunicki *et al.* suggested that TTR levels in CSF were not age nor gender dependent (Kunicki et al., 1998). Regarding gender, Han *et al.* found that men presented significantly higher serum

TTR levels than women in the control group, whereas no differences were detected between genders in the AD patients (Han et al., 2011). In our work, TTR concentration was not statistically different between men and women in the control group. However, in the patient groups, the decrease observed in TTR levels was different in the two genders, with women contributing substantially to the final result: women from each patient group displayed lower TTR levels than the respective men patient groups, and controls. TTR levels did not correlate with age among females from both patient groups, whereas a negative correlation was obtained for males from the AD group. This data can help to explain the reason why women are at a greater risk for developing AD (Henderson, 1997), compared to men. Our results further support a possible role for TTR in AD, and provide evidence for its different behavior in the two genders: a greater decline in plasma TTR in women would increase their risk for developing AD. Indeed, our work is the first to demonstrate that there are gender differences in the plasma TTR levels of AD patients. In fact, Velayudhan *et al.* (Velayudhan et al., 2011) have not done any gender analyses and, contrarily to their study, we used a balanced number of men and women samples. Very recently by Oliveira et al., in a study using transgenic APP/presinilin (PS) mice with or without endogenous TTR, besides reporting higher plaque burden and Aβ levels in females than in males, also described higher levels of circulating TTR in AD male mice compared to AD females littermates and further, they showed that TTR levels were decreased in an initial stage of the disease, when compared to the control group (Oliveira et al., 2011).

TTR decrease in CSF of AD patients has been established by several authors but whether this decline is a cause or a consequence of the disease, i.e. if it is an early or late pathological event, is not known. Our results suggest that TTR decline starts before a probable AD patient is diagnosed, since aMCI patients presented reduced plasma TTR levels. Although it is not certain that all aMCI patients will evolve to AD, a significant percentage does evolve to this condition. Locascio *et al.* hypothesized that before Aβ deposition initiates in the brain, high plasma Aβ levels may reflect a genetic predisposition for increased Aβ production, or reduced clearance (Locascio et al., 2008). With the initiation of Aβ deposition, plasma Aβ levels decline and, once the disease is established, the lowest Aβ levels seem to predict a more rapid progression (Locascio et al., 2008). One might suggest that sequestration of Aβ in neuritic plaques may explain the low Aβ (1–42) levels in CSF of AD patients and we hypothesized that an early decrease in TTR levels might result in increased Aβ levels; as the disease progresses, Aβ deposits and levels diminish since TTR continues to decrease, as indicated by our results, in particular from AD women, with moderate/severe cases presenting significantly lower plasma TTR levels than mild cases, making this protein an interesting biomarker for AD diagnosis and

staging. Previous work using CSF from AD patients also concluded that TTR decline in AD correlates negatively with disease severity (Gloeckner et al., 2008) and senile plaque burden (Merched et al., 1998). Moreover, the report performed by Velayudhan *et al.* also described that plasma TTR is decreased in subjects with moderate-severe AD, adding that TTR levels in plasma are significantly reduced in patients with a rapid cognitive decline (Velayudhan et al., 2011). In the future it would be important to measure plasma Aβ in the same AD samples and correlate Aβ levels with TTR concentrations in order to investigate the relation Aβ/TTR levels in AD longitudinally over the course of the disease. It would also be important to follow up the aMCI patients here investigated, as well as the AD subjects, by measuring simultaneously TTR and Aβ levels over the years.

The importance of ApoE and its isoforms has already been established in AD. While the ε2 allele has been described as protective, the ε4 is known to increase the risk for late onset AD (Roses, 1996). This apoliprotein plays determinant roles in the regulation of Aβ levels, in its deposition and clearance, (Holtzman, 2001, Deane et al., 2008) and was also described as a promoter of proteolytic degradation of *Aβ* peptide through neprilysin and other enzymes. These effects are dependent on its lipidation status and, fundamentally, dependent on the isoform that is present. For instance, ApoE ε4 isoform exhibits an impaired ability to promote Aβ proteolysis compared to both ApoE ε2 and ApoE ε3 isoforms (Jiang et al., 2008). Since *in vitro* studies showed TTR protection against Aβ toxicity through its proteolytic degradation (Costa et al., 2008), we investigated the relation between the presence of the ApoE ε4 allele and TTR levels; no differences were found in neither of the patient groups, as also described by Velayudhan *et al.* (Velayudhan et al., 2011).

T119M TTR is a non-amyloidogenic variant previously described to attenuate the symptoms in heterozygous carriers of the amyloidogenic V30M mutation associated with FAP (T119M/V30M). Furthermore, this variant presents higher resistance to dissociation into monomers than WT TTR (Quintas et al., 1997). Heterotetrameric T119M/V30M TTR is cleared slower, resulting in higher circulating levels, than homotetrameric V30M TTR which presents faster clearance, explaining the lower plasma levels (Longo Alves et al., 1997). The T119M mutation is located in the H strand and participates in the binding to T_4, resulting in increased affinity for the hormone; in contrast, V30M TTR presents reduced affinity to the hormone (Almeida et al., 1997). Hence, T_4 binding can be used as a measure of protein functionality. Our results showed that TTR from aMCI and AD patients bound less T_4 than TTR from the control group which is suggestive of increased free T_4 levels. In this context, Stuerenburg and colleagues reported that high plasma free T_4 levels correlated with impaired cognitive function in AD (Stuerenburg et al., 2006). Total

and free T_4 levels, T_4 affinity to TTR and T_4 binding to other hormone transporters (TBG and albumin) should be measured to confirm the present results. Our results may indicate that TTR decline observed in this disorder reflects a protein loss of function, possibly resulting in accelerated clearance and consequent decreased levels, as observed when TTR is mutated in FAP. However, other factors can be ascribed to different possibilities: i) TTR might bind to other ligands: e.g. the presence of an aberrant complex between TTR and prostaglandin-d-synthase in the CSF of AD patients has been reported (Lovell et al., 2008); this or other types of complexes may exist in plasma of AD patients and should be explored in the future; ii) protein modifications might affect TTR behavior, as already be investigated in the AD context; finally, iii) there might exist still unknown metabolites in plasma that could displace T_4 from the TTR binding site.

One aspect that is pointed out for the fact AD affects more women than men is the hormone levels and recent literature showed that TTR is up-regulated by sex hormones (Goncalves et al., 2008). With regard 17β-estradiol, the predominant sex hormone present in females, it has been shown that it induces TTR expression in the choroid plexus (Quintela et al., 2009), prompting this hormone as a key target in AD. Furthermore, an early-onset AD transgenic mouse model expressing the double-mutant form of human precursor protein (APP) treated with 17β-estradiol, resulted in: i) significantly lower APP processing through β-secretase; ii) enhanced alpha-secretase activity; iii) decreased Aβ (1-42) levels and plaque burden, and 4) elevated brain levels of TTR (Amtul et al., 2010). Estradiol assumes an important role in AD: APP processing, Aβ levels and factors that contribute to its clearance, like TTR levels. Here, we showed that both aMCI and AD women had lower estradiol levels in plasma as compared with controls. It is possible that in AD, through mechanisms that are not yet elucidated, the normal decline of estrogens in women after menopause is greater than in non-AD women leading to a lower expression of TTR by 17β-estradiol, therefore explaining the higher disease prevalence in women compared to men (figure 4). We did not find any relation between estradiol and TTR levels in plasma probably because TTR liver expression is not solely affected by estrogens. In fact, other conditions such as inflammation and nutritional status have been shown to affect protein levels (Johnson, 1999). Measurement of estrogen levels in CSF samples of AD patients can help to clarify this issue and should be performed.

In summary, our study presented for the first time data on TTR as an early staged AD plasma marker. TTR decline was most prominent in women, suggesting that the putative TTR modulatory role in AD is gender dependent. The estradiol decrease observed in women from the AD group when compared to controls can partially account for lower TTR levels, thus explaining the higher propensity that women show to develop this disorder. Furthermore, our study is pioneering in the analysis of the functionality of

TTR, demonstrating that not only the levels are decreased in AD, but the protein itself has one of its main functions compromised, such as its ability to carry T_4, which may be leading to an inefficient clearance of Aβ (figure 4). In the future, would be important to understand the intervening mechanisms downstream of TTR reduction, and why men are, in general, more protected.

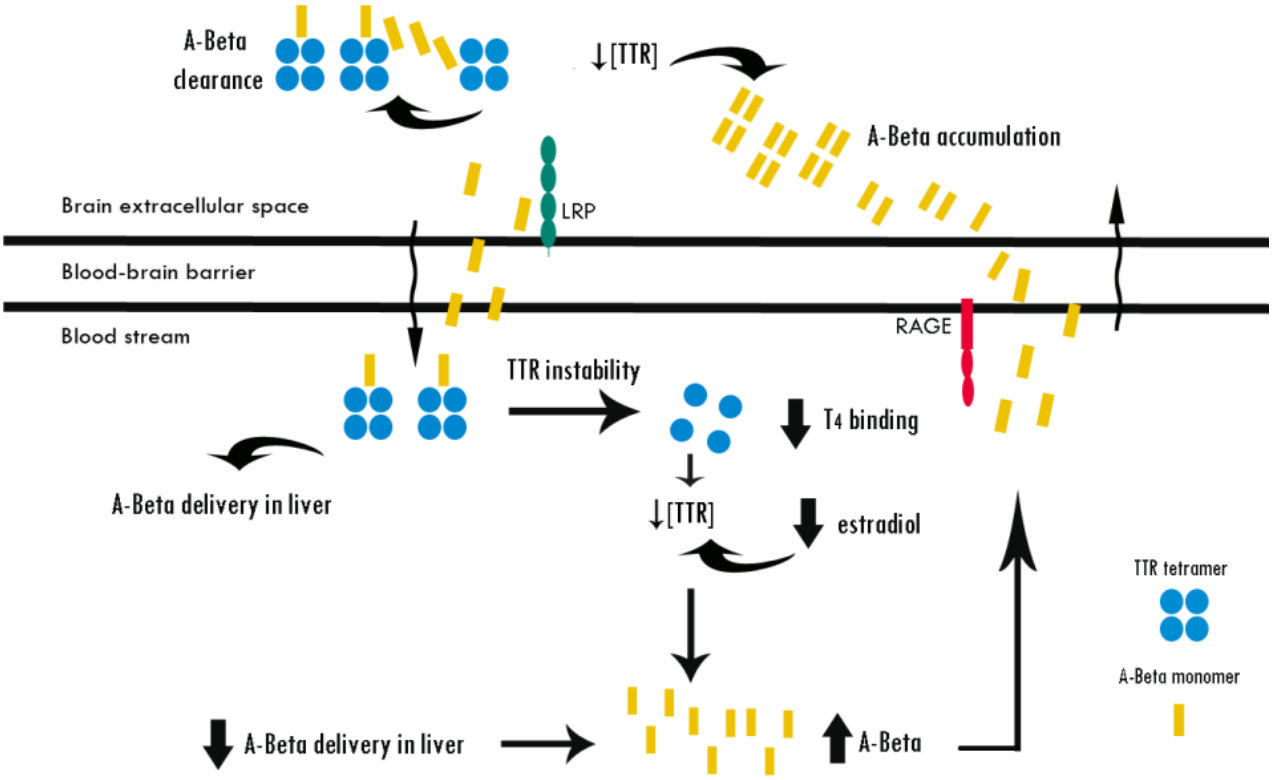

Figure 4 – Transthyretin contribution to maintenance of A-Beta (Aβ) levels in the brain.
The steady-state level of Aβ depends on the balance between production and clearance. In normal conditions, TTR binds Aβ in the CSF preventing its accumulation and deposition in the brain. The lipoprotein receptor-related protein (LRP) mediates Aβ transport from the brain to the periphery, where TTR can bind the peptide promoting its degradation in the liver. TTR destabilization results in impaired ability to carry thyroxine, increased clearance and decreased levels of the protein, both in the brain and in plasma. As a consequence, increased Aβ in the periphery disrupts the Aβ equilibrium between the brain and the blood, promoting its influx to the brain, mediated by the receptor for advanced glycation end products (RAGE).

Acknowledgments

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CHAPTER II

Stability of the Transthyretin molecule as a key factor in the interaction with Aβ peptide - relevance in Alzheimer's disease

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Abstract

Transthyretin (TTR) protects against Aβ toxicity by binding the peptide thus inhibiting its aggregation. More recently, we showed that TTR not only avoids Aβ aggregation but it also disrupts fibrils derived from the peptide. In this work we investigated the possibility of TTR being a universal fibril disrupter and tested fibrils from amylin, insulin and β2-microglobulin.Previous work showed different TTR mutations interact differently with Aβ, with increasing affinities correlating with decreasing amyloidogenecity of the TTR mutant; this did not impact on the levels of inhibition of Aβ aggregation, as assessed by transmission electron microscopy. Our work aimed at probing differences in binding to Aβ by WT, T119M and L55P TTR using quantitative assays, and at identifying factors affecting this interaction. We addressed the impact of such factors in TTR ability to degrade Aβ. Using a dot blot approach with the anti-oligomeric antibody A11, we showed that Aβ formed oligomers transiently, indicating aggregation and fibril formation, whereas in the presence of WT and T119M TTR the oligomers persisted longer, indicative that these variants avoided further aggregation into fibrils. In contrast, L55PTTR was not able to inhibit oligomerization or to prevent evolution to aggregates and fibrils. Furthermore, apoptosis assessment showed WT and T119M TTR were able to protect against Aβ toxicity. Because the amyloidogenic potential of TTR is inversely correlated with its stability, the use of drugs able to stabilize TTR tetrameric fold could result in increased TTR/Aβ binding. Here we showed that iododiflunisal, 3-dinitrophenol, resveratrol, [2-(3,5-dichlorophenyl)amino] (DCPA) and [4-(3,5-difluorophenyl)] (DFPB) were able to increase TTR binding to Aβ; however only DCPA and DFPB improved TTR proteolytic activity. Thyroxine, a TTR ligand, did not influence TTR/Aβ interaction and Aβ degradation by TTR, whereas RBP, another TTR ligand, not only obstructed the interaction but also inhibited TTR proteolytic activity. Our results showed differences between WT and T119M TTR, and L55PTTR mutant regarding their interaction with Aβ and prompt the stability of TTR as a key factor in this interaction, which may be relevant in AD pathogenesis and for the design of therapeutic TTR-based therapies.

Introduction

Alzheimer's disease (AD) is nowadays responsible by 50 to 80 percent of dementia cases (Association, 2010) and is mainly characterized by two types of lesions: neurofibrillary tangles (NFTs) and neuritic plaques. NFTs (Alzheimer et al., 1995) are bundles of abnormal filaments composed of highly phosphorylated tau protein (Boutajangout et al., 2002). Neuritic plaques are extracellular amyloid deposits found in the brain and are mainly constituted by β-amyloid (Aβ) peptide. Accumulation of Aβ is due to deregulated proteolytic processing of its precursor, the Amyloid Precursor Protein (APP).

Schwarzman *et al.* described that normal cerebrospinal fluid (CSF) inhibits amyloid formation (Schwarzman et al., 1994) and concluded that transthyretin (TTR) was the major Aβ binding protein in CSF, leading to a decrease in the aggregation state of the peptide (Schwarzman et al., 1994). More recently it has been shown that deletion of the TTR gene in a mouse APP transgenic model results in increased of Aβ deposition (Choi et al., 2007). Our group characterized the TTR/Aβ interaction showing that TTR is capable of interfering with Aβ fibrilization by both inhibiting and disrupting fibril formation (Sciarrone et al., 2008). We also demonstrated that TTR, either recombinant or isolated from human sera, was able to proteolytically process Aβ. Furthermore, Aβ new peptides (1-14) and (15-42), generated upon cleavage by TTR, showed lower amyloidogenic potential than the full length counterpart (Costa et al., 2008).

TTR is a homotetramer and it is thought that the first step in the cascade that results in amyloid formation, is the dissociation of the tetramer into monomers (Lashuel et al., 1998). TTR is responsible for the transport of thyroxine (T_4) and retinol, through binding to the retinol binding protein (RBP). The four monomers within a TTR tetramer, form an open channel where T_4 binds (Blake et al., 1971, Nilsson and Peterson, 1971) while retinol interacts with only one of the dimers, at the surface (Monaco et al., 1995). TTR, when mutated, is associated to another amyloidotic disorder, Familial Amyloid Polyneuropathy (FAP), characterized by the extracellular deposition of TTR in several organs with a special emphasis in the peripheral nerve.

Particular mutations in the protein lead to instability of the native fold, increasing its propensity to precipitate and aggregate. For instance, it was suggested that mutated L55P TTR significantly alters tetramer stability and increases amyloidogenicity (McCutchen et al., 1993). The authors described that L55P TTR tetramer was more unstable than the WT TTR and that the ability of L55P TTR to develop to an amyloidogenic intermediate occurred at higher pHs than the wild-type protein; this could explain why some TTR variants form amyloid fibrils, while the WT TTR remains stable and nonamyloidogenic (McCutchen et al., 1993). Furthermore, Longo Alves *et al.* compared the stability and

clearance of V30M TTR and T119M TTR and described that the more stable properties of T199M variant could be involved in the protective clinical effect of the T119M mutation in FAP. Baures *et al.* and Oza *et al.*, demonstrated several small compounds sharing molecular structural similarities with TTR natural ligand - T_4 - that can bind in the T_4 binding sites, proposing them as inhibitors of TTR fibril formation *in vitro* (Baures et al., 1998, Oza et al., 2002). In addition and related to the stabilization of TTR, Costa *et al.* described that TTR mutations, such T119M, Y78F, V30M and L55P bound differently to Aβ. Additionally, an inverse relation between the amyloidogenic potential of TTR and the affinity to Aβ peptide was suggestive of a direct relationship with TTR stability (Sciarrone et al., 2008).

In the present work we further explore TTR/Aβ interaction, in particular the influence of TTR stability and ligands, on binding properties.

Materials and Methods

TTR production and purification

Recombinant TTRs were produced in a bacterial expression system using *Escherichia coli* BL21 (Furuya et al., 1991) and purified as previously described (Almeida et al., 1997). Briefly, after growing the bacteria, the protein was isolated and purified by preparative gel electrophoresis after ion exchange chromatography. Protein concentration was determined using the Bio-Rad assay kit (Bio-Rad), using bovine serum albumin (BSA) as standard.

Fibrils formation and effect of TTR

Human Amylin was bought to Bachem and dissolved in Tris 10mM solution pH=7.5 at a final concentration of 250mM and incubated at 37ºC for fibril formation. Insulin fibrils were formed by dissolving insulin (Sigma) at 10 mg/ml in acetic acid/HCl pH 2.0, and incubated at 85ºC for 2 hours. Then, the sample was frozen (-70ºC)/defrosted (50 ºC) 5 times. Finally, the sample was centrifuged (15000 g) for 30 minutes and the pellet resuspended in water. Beta 2-microglobulin (100uM) was incubated at 37ºC in 50 mM phosphate buffer, 100 mM NaCl (pH=7.4), 20% (v/v) TFE and with 20 µg/ml of Beta 2-microglobulin seeds. WT TTR (10 µM) was added to the samples and incubated for periods of 1, 3 and 5 days and observed by Transmission Electron Microscopy (TEM).

Transmission electron microscopy (TEM)

Sample aliquots were negatively stained with 1% uranyl acetate. The grids were visualized with a Zeiss microscope (EM10C), operated at 60 kV.

Production of Aβ species

The peptide was purchased from Genscript, dissolved in Hexafluoroisopropanol (HFIP) and kept at room temperature overnight. The HFIP was removed under a stream of nitrogen and the residue was then dissolved in Dimethyl sulfoxide (DMSO) at 2 mM. For experiments, Aβ was diluted in the buffers mentioned below at the indicated concentrations.

A11 antibody assay

Soluble Aβ alone (100µM) or mixed with WT TTR, T119M or L55P TTR (10 µM), or TTR alone, were incubated at 37ºC and aliquots of 0.45 µg Aβ were removed over time and immobilized onto a nitrocellulose membrane. Detection of oligomers was performed using the A11 antibody (1:500, Biosource), followed by an anti-rabbit HRP (horseradish peroxidase) conjugate (1:500) as the secondary antibody. Detection was performed with ECL® (enhanced chemiluminescence; GE Healthcare).

Cell culture and caspase-3 assay

SH-SY5Y cells (human neuroblastoma cell line; (European Collection of Cell Cultures)) (Magalhaes et al., 2010), were propagated in 25-cm flasks and maintained at 37°C in a humidified atmosphere of 95% and 5% Carbon dioxide (CO_2) and grown in MEM/F12 (1:1) (Lonza) supplemented with 15% fetal bovine serum (FBS) (Gibco BRL). Activation of caspase-3 was measured using the CaspACE fluorimetric 96-well plate assay system (Sigma), following the manufacturer's instructions. Briefly, 80% confluent cells were cultured in 6-well plates and were grown for 24 hours. Then, the medium was renewed 3-5 hours later, and 10 µM Aβ peptide, previously incubated with and 2 µM TTR, WT, T119M or L55P, for 48 hours at 4ºC in F12 media (Lonza), was added to the cells. Subsequently, each well was trypsinized and the cell pellet was lysed in 100 µl of hypotonic lysis buffer (Sigma). 40 µl of each cell lysate was used in duplicates for determination of caspase-3 activation. The remaining cell lysate was used to measure total cellular protein concentration with the Bio-Rad protein assay kit (Bio-Rad), using BSA as standard. Experiments were repeated at least twice; values shown are the mean of duplicates.

Chemical Compounds

Tri-iodophenol (TIP), Flufenamic Acid (Fluf), Diflunisal (DIF), Resveratrol (Resv), 2-((3,5-Dichlorophenyl)amino)benzoic acid (DCPA), Dinitrophenol (DNP), 4-(3,5-difluorophenyl) (DFPB), Genistein, Epigallocatechin gallate (EGCG) and thyroxine (T_4) were from Sigma. The diflunisal derivative, iododiflunisal (IDIF, Mr 376.1) was kindly provided by Dr. Gregorio Valencia, CSIC, Barcelona (Almeida et al., 2004). Retinol Binding Protein (RBP) was isolated from serum by affinity in a TTR column and saturated with 3.3 mg/ml all-*trans* retinol (Sigma) in ethanol as follows: (i) 25 µl of all-*trans*-retinol were incubated with 800 µl of RBP (1 mg/ml) at 37 °C in the dark for 1 h; and (ii) excess retinol was separated from RBP by gel filtration in 10-ml Biogel P-6 DG columns (Bio-Rad) (Liz et al., 2004).

Competition binding assays

Recombinant TTRs were iodinated with $Na^{125}I$ (NEN) using the Iodogen (Sigma) method, following the supplier's instructions. The reaction was desalted by Sephadex G50 gel filtration; 96 well plates (Maxisorp, Nunc) were coated with Aβ (5µg/well) in coating buffer (Na_2CO_3/$NaHCO_3$ pH 9.0) and incubated overnight at 4ºC. Unoccupied sites were blocked with 5% non-fat dried milk in PBS for 2 hours at 37ºC. For competition studies, different concentrations of cold TTR (0x to 100x molar excess), alone or pre-incubated for 12 hours with a 5x molar excess of RBP or different compounds included: TIP, Fluf, DIF, IDIF, Resv, DCPA, DNP, DFPB, Genistein and EGCG were used and mixed with a constant amount of ^{125}I-WT TTR (500000 cpm) and incubated for two hours at 37ºC. Binding was determined after five washes in ice cold phosphate-buffered saline with 0.05% tween20 (PBS-T). Then, 0.1 ml of elution buffer (NaCl 0.1M containing Nonidet P40 (NP40) 1%) was added for 5 minutes at 37ºC and the contents of the wells aspirated and counted in a rackgamma counter. Experiments were repeated at least twice in quadruplicates.

Aβ proteolysis assay

A fluorogenic peptide encompassing 6 residues of the Aβ peptide sequence containing one of the TTR cleavage sites previously described (Costa et al., 2008) was used (Abz-VHHQKL-EDDnp, Genscript); this substrate is an internally quenched fluorescent peptide in which Abz (*ortho*-aminobenzoic acid) is the fluorescent donor and EDDnp [*N*-(ethylenediamine)-2,4-dinitrophenyl amide] is the fluorescent quencher. Hydrolysis of the fluorogenic substrate was monitored by measuring fluorescence at λ_{em} = 420 nm and λ_{ex} = 320 nm in a *f*max plate reader (Molecular Devices). The kinetics of the reaction was followed for 1 h at 37 °C. To determine the effect of different TTR ligands on its ability to cleave Aβ peptide, 5µM of TTR was pre-incubated (for 1 hour) with the compounds described above (till 10 fold molar excess) at 37ºC with 50mM Tris pH 7.5 and then 5µM of substrate was added in a final volume of 100µL. Experiments were repeated at least three times.

Assessment of tetrameric TTR stability by IEF (isoelectric focusing) in semi-denaturing conditions

The conditions used for the IEF of plasma TTR have been described previously by Altland et al. (Altland et al., 1981) and Almeida et al. (Almeida et al., 2004). Briefly, 12 µg of recombinant L55P TTR were incubated at 4°C overnight, with 10 mM solution of the tested compounds. The preparations were then subjected to native PAGE to isolate TTR. The gel band containing TTR was excised and subjected to IEF in a gel with 4 M urea and 5% ampholytes (pH 4–6.5) at 1200 V for 6 h. Proteins in the gel were stained with Coomassie Blue. These semi-denaturing conditions allow the visualization of bands corresponding to the TTR monomer and tetramer and also to an oxidized form of the monomer. Gels were scanned and bands were quantified using the Quantity One program. Experiments were repeated at least two times and data shown are representative of the results obtained.

Statistical analysis

All data were expressed as mean values ± standard error of the mean (SEM). One way ANOVA with Bonferroni's post-test was performed using GraphPad Prism, version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Values of $p<0.05$ were considered to be significant.

Results

Specificity of the TTR fibril disrupter activity towards Aβ fibrils

It has been previously shown that TTR binds Aβ, inhibiting fibril formation and abrogating the toxicity associated to the peptide. Also, TTR is able to disaggregate Aβ mature fibrils acting as a fibril disrupter.

It is known that amyloid fibrils display common structural and tinctorial features, independent of their protein precursor. Thus, it is reasonable to think that TTR could also disrupt other amyloid fibrils besides the ones derived from Aβ peptide. In this work we studied the effect of WT TTR in fibrils derived from amylin, insulin and β2-microglobulin, by TEM; addition of TTR to the pre-formed fibrils had no effect, and fibrils similar to the control preparations were observed, as depicted in figure 1, suggesting that TTR is not a universal disrupter, but rather is specific for Aβ

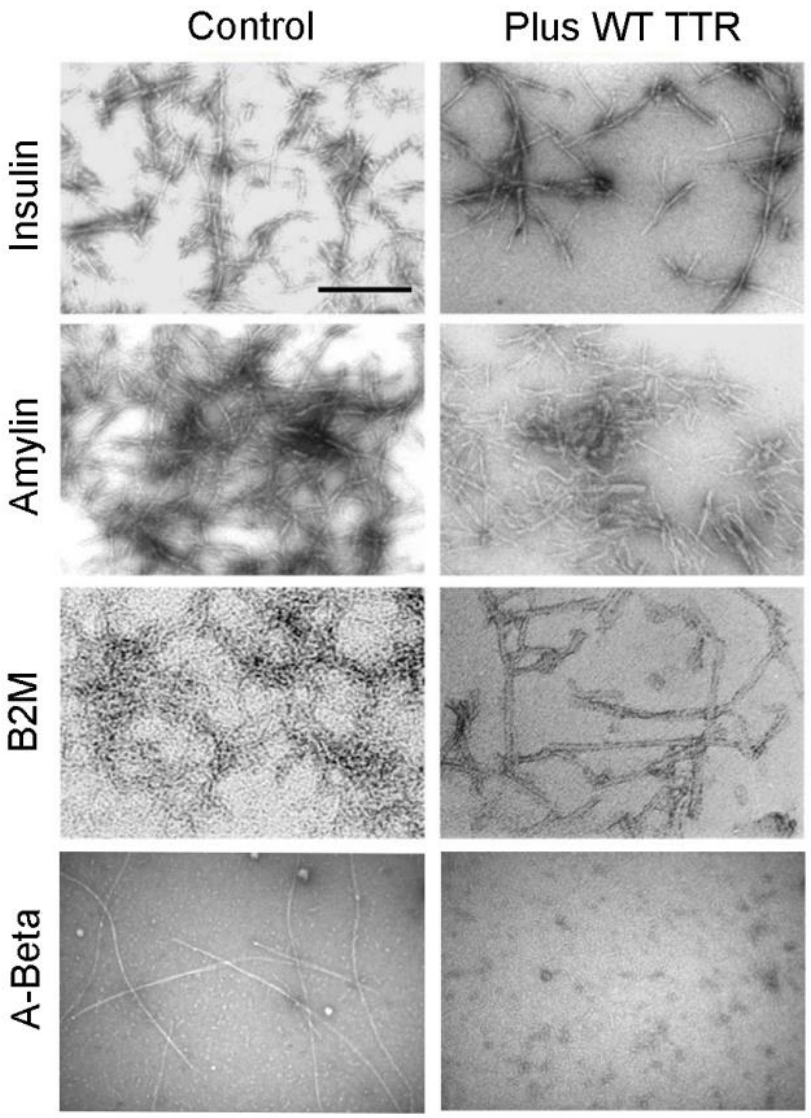

Figure 1 - TEM analysis of amyloid fibrils from different precursors incubated with WT TTR. On the left Insulin, Amylin, β2-microglobulin and Aβ (control) fibrils incubated alone for 5 days. On right, the same fibrils incubated in the presence of TTR for the same period of time. Scale bar = 100 nm.

Differences in binding to Aβ peptide by WT, T119M and L55P TTRs using quantitative assays

a. Effect on Aβ peptide oligomerization

Previous results indicated that TTR mutants bind differently to Aβ peptide, with the following trend: T119M > WT > V30M > Y78F > L55P TTR, suggesting that TTR amyloidogenecity influences its binding to the peptide; at the level of Aβ aggregation, we did not detect any differences between TTR mutants, as both non-amyloidogenic and amyloidogenic variants were able to prevent Aβ fibrilization with approximately the same extent, as evaluated by transmission electron microscopy (TEM) (Sciarrone et al., 2008). In the present work, we further compared if different TTR variants differently affect Aβ fibrillogenesis, making use of a dot blot assay with the A11 antibody, described as specific for oligomeric species. This antibody has the ability to recognize an amyloidogenic conformation present in intermediate species from different amyloidogenic precursors; however, it does not recognize their soluble, aggregated or fibrillar counterparts (Kayed et al., 2007).

Soluble Aβ alone or mixed with WT, TT119M or L55P TTR mutant were incubated at 37ºC and aliquots of 0.45 µg were removed over time and immobilized onto a nitrocellulose membrane. For the incubation of Aβ alone, detection with the A11 antibody showed the presence of Aβ oligomers starting at day 0 (not shown) which continued until day 8 (figure 2). At day 10 the A11 signal disappeared completely, suggesting that Aβ oligomers evolved to other species such as aggregates and fibrils which are not recognized by this antibody. Co-incubation of the peptide with WT TTR produced a different profile as the presence of oligomers was prolonged until at least day 10 (last time point analysed) (figure 2), indicative that the WT protein avoided further aggregation of the peptide into fibrils, by arresting the oligomer stage. T119M TTR, a protective mutation in FAP patients, was also able to avoid further aggregation of Aβ peptide, producing an Aβ profile similar to the one treated with WT TTR.

On the contrary, L55P TTR was not able to inhibit oligomerization nor to prevent evolution to aggregates and fibrils (figure 2), resulting in a fainter A11 signal in the last days analyzed, indicating that Aβ aggregated and evolved to aggregates and fibrils. The same amount of WT, T119M and L55P TTR were also incubated alone and analysed over time,

as above; detection with the A11 antibody revealed no reaction confirming that the signal observed previously was from Aβ (not shown).

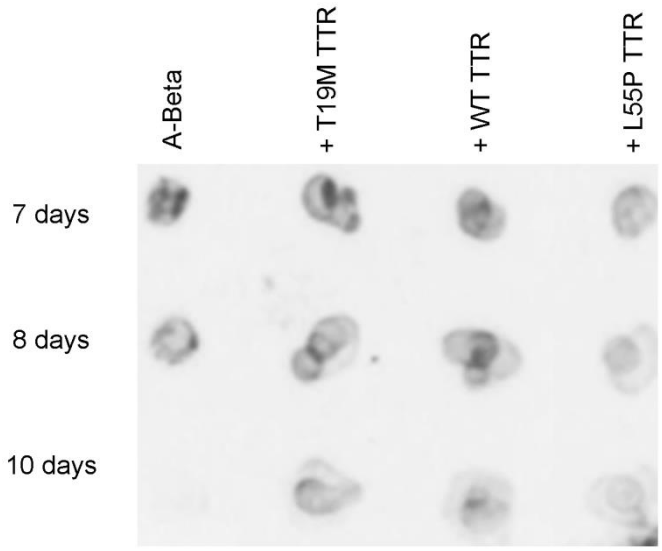

Figure 2 - Immunodot blot of Aβ alone or mixed with WT TTR, T119M or L55P TTR, incubated at 37ºC for different periods of time, and analyzed with the anti-oligomeric antibody A11. WT and T119M TTRs interfered with Aβ oligomerization resulting prolonged presence of oligomers, compared to Aβ alone which evolved to aggregates and fibrils. In opposition, L55P TTR had impaired ability to avoid fibrillization of the peptide.

b. Effect on Aβ toxicity

We next analyzed the effect of TTR on Aβ toxicity, using the same TTR variants, WT, T119M and L55P TTR. Caspase 3 activation was measured in SH-SY5Y cells treated with Aβ previously oligomerized for 48 hours, alone or in the presence of the selected TTR variant; controls of TTRs alone were also included. Figure 3 shows that WT TTR was able to prevent the toxicity associated to Aβ oligomers, as previously shown (Sciarrone et al., 2008); Moreover, the non-amyloidogenic variant T119M also significantly inhibited caspase 3 activation, therefore preventing Aβ toxicity, whereas Aβ pre-incubated with L55P TTR produced similar levels of active caspase 3 as found in cells treated with Aβ alone.

Altogether, the oligomerization and toxicity studies indicated differences between the non-amyloidogenic TTR variants WT and T119M, and the highly amyloidogenic mutant L55P TTR regarding their ability to interfere with Aβ peptide amyloidogenic properties.

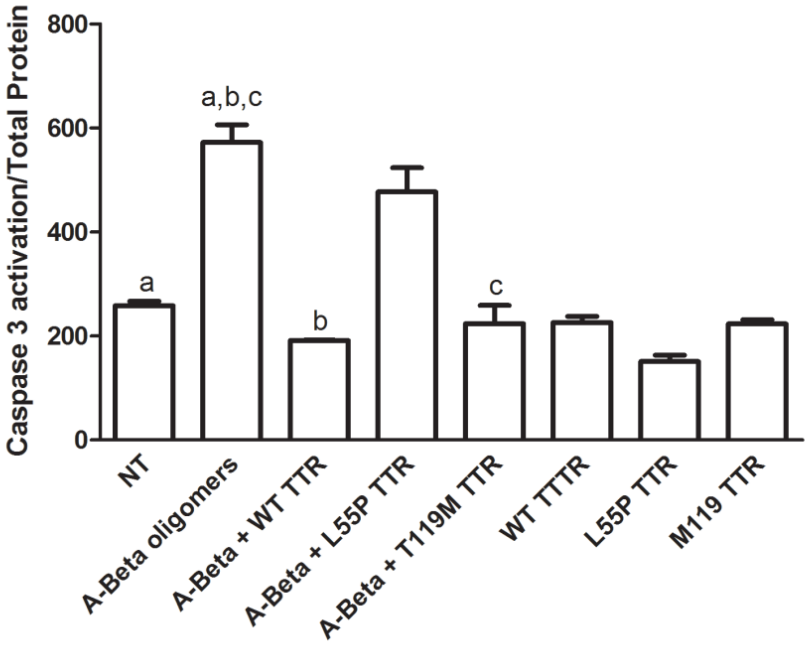

Figure 3 - Caspase 3 activation in SH-SY5Y cells. Aβ without or with TTR (WT TTR, T119M or L55P TTR) incubated at 4ºC for 48h, was then added to SH-SY5Y cultured cells and further incubated for another 48h at 37ºC. Aβ was used at a final concentration of 10μM and TTR at 2μM. Significant caspase 3 activation was observed in the presence of Aβ peptide alone and when pre-incubated with L55P TTR. WT and T119M were able to protect against Aβ toxicity and lower levels of caspase 3, similar to untreated cells, were observed. a,b,c $p < 0,001$

Importance of TTR stability in its interaction with Aβ peptide

a. Effect of TTR stabilizers in Aβ binding

We tested whether small compounds known to stabilize the TTR tetrameric fold should restore the capacity of TTR to bind Aβ, in particular the L55P TTR variant with low binding capacity. To test this hypothesis, we used competition binding assays as this technique allows not only the assessment of the interaction between molecules but also to infer on the strength of the interaction. We started by using DIF, a non-steroid anti-inflammatory (NSAIDs), and its derivative, IDIF. The latter has been shown to highly stabilize the TTR tetramer structure, *in vitro* and *ex vivo*, whereas DIF could only produce a mild effect, under the same conditions (Almeida et al., 2004).

Using competition binding assays after pre-incubation of WT TTR with the referred drugs, we observed that the presence of IDIF improved TTR binding to Aβ peptide, whereas DIF, on the contrary, worsened the binding (figure 4). Other small compounds known to

stabilize the TTR tetrameric fold such as Resv, DNP, DCPA, DFPB, EGCG, flufenamic acid, genistein, benzo and TIP (Cardoso et al., 2007) were also assayed but we did not observe improvement in the WT TTR/Aβ binding (data not shown)

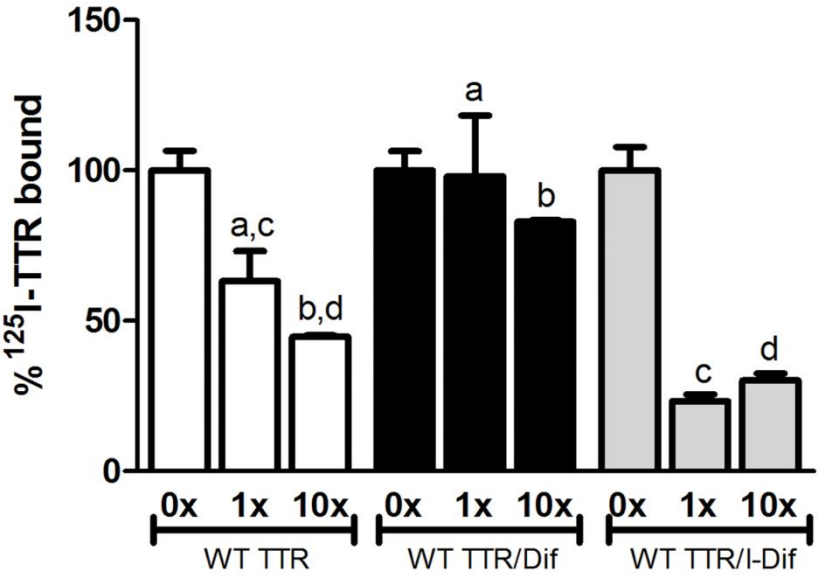

Figure 4 – Aβ peptide immobilized onto a microtiter plate was incubated with a constant amount of ^{125}I-WT TTR and increasing concentrations of unlabeled WT TTR, and binding was evaluated. Alternatively and to investigate the ability certain compounds to improve TTR/Aβ interaction, unlabeled TTR was pre-incubated with I-Dif or Dif, and further added to the immobilized Aβ peptide. I-Dif, but not Dif, was able to improve TTR/Aβ interaction. $^{a}p < 0.05$; $^{b}p < 0.05$; $^{c}p < 0.05$, $^{d}p<0.05$.

We reasoned that drugs with more modest effects, not as striking as IDIF, may not produce visible impact on WT TTR/Aβ binding, but could produce a profound effect if applied to the L55P TTR variant. We started by performing IEF assays to investigate if this variant was also stabilized by the same compounds as the WT and V30M counterparts did (Cardoso et al., 2007), and found no differences in the degree of stabilization among the three proteins (i.e. tetramer/monomer ratio) for the different tetramer stabilizing drugs (figure 5).

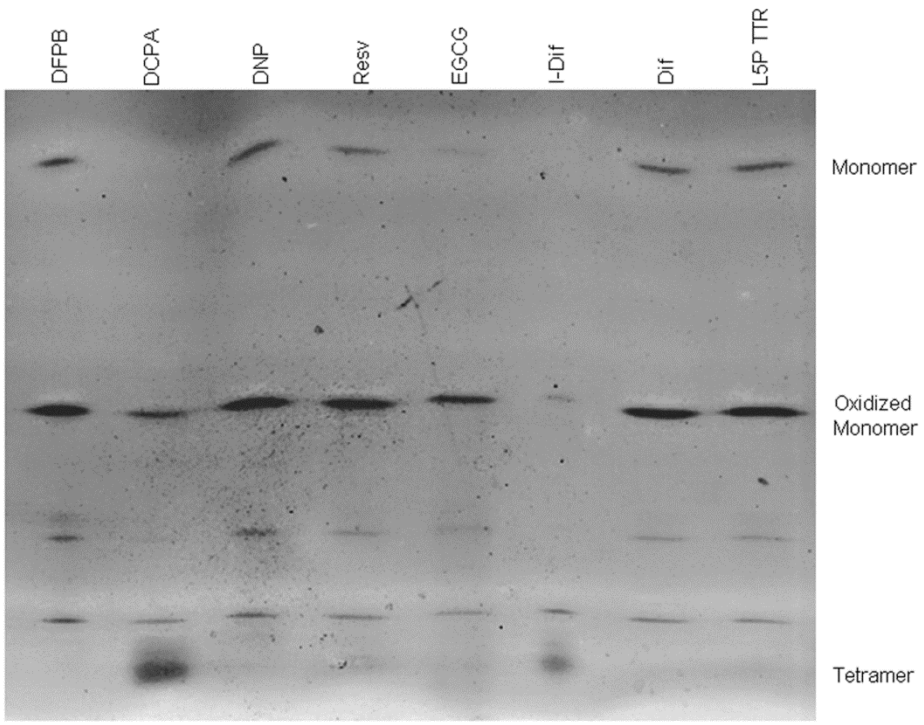

Figure 5 - IEF analysis of L55P alone or in the presence of different compounds known as TTR stabilizers. Similarly to previous results obtained with WT and V30M TTR (22, 23), DCPA and I-Dif were able to stabilize the L55P fold, resulting in increased amounts of tetramer and decreased quantity of monomer; compounds such as DFPB, DNP, Resv, EGCG and Dif did not produce any effect and levels of tetramer and monomer were similar to the L55P protein alone.

We then repeated the Aβ binding assays with L55P and, as expected, this variant bound poorly to the peptide since no differences in binding were observed in the presence of the different cold TTR concentrations used (Figure 6, white bars). Nevertheless, in our assays we detected a decrease in binding when comparing ^{125}I-TTR L55P alone to ^{125}I-TTR L55P mixed with the different cold TTR concentrations, which may reflect interference from the ^{125}I atom. On the contrary, WT TTR clearly bound Aβ peptide as concluded by the increased level of competition between ^{125}I-TTR and increasing cold TTR concentrations (figure 4).

After drug pre-incubation with TTR L55P we observed increased TTR/Aβ interaction using DCPA, DFPB, Resv and DNP similarly to the positive control (IDIF) (figure 6). However, DIF (negative control) and EGCG persisted failing to produce any effect (figure 3B), as well as other compounds such as flufenamic acid, genistein, benzo and TIP (data not shown).

For some of the compounds, being particularly pronounced for DCPA, there is a marked increase in binding at higher concentrations (100x). Usually, this high concentration is used to assess non-specific binding.

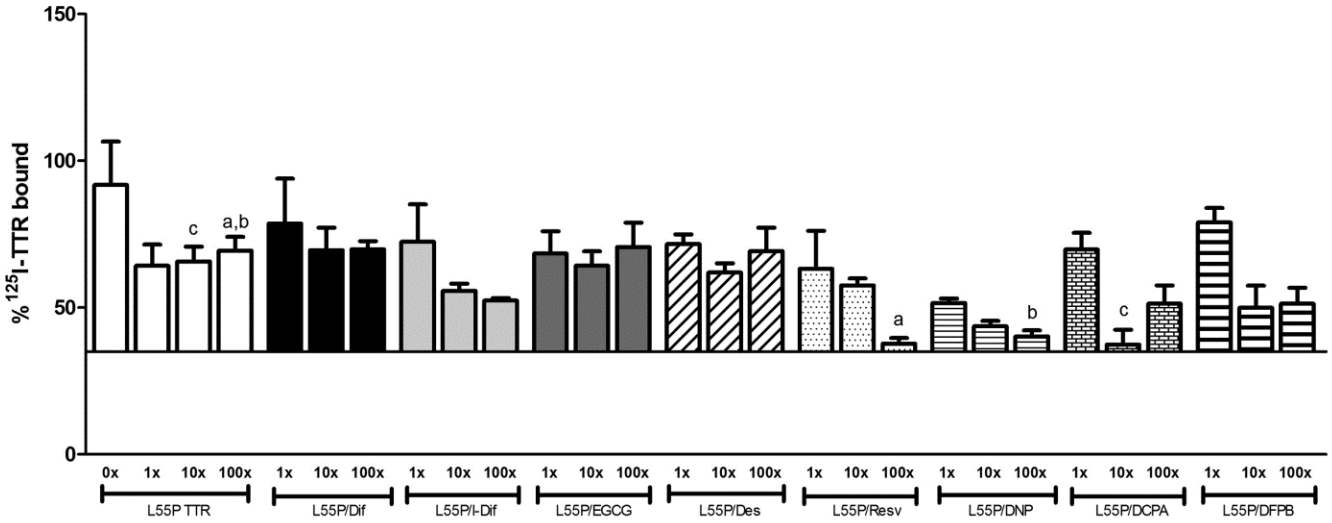

Figure 6 – Aβ peptide immobilized onto a microtiter plate was incubated with a constant amount of ^{125}I-L55P TTR and increasing concentrations of unlabeled L55P TTR, and binding was evaluated. Alternatively and to investigate the ability certain compounds to improve TTR/Aβ interaction, unlabeled TTR was pre-incubated with I-Dif or Dif, EGCG, resv, DNP, DCPA and DFPB, and further added to the immobilized Aβ peptide. Using this variant, I-Dif, Resv, DNP, DCPA and DFPB were selected as improving TTR/Aβ interaction, whereas Dif, EGCG and DES had no effect. $^{a}p < 0.05$; $^{b}p < 0.05$; $^{c}p < 0.05$.

b. Effect of TTR stabilizers in Aβ proteolysis

It has been previously shown in our laboratory that TTR is able to proteolytically process the Aβ peptide, contributing to reduction of its toxicity, as the generated peptides are less amyloidogenic than the full-length peptide (Costa et al., 2008). This finding suggested Aβ proteolysis by TTR as a protective mechanism in AD.

Because it is not known if binding to Aβ peptide and its degradation by TTR is part of the same protective mechanism, it was important to assess the impact of the above selected drugs on TTR ability to degrade Aβ, thus ascertaining if TTR stability is directly related to its proteolytic function.

Using a fluorescent peptide containing part of the Aβ sequence and one of the TTR cleavage sites described for Aβ (Costa et al., 2008), we observed that only DCPA and DFPB showed increase of 15% in TTR proteolytic ability to degrade Aβ peptide (figure 7) whereas the remaining, like IDIF, DNP (figure 3C), DIF, Resv, EGCG, flufenamic acid, genistein, benzo and TIP, produced no effect (data not shown).

Our results suggested TTR stability as a key factor in the interaction with Aβ. Proteolysis can also be modulated by small compounds known to affect TTR stability/aggregation; however, different drugs have different effects at the binding and at the proteolysis levels, suggesting that these are separate events or that the TTR regions involved, in the two activities, are different.

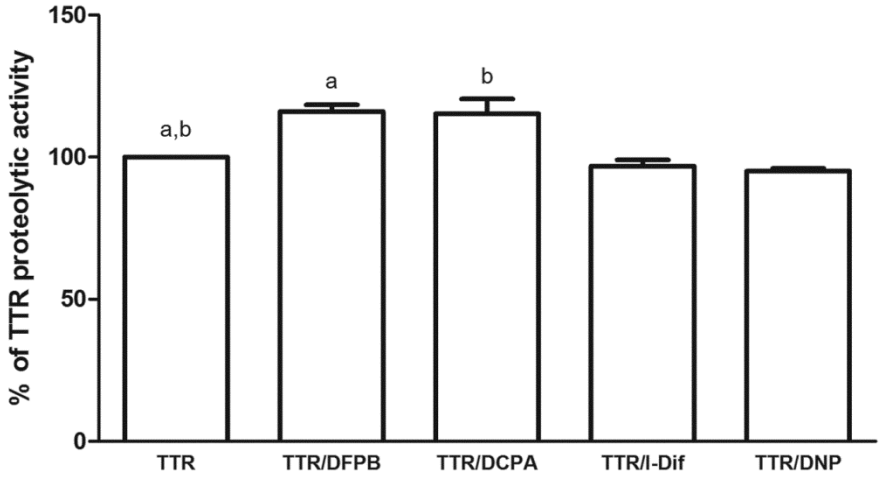

Figure 7 - Effects of selected compounds on Aβ proteolysis by TTR, showing that DCPA and DFPB improved the TTR proteolytic activity by approximately 15%, whereas other compounds such as I-Dif and DNP produced no effect. $^{a,b}p<0.05$

Effect of TTR natural ligands on TTR binding to Aβ and impact in Aβ proteolysis

Previous work by competition binding assays showed that Aβ peptide did not compete with T_4 for binding to TTR (Sciarrone et al., 2008); here we show that, on the contrary, Aβ displayed decreased binding to TTR saturated with RBP, as no significant competition was observed between ^{125}I-WT TTR and cold (WT TTR+RBP) (figure 8).

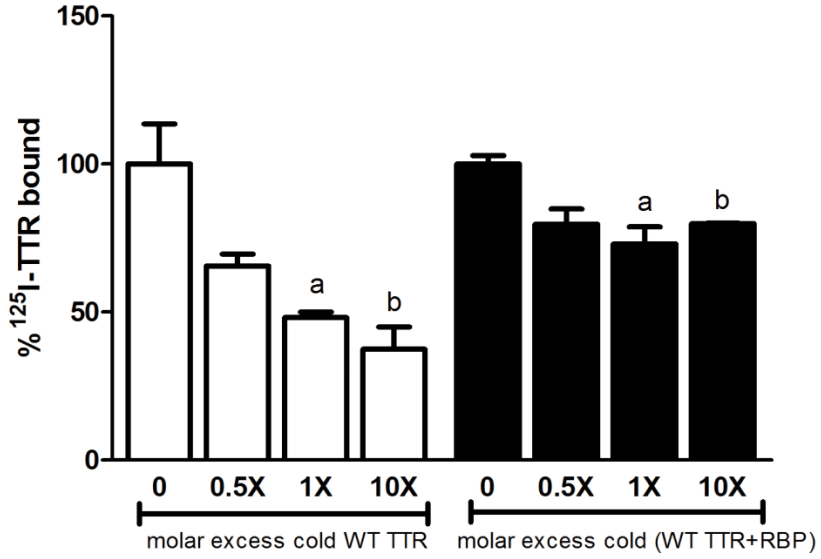

Figure 8 - Competition binding assay between Aβ and ^{125}I-TTR in the presence of increasing concentrations of cold TTR or cold TTR saturated with RBP, showing that the presence of RBP abrogated binding.

We next investigated the impact of the natural ligands (i.e. T_4 and RBP) in the proteolytic capacity of TTR to degrade Aβ peptide. We found that T_4 did not affect the TTR ability to cleave Aβ peptide; in contrast, when TTR is complexed with RBP, it drastically reduced its capacity to cleave Aβ peptide, in as much as 82% (figure 9).

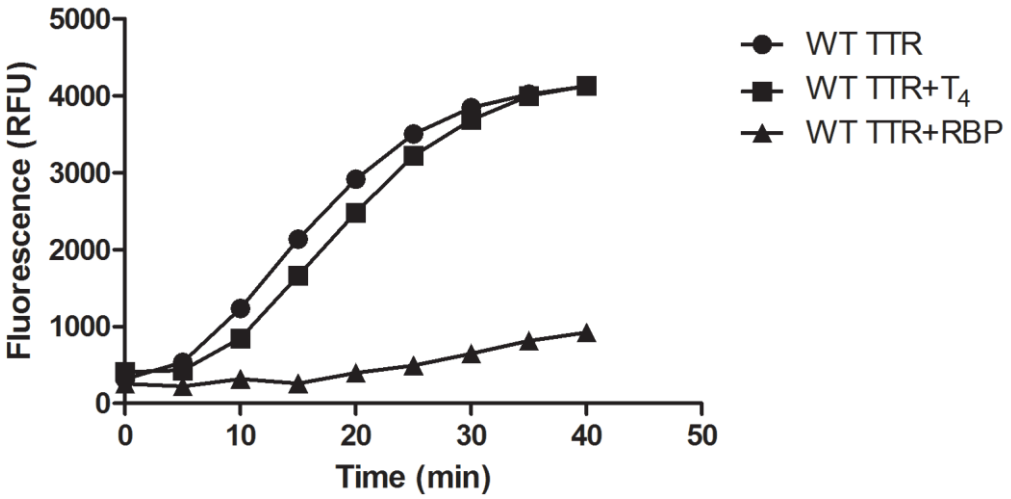

Figure 9 - Effect of T_4 and RBP in Aβ degradation by TTR using a fluorescent peptide containing an Aβ sequence. Similarly to binding, TTR proteolytic activity is abolished in the presence of RBP, while T_4 does not interfere with this activity.

Discussion

Apart from its function as a carrier of T_4 and retinol, TTR has taken more recently a very important role in amyloidogenic diseases and it is one of the 30 human proteins associated with amyloidosis, in particularly FAP. Although, clinically, TTR concentration in serum can be utilized as a marker of nutritional/inflammatory status (Potter and Luxton, 1999) its reduction in CSF and plasma has been associated with AD development (Serot et al., 1997, Velayudhan et al., 2011). In fact, TTR has been described as the major Aβ binding protein in CSF avoiding its aggregation and toxicity (Schwarzman et al., 1994, Schwarzman and Goldgaber, 1996). Furthermore, investigation using *in vivo* models has established TTR as a survival molecule since increased levels of TTR are observed in transgenic mice for APP (Stein and Johnson, 2002). Moreover, disruption of the TTR gene results in increased and earlier Aβ deposition (Choi et al., 2007). However, the mechanism by which TTR protects against Aβ toxicity, the pathways and molecules involved are not identified. Recently, we proposed that TTR can proteolytically process Aβ peptide, generating smaller and less amyloidogenic new peptides enabling cells to eliminate them (Costa et al., 2008).

Furthermore, It was described that TTR is able to inhibit Aβ fibril formation and to disrupt Aβ pre-formed fibrils (Sciarrone et al., 2008). A common structure is attributed to amyloid, independently of its protein precursor: characteristically fibrilar, with an unbranched appearance, consisting of a number of filaments aggregated side-by-side, forming fibres 75±100A in diameter and with variable length. There is a predominant beta-pleated sheet structure and extensive antiparallel strands with their axes arranged perpendicular to the longitudinal axes of the fibre. All the deposits bind the dye Congo Red resulting in a very characteristic positive green birefringence when viewed under polarized light and yellow± green fluorescence upon staining with Thioflavin S. Thioflavin T (Th T) interacts specifically with amyloid fibrils generating a characteristic fluorescent signal. Due to these facts, we reasoned that TTR could also disrupt amyloid fibrils other than the ones derived from the Aβ peptide. However, TTR add no effect on amylin, insulin and β2-microglobulin fibrils, suggesting that TTR is recognizing a sequence and not a structural motif, further supporting the proteolytic hypothesis.

Previous work, established that different TTR variants bind Aβ peptide with different affinities; however no differences were found in their capacity to avoid Aβ aggregation as qualitatively determined by TEM analysis,. In the present work, using quantitative assays, we showed differences between WT and T119M TTR and the less stable L55P TTR mutant regarding their interaction with Aβ. We described that L55P TTR, contrarily to the WT and T119M counterparts, when incubated with Aβ peptide was not able to prevent Aβ

aggregation and fibrillization; in addition, this variant was also not capable of avoiding Aβ oligomers toxicity. In addition, we showed (chapter I) that plasma TTR from aMCI and AD patients binds less T_4 than age-matched controls, thus suggesting TTR impaired function and protein instability.

One of the therapeutic strategies for TTR-related amyloidosis aims at stabilizing the TTR tetrameric fold using small molecules which can either i) bind the T_4 binding central channel or ii) bind outside the T_4 binding channel. In group I, drugs such as iododiflunisal (Almeida et al., 2004), TIP (Miroy et al., 1996), DCPA and DFPB (Almeida, MR., personal communication, 2011), diclonisal (Sekijima et al., 2006), benzoxazole, DES (Morais-de-Sa et al., 2004), flufenamic acid (Peterson et al., 1998, Baures et al., 1999), DNP (Raghu et al., 2002) and genistein (Trivella et al., 2010) have been identified. Previous work by Cardoso et al (Cardoso et al., 2007) showed that TTR stabilization may be achieved by mechanisms other that binding to the T_4 channel (group II) and compounds such as diclofenac (Almeida et al., 2004), resveratrol (Morais-de-Sa et al., 2004), DIF (Almeida et al., 2004), daidzein (Radovic et al., 2006) were identified to avoid TTR aggregation in cellular studies (Cardoso et al., 2007) and in *in vivo* studies using an FAP mice model (Macedo et al., 2008, Cardoso et al., 2010).

We reasoned that stabilization of TTR by these two types of compounds could also improve binding to Aβ. If true, poor TTR/Aβ binding of L55P TTR would be rescued by TTR stabilizing drugs. For compounds belonging to group I, we used IDIF and DIF as controls, positive and negative, respectively. Our hypothesis was confirmed as, in fact, DCPA - a strong T_4 competitor - was also able to significantly improve TTR/Aβ binding. We also investigated the effect of drugs belonging to group II (non-T_4 competing drugs) on TTR/Aβ binding and found, among the compounds tested that resv also increased TTR/Aβ interaction, further confirming our hypothesis. DFPB was shown to compete moderately with T_4 for TTR binding (Almeida M.R., personal communication, 2011), but was able to improve TTR/Aβ interaction as much as DCPA and I-Dif. DFPB has been shown to greatly avoid TTR aggregation using a cellular model (Cardoso et al., 2007), however, by IEF, DFPB does not produce the expected increase in tetramer/monomer TTR ratio (Cardoso et al., 2007) and thus its TTR-stabilizing properties must be tetramer-independent. To further understand how DFPB stabilizes TTR, it is important to determine the structure of the complex.

Recently, it has been shown that EGCG is able to avoid TTR aggregation *in vitro and in vivo* (Ferreira et al., 2009, Ferreira et al., 2012a) and to disaggregate pre-formed TTR amyloid fibrils (Ferreira et al., 2011). Although EGCG does not compete with T_4, its binding at the surface of the protein results in TTR tetrameric stabilization as assessed by IEF (Ferreira et al., 2009). In fact, Miyata et al. determined the crystal structure of the

EGCG-V30M TTR complex and showed that there are three EGCG binding sites in TTR that are not related with T_4 binding sites. Although, it is not clear which residues are crucial for EGCG/TTR binding, they described that EGCG binds in the interface of both dimers, resulting in stabilization of the tetramer (Miyata et al., 2010). In the present studies, EGCG as well as other tested compounds had no effect or even worsened TTR/Aβ binding, which can be explained if these drugs bind in or near the Aβ binding in TTR, thus masking their ability to improve TTR stability and consequent increase in Aβ binding.

To further ascertain the influence of stability in the role of TTR in AD, namely on proteolysis, we quantified TTR proteolytic activity after incubation with a fluorogenic peptide encompassing 6 residues of the Aβ peptide sequence containing one of the TTR cleavage sites as previously described (Costa et al., 2008). In our assay, only DCPA and DFPB facilitated Aβ proteolysis by TTR, raising the hypothesis that binding and proteolysis are two distinct mechanisms through which TTR protects against AD. At this point there is no information neither on the TTR region that binds Aβ nor on the residues important for its proteolytic activity, which was firstly described for ApoAI (Liz et al., 2004) and more recently for NPY (Liz et al., 2009). Possibly, the two events are directly related, and depend on each other (for instance binding preceding proteolysis), but with different regions of the TTR molecule responsible for the two activities.

TTR natural ligands showed different effects in TTR binding and proteolytic activity towards Aβ peptide. While T_4 did not affect TTR/Aβ interaction (Sciarrone et al., 2008) and TTR proteolytic activity, RBP not only prevented TTR binding to Aβ but also abolished TTR ability to cleave the peptide. Liz et al. using a fluorogenic substrate corresponding to an apoA-I fragment encompassing amino acid residues 223–228, established that while TTR proteolytic activity was only slightly affected when complexed with T_4, TTR complexed to RBP lost its proteolytic activity (Liz et al., 2004). Because not all TTR is bound to RBP (the ratio RBP: TTR in plasma is approximately 0.3 in healthy individuals indicating that most of the circulating TTR remains free of ligand (Shoji and Nakagawa, 1988, Filteau et al., 2000)), it is expected that TTR proteolytic activity is not affected by RBP *in vivo*.

In summary, this work showed that mutations in TTR affect its ability to bind Aβ peptide, interfere with Aβ aggregation, and its noxious effects, at the cellular level. TTR protection in AD is probably dependent on its stability, and although genetic tests performed so far did not reveal mutations in the TTR gene of AD patients (Palha et al., 1996), destabilization of the protein may result from other events, such as metal ions concentration and interaction with other proteins. Small compounds known as TTR

tetrameric stabilizers are able to improve TTR/Aβ interaction; proteolysis also participates in TTR protection against Aβ toxicity and may also be modulated by small TTR stabilizers. These results may be of relevance for the design of therapeutic drugs that might improve TTR/Aβ binding and TTR proteolytic capacity to decrease or inhibit Aβ deposition in AD.

Acknowledgements

We thank Paul Moreira for his assistance in TTR production.

CHAPTER III

Transthyretin stabilization by Iodo-diflunisal promotes Aβ peptide clearance, decreases its deposition and ameliorates cognitive deficits in an AD mouse model

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Abstract

Alzheimer's disease (AD) is the most common form of dementia and represents, nowadays, 50-70% of total dementia cases. Over the last two decades, transthyretin (TTR) has been associated with AD and, very recently, a novel concept of TTR stability has been established *in vitro* as a key factor in TTR/Aβ interaction. Small compounds- TTR stabilizers- usually non-steroid anti-inflammatory drugs (NSAIDs), bind to the thyroxine (T_4) central binding channel, increasing TTR tetrameric stability and TTR/Aβ interaction.

In this work, we evaluated *in vivo* the effects of one of the TTR stabilizers identified as improving TTR/Aβ interaction – iodo-diflunisal (IDIF) – in Aβ deposition and other AD features, using an AD mouse model available and characterized in our laboratory. The results showed that IDIF administered orally bound TTR in plasma and stabilized the protein, as assessed by T_4 displacement assays, and was able to enter the brain as revealed by mass spectrometry analysis of CSF. TTR levels, both in plasma and CSF, were not altered. Further, IDIF administration resulted not only in decreased brain Aβ levels and deposition but also in improved cognitive function associated to the AD-like neuropathology in this mouse model. Aβ levels were reduced in plasma and presented a trend for reduction in the CSF, suggesting TTR promoted Aβ clearance from the brain and from the periphery. Taken together, these results strengthen the importance of TTR stability in the design of therapeutic drugs, highlighting the capacity of IDIF to be used in AD treatment to prevent and to slow the progression of the disease.

Keywords: Alzheimer's disease; amyloid-beta peptide; behaviour; cerebrospinal fluid; iodo-diflunisal; plasma; transgenic mouse; transthyretin.

Introduction

Alzheimer's Disease (AD), described for the first time by Alois Alzheimer in 1906, is characterized by progressive loss of cognitive functions ultimately leading to death (Alzheimer et al., 1995). Pathologically, the disease is characterized by the presence of extraneuronal amyloid plaques consisting of aggregates of amyloid-beta (Aβ) peptide, and neurofibrillary tangles (NFTs) which are intracellular aggregates of abnormally hyperphosphorylated tau protein (Pimplikar, 2009).

In the mid-nineties Schwarzman and colleagues showed, for the first time, a relationship between AD and a cerebrospinal (CSF) and plasma circulating protein named transthyretin (TTR) (Seibert and Nelson, 1942, Schwarzman et al., 1994). TTR is a 55 kDa homotetrameric protein involved in the transport of thyroid hormones and retinol through binding to retinol-binding protein (RBP). TTR is mainly synthetized in the liver and choroid plexus, but other sites of synthesis have been described in mammals (Oliveira et al., 2012). In human plasma, TTR, thyroxine-binding globulin (TBG) and albumin are responsible for the delivery of thyroxine (T_4) into target tissues (Robbins, 1991). Although TBG is much less concentrated in the plasma than TTR, it presents the highest affinity constant for T_4 and transports about 70% of plasma T_4. TTR has an intermediate affinity for T_4 transporting about 15% of the hormone whereas albumin presents the lowest binding affinity (Robbins, 1991, Loun and Hage, 1992). In rodents, however, TTR is the main circulating T_4-binding protein transporting approximately 50% of the hormone (Oliveira et al., 2012).

Schwarzman and co-workers described TTR as the major Aβ binding protein in CSF, able to inhibit Aβ aggregation and toxicity, suggesting that when TTR fails Aβ sequestration, amyloid formation occurs (Schwarzman et al., 1994, Schwarzman and Goldgaber, 1996). Supporting the importance of TTR in AD is the observation that TTR is decreased in CSF of AD patients (Hansson et al., 2009); very recently, we and other authors showed that TTR is also decreased in sera from AD patients (Han et al., 2011, Velayudhan et al., 2011, Ribeiro et al., 2012a). In addition, data from transgenic mice showed that APPswe/PS1ΔE9 mice exposed to an enriched environment presented reduced signs of AD-like neuropathology and altered expression of several genes including upregulation of TTR (Lazarov et al., 2005). Moreover, AD transgenic mouse models with genetically altered TTR levels provided evidence (although sometimes conflicting) for a critical role of TTR in AD (Choi et al., 2007, Buxbaum et al., 2008, Wati et al., 2009, Doggui et al., 2010, Oliveira et al., 2011).

TTR mutations are associated with Familial Amyloid Polyneuropathy (FAP), a systemic amyloidosis with a special involvement of the peripheral nerve (Benson and

Kincaid, 2007). It is believed that the amyloidogenic potential of the TTR variants is related to a decrease in tetrameric stability (Quintas et al., 1997) and it is thought that dissociation of the tetramer into monomers is the basis of a series of events that lead to the formation of TTR amyloid (Cardoso et al., 2002, Almeida and Saraiva, 2012). 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 (Almeida et al., 2005, Bulawa et al., 2012). Such stabilization can be achieved through the use of small compounds sharing molecular structural similarities with T_4 and binding in the T_4 central binding channel (AlmeidaMiroy et al., 1996, Baures et al., 1999, Raghu et al., 2002, Almeida et al., 2004, Morais-de-Sa et al., 2004).

To further gain insights into the factors affecting TTR decrease in AD, we previously assessed plasma TTR binding to T_4 and described decrease ability of the protein to carry T_4 in AD patients, indicating that this function of TTR is impaired (Ribeiro et al., 2012a). We hypothesized that TTR stabilization may be a key factor in the TTR/Aβ interaction since previous works showed that TTR amyloidogenic variants bind less to Aβ peptide (Schwarzman et al., 2004, Sciarrone et al., 2008). We then postulated that the use of small compounds known to stabilize the TTR tetrameric fold should result in improved TTR binding to Aβ, and could be used as potential therapeutic strategies in AD; indeed we were able to identify drugs such as iodo-diflunisal (IDIF), resveratrol, dinitrophenol (DNP), 2-((3,5Dichlorophenyl)amino)benzoic acid (DCPA) and 4-(3,5-difluorophenyl) (DFPB), that strengthened TTR/Aβ interaction (Ribeiro et al., 2012b).

The present work aimed at testing in vivo the effect of one of the drugs identified as improving TTR/Aβ interaction- IDIF- in Aβ deposition and other AD features, using an AD mouse model previously characterized and shown to present gender-associated modulation of brain Aβ levels by TTR (Oliveira et al., 2011). IDIF has been described as a potent TTR tetrameric stabilizer *in vitro* (Cardoso et al., 2007) in the context of FAP, and was shown to be one of the best at improving TTR/Aβ interaction, among the tested compounds (Ribeiro et al., 2012b).

We are raising for the first time the hypothesis that restoring or improving TTR/Aβ binding can be a therapeutic avenue in AD.

Materials and Methods

Animals

The mouse model AβPPswe/PS1A246E/TTR used in this study was generated by crossing AβPPswe/PS1A246E transgenic mice (Borchelt et al., 1997) (B6/C3H background) purchased from The Jackson Laboratoy with TTR-null mice ($TTR^{-/-}$) (SV129 background) (Episkopou et al., 1993) as previously described (Oliveira et al., 2011). In this study, we used cohorts of littermates AβPPswe/PS1A246E/$TTR^{+/+}$ and AβPPswe/PS1A246E/$TTR^{+/-}$ female mice aged 5 or 7 months.

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 the next sections, the AβPPswe/PS1A246E/TTR colony will be referred to as AD/TTR, and the different genotypes AβPPswe/PS1A246E/$TTR^{+/+}$ and AβPPswe/PS1A246E/$TTR^{+/-}$ referred to as AD/$TTR^{+/+}$ and AD/$TTR^{+/-}$, respectively.

Synthesis of Iododiflunisal meglumine salt

To 2 mL of water 1.22 g (6.23 mmol, 1.0 equiv.) of *N*-methyl-D-glucamine (meglumine) were added. Then 0.5 mL of ethanol was added. To this solution 2,342 g (4.23 mmol, 1 equiv.) of iododiflunisal were added in small portions during 15 min. The suspension was stirred for 30 min till becoming a homogeneous solution. After 2 h the solution was evaporated under reduced pressure, water was added and the mixture was frozen-down to yield 3.7 g of a yellowish solid. The residue was dried on a desiccator over P_2O_5.

^{1}H-RMN (400.1 MHz; DMSO-d_6): 2.52 (s, 3H), 2.86-3.05 (m, 2H), 3.20-3.51 (m, 5H), 3.56 (dd, *J* = 10.7, 3.2, 1H), 3.63 (dd, *J* = 5.2, 1.6, 1H), 3.83 (ddd, *J* = 8.9, 5.1, 3.3, 1H), 4.33 (br s, 1H), 4.57 (br s, 1H), 5.39 (br s, 1H), 7.07 (tdd, *J* = 8.6, 2.7, 1.1, 1H), 7.24 (ddd, *J* = 11.5, 9.3, 2.6, 1H), 7.46 (td, *J* = 9.0, 6.6, 1H), 7.74 (dd, *J* = 2.4, 1.5, 1H), 7.79 (t, *J* = 2.1, 1H). **^{13}C-RMN (100.6 MHz, DMSO-d_6):** 33.1, 50.9, 63.4, 68.4, 70.2, 70.5, 71.4, 87.3, 104.4 (dd, *J* = 27.3, 25.7), 111.9 (dd, *J* = 21.0, 3.7), 119.1, 122.9 (d, *J* = 1.4), 124.0 (dd, *J* = 13.4, 3.8), 130.4 (d, *J* = 3.1), 158.9 (dd, *J* = 247.5, 12.2), 161.1 (dd, 246.0, 12.2), 163.8, 170.3.

^{1}H-RMN (400.1 MHz; D_2O): 2.72 (s, 3H), 3.08-3.28 (m, 2H), 3.52-3.92 (m, 5H), 4.03-4.18 (m, 1H), 6.55.6.76 (m, 2H), 6.81-6.98 (m, 1H), 7.46 (s, 1H), 7.64 (s, 1H).

(Reference procedure: PATENT **US 4748174 A: Water soluble salts of an NSAID with meglumine/glucamine)**

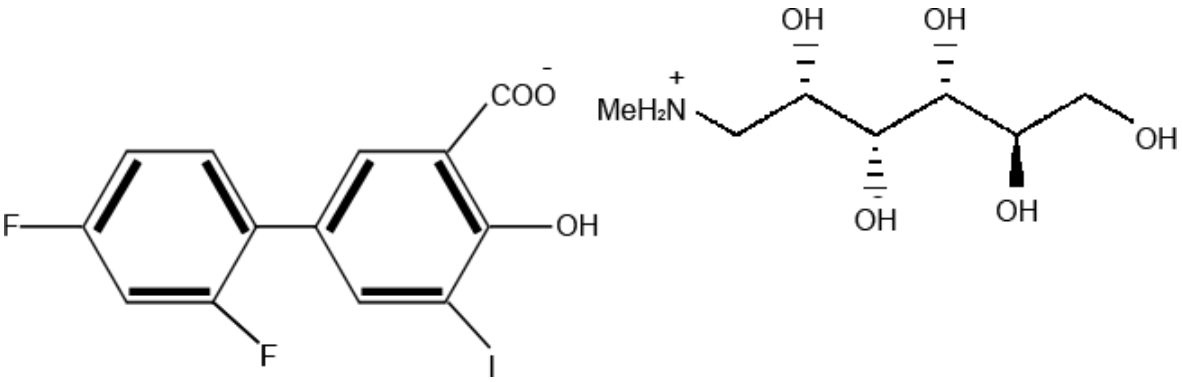

Figure 1- Structure of the Iododiflunisal meglumine salt (MW 571.31)

Chronic IDIF administration

Meglumine IDIF salt was dissolved in water and administered in the drinking water (2.8 mg drug/rodent/day – 575 ppm) over two months ($AD/TTR^{+/+}$ n=7 and $AD/TTR^{+/-}$ n=9; mice aged 5 months).

Age matched control mice were maintained in the same conditions but given water alone ($AD/TTR^{+/+}$ n=7 and $AD/TTR^{+/-}$ n=9).

Acute IDIF administration

To confirm the presence of IDIF salt in the CSF, acute treatment using a higher concentration of IDIF was performed.

The drug was dissolved in water as before and administered in the drinking water at a higher dose (28 mg drug/rodent/day – 5750 ppm) for 3 days ($AD/TTR^{+/+}$ n=4; mice aged 7 months). Age matched control animals were maintained in the same conditions but given water alone ($AD/TTR^{+/+}$ n=4).

Tissue processing

After IDIF administration, animals were sacrificed following anaesthesia with a mixture of ketamine (75mg/kg) and medetomidine (1mg/kg) administrated by intraperitoneal injection. CSF was collected from the cisterna magna, assessed for blood contamination analysis as previously described (Huang et al., 1995) and stored at -80ºC. Blood was collected from the inferior vena cava in syringes containing EDTA as anticoagulant, followed by centrifugation at 1000 × *g* for 15 min at room temperature (RT). Plasma

samples were then collected and stored at -80ºC. Brains were removed and bisected longitudinally; each half was either immediately frozen for biochemical analyses, or fixed for 24h at 4ºC in 10% neutral buffered formalin and then transferred to a 30% sucrose solution for cryoprotection before cryostat sectioning and immunohistochemical analyses.

Thyroxine binding assays

Qualitative studies on the displacement of T_4 from plasma TTR by IDIF were performed by incubation of 5µl of mouse plasma (treated and non-treated animals) with [^{125}I]T_4 (specific radioactivity 1250 µCi/µg; concentration 320 µCi/ml; Perkin Elmer, Boston, MA, U.S.A.) o/n at 4ºC. Plasma proteins were separated using a native PAGE protocol as previously described (Saraiva et al., 1988). Finally, the gel was dried, exposed to phosphor imaging (Typhoon 8600; Molecular Diagnostics, Amersham Biosciences), and analyzed using Image J 1.42q software (Wayne Rasband, National Institutes of Health, USA).

LC-DAD-ESI-MS/MS Analysis of IDIF

The identification of IDIF was confirmed by HPLC online coupled with electrospray ionization tandem mass spectrometry. The HPLC system (Finnigan, Thermo Electron Corporation, San Jose, CA) consisted of a low-pressure quaternary pump (Thermo Finnigan Surveyor), an auto-sampler (Thermo Finnigan Surveyor) with 200-vial capacity sample and a PDA (photodiode array) detector (Thermo Finnigan Surveyor). Separations were carried out on a Kinetex 2.6 µm C18 100 Å, LC Column 150 x 4.6 mm (Phenomenex Inc., USA) with isocratic elution of 50% acetonitile containing 0.1% TFA and 50% water containing 0.1% TFA, at a flow rate of 0.5 mL/min. A total of 25 µL (IDIF standards) or 10 µL (CSF samples) was injected onto the column which was kept at 20ºC.

An ion-trap mass spectrometer (Finnigan LCQ Deca XP Plus, San Jose, CA) equipped with electrospray ionization (ESI) source was used. Simultaneous acquisition of mass spectral data and photodiode array (PDA) data was processed by using Xcalibur software version 1.4 (Finnigan, San Jose, CA). Optimal operating parameters of the ESI interface and quadrupole/ion trap were found by infusing a standard solution of IDIF (0.02 mM in water/acetonitrile) at 3 µL/min using a Finnigan syringe pump. The optimum conditions of the interface were selected as follows: source voltage, 5.0 kV; source current, 0.05 mA; capillary voltage, -37.0 V; capillary temperature, 325 ºC; sheath gas flow, 90 arbitrary units; auxiliary gas flow, 25 arbitrary units; collision energy for fragmentation, 45 (normalized collision energy). Acquisition of the mass data was performed between m/z 50.00 and 2000.00. The pseudomolecular ions were fragmented by collision-induced dissociation (CID) with the nitrogen collision gas in the ion trap (trap

CID). The negative ion mode was selected in this work due to a better signal-to-noise ratio in comparison with positive ion mode. Pools of CSF (15 µl) from control mice, mice treated with IDIF for 2 months (chronic treatment) or from mice treated for 3 days (acute treatment) were thus analyzed.

CSF and Plasma TTR levels determination

CSF and plasma TTR levels were quantified using Mouse Prealbumin ELISA Kit (MyBioSource) according to the manufacturer's instructions. Data were expressed in mg/mL.

Brain Aβ40 and Aβ42 levels determination

Aβ levels in brain extracts (detergent-soluble and formic acid (FA)-soluble Aβ) were evaluated using sandwich ELISA analysis as previously described (Oliveira et al., 2011). Each half brain was homogenized in 1 mL of 0.1% Triton X-100 and 2 mM EDTA in 50 mM Tris-buffered saline (TBS) (pH 7.4) with protease inhibitors (Amersham Biosciences), and centrifuged at 21500 × *g* for 15 min at 4°C. The supernatant was collected, aliquoted, and frozen at -80°C for subsequent analysis - detergent-soluble fraction of brain Aβ. The FA-soluble fraction of brain Aβ was obtained by homogenization of the pellet with 1mL 70% FA in distilled water (dH_2O) and centrifugation as described before. The supernatant was collected and neutralized with 1 M Tris (pH 11.0) (1/20 dilution), aliquoted and frozen at -80°C. Sandwich ELISA analyses of Aβ40 and Aβ42 in the obtained fractions were performed using Human Aβ40 and Human Aβ42 ELISA Kits (Invitrogen) according to the manufacturer's instructions. Data were expressed in pmol/g wet tissue.

Aβ immunohistochemistry

Aβ plaque burden was evaluated by using a monoclonal biotinylated Aβ1-16 antibody (6E10) (Covance Research Products, Inc.) to perform free-floating immunohistochemistry of 30 µm-thick cryostat coronal brain sections. According to a previously described protocol by Oliveira et al. (Oliveira et al., 2011), free-floating brain sections were washed twice in phosphate-buffered saline (PBS), and once in dH_2O. For partial amyloid denaturation, 70% formic acid (FA) was used for 15 min at RT, with gentle agitation. After washing in dH_2O and then PBS, endogenous peroxidase activity was inhibited with 1% hydrogen peroxide (H_2O_2) in PBS for 20 minutes. Following PBS washes, sections were

blocked in blocking solution (10% fetal bovine serum (FBS) and 0.5% Triton X-100) for 1 h at RT and then incubated with biotinylated 6E10 primary antibody (diluted 1/750 in blocking buffer) overnight (O/N) at 4°C, with gentle agitation. Sections were washed with PBS and incubated in Vectastain® Elite ABC Reagent (Vector Laboratories, Inc.). Sections were once more washed in PBS followed by development with diaminobenzidine (Sigma-Aldrich, Inc.), mounted on 0.1% gelatin-coated slides and were left to dry O/N at RT. After dehydration, slides were coverslipped under Entellan® (Merck & Co., Inc.). Sections were examined with an Olympus BX50 light microscope.

Aβ plaque burden was evaluated using Image-Pro Plus software, by analyzing the immunostained area fraction in the hippocampus and cortex (expressed as percentage of analyzed area) of three sections *per* animal visualized by microscopy (Olympus DP71 microscope).

CSF and plasma Aβ42 levels determination

Sandwich ELISA analyses of Aβ42 in CSF and plasma were performed using Human Aβ42 ELISA Kits (Invitrogen) according to the manufacturer's instructions. Data were expressed in pg/ml.

Morris water maze test (MWM)

Prior to the beginning of the behavioral tests, mice were allowed a 2-week adaptation period to their new surroundings. Tests were conducted in the dark (active) phase.

A circular pool (110 cm in diameter, 30 cm deep) filled with water (27±2°C) to a depth of 18.5 cm was placed in a quiet room decorated with contrast visual cues. Water was made opaque by the addition of white non-toxic ink. Abstractly, the pool was divided into four quadrants, and eight start locations were defined—north (N), south (S), east (E), west (W), northeast (NE), southeast (SE), (northwest) NW and southwest (SW)—at equal distances to the center. An escape platform (10 x 10 cm) was immersed 0.5 cm bellow the water line. On the first two days, mice were subjected to cued learning in order to test them for their ability to learn to swim to a cued goal. For this procedure, curtains were closed around the maze to reduce the availability of distal cues and a flag was attached to the hidden platform. Animals were given four 60-second trials per day, each trial with different start and goal positions. Between each trial, mice were allowed to stay on the platform for 15 seconds. After the cued learning, mice were tested for their visual acuity. For this, a large plastic cue was placed on the platform and each mouse was scored for its latency to reach the platform. Twenty-four hours before this test, an identical plastic cue

was placed in each of the mouse housing cages to minimize the possible effects of exposure to a novel object. After the cued learning, a 7-day hidden-platform learning phase was initiated. The platform was placed in the SW quadrant and the animals were scored for their latency to find the hidden platform. Mice were given four 60-second trials per day, each trial with different start locations, and inter-trial intervals on the platform of 30 and 15 seconds on days 1 and 2-7, respectively. Twenty-four hours following day 7 of the hidden-platform learning phase, the platform was removed and each mouse was subjected to a 30-second probe trial starting 180° (NE) from the original platform position (SW). The number of platform-site crossovers, the latency to first target-site crossover, the percent time spent in the target quadrant (and also in the opposite quadrant) compared with the other quadrants were evaluated using SMART software.

Open field test

Before the MWM testing, general locomotor activity levels were evaluated by performing the Open field test. Each mouse was individually placed in the center of an acrylic cubic open field arena (40 x 40 x 40 cm) equipped with two parallel arrays of photocells (San Diego Instruments, San Diego, CA), and data were collected at 1 minute intervals over a 30-minute session. The total activity of each animal was automatically registered.

Statistical analyses

All data were expressed as mean±SE. D'Agostino and Pearson tests were used to evaluate normal distributions. The differences in plasma TTR levels, Aβ levels and plaque burden were analyzed using one way analysis of variance (ANOVA) with Bonferroni's post hoc tests. The differences in the behavior test (MWM) were analyzed using a T-Test analysis. A $p<0.05$ was considered significant for all analyses. GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com, was the statistical software used.

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 (Oliveira et al., 2011), as elevated brain levels of Aβ42 were observed in particular in AD/TTR$^{+/-}$ female mice as compared to their AD/TTR $^{+/+}$ counterparts. In this model the onset of Aβ deposition occurs around 6 months of age, thus, IDIF administration began at the age of 5 months, before the onset of deposition, in AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ female mice. Treatment lasted for 2 months and thus animals were sacrificed at 7 months of age, after the start of Aβ deposition.

IDIF binds TTR *in vivo* displacing T_4

Our approach to investigate if IDIF was able to stabilize mouse TTR (moTTR) *in vivo* was to perform T_4 binding assays in order to determine the drug ability to compete with T_4 for TTR binding in plasma (Figure 2). In AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ control samples, in the absence of IDIF two main proteins were shown to bind T_4: albumin and moTTR. However, it is important to mention that under non-denaturing conditions moTTR does not fully separate from albumin, and thus a single band is visualized: the upper part of the band corresponds to albumin, whereas the lower part corresponds to TTR. Unlike humans, mice TBG binds poorly to T_4, and, as a result, a much weaker band was observed. In the presence of IDIF (AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ treated samples), T_4 was displaced from TTR as deduced from the upwards shift of the larger band (albumin component of the band), resulting in increased TBG binding to T_4.

Taken together, these results suggest that orally administered IDIF was able to bind and stabilize TTR in plasma.

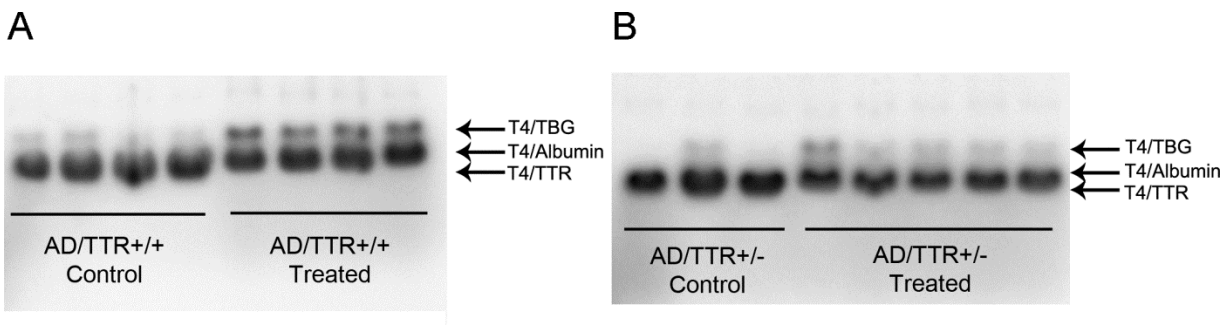

Figure 2- T_4 binding gel electrophoresis of plasma samples from AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ littermate mice control or treated with IDIF, incubated with ^{125}I-T_4. In the absence of IDIF, T_4 bound mainly TTR and albumin (represented by a single band) and, at a much lower extent, TBG. IDIF was able to promote the displacement of T_4 from TTR both in TTR/AD$^{+/+}$ (A) and TTR/AD$^{+/-}$ mice (B), as evidenced by the upwards shift of the larger band and increase of the TBG band.

IDIF enters the brain

We have recently shown that plasma TTR levels are decreased early in AD which may reflect disease disturbances in AD patients, prompting this protein to be considered a biomarker (Ribeiro et al., 2012a). Because plasma TTR binds Aβ, it might interfere with the peripheral transport and elimination of the peptide. Hence, IDIF administration in the drinking water was reasoned based on this hypothesis and its ability to cross the BBB was not mandatory. Nevertheless, as TTR is also produced in the brain, and its importance in AD has been well established, we considered important to determine if the drug entered the brain.

IDIF used in this work was formulated as the meglumine salt (*N*-methylglucamine), which is an amino sugar derived from sorbitol. Charged analytes can be separated on a reversed-phase column by the use of ion-pairing. Reversed-phase ion-pairing chromatography relies upon the addition of a counter ion to the mobile phase in order to promote the formation of ion-pairs with charged analytes. Trifluoroacetic acid (TFA) was used here as an ion-pairing reagent, in order to selectively increase the retention time of the charged analyte. TFA is the most commonly used ion-pairing agent for use in reversed-phase HPLC separations of charged analytes because it sharpens peaks and improves resolution, is volatile and easily removed.

Liquid chromatography coupled to electrospray mass spectrometry enabled us to identify the protonated molecule in positive ion mode: $[MH]^+$ ion of N-methylglucamine at m/z 196.13 ($C_7H_{18}NO_5^+$). The positively charged part of the molecule elutes early in the column (retention time 2.71 min). The negatively charged ion (2',4'-difluoro-4-hydroxy-5-iodo-[1,1'-biphenyl]-3-carboxylate), following ion-pair formation with TFA, showed the

longest elution time (retention time 41.6 min, Figure 3A) and originates the deprotonated molecule in negative ion mode: $[M-H]^-$ ion at m/z 375.13 ($C_{13}H_6F_2IO_3^-$) (Figure 3C). The observed m/z 488.53 (Figure 3C) may be ascribed to the addition of TFA (375.13 + 114.02). Loss of CO_2 and the iodide atom explains the presence, respectively, of the fragments with m/z 331.07 ([M-H-CO_2]-) and m/z 127.07 (I^-) on collisional activation in MS-MS experiments showed in Figure 3D.

Analysis of treated CSF sample obtained from mice that underwent chronic treatment with IDIF, showed a small peak which could be detected at 41.67 minutes, absent in control CSF samples (data not shown). To confirm the presence of the drug in the CSF, we performed a second treatment using a higher concentration of IDIF (acute treatment). The chromatograms obtained for control and treated CSF are shown in Figure 3E and 3G, respectively. The presence of a chromatographic peak with retention time 41.82 min was noticed for the treated sample (Figure 3E), whereas this peak was absent in control sample (Figure 3G). This compound exhibits two absorption bands (226 nm and 325 nm, Figure 3F) practically coincident with those observed for the IDIF standard (229 nm and 325 nm, Figure 3B). Total ion current (TIC) and selected reaction monitoring (SRM) scan modes were persistently applied in order to gather data which would enable us to determine the molecular weight of this compound. Its low concentration along with the ion suppression effect of TFA (ion-pairing agents are well known signal suppressors, tolerable in small amount) precluded its unambiguous identification. Furthermore, the described interactions between IDIF and TTR, including intermonomer hydrogen bonds (Gales et al., 2005) could be destabilized and dissociated in the presence of an ion-pairing agent, such as TFA. Based on the UV data and the retention time, this peak might be attributed to the negatively charged ion 2',4'-difluoro-4-hydroxy-5-iodo-[1,1'-biphenyl]-3-carboxylate with a high degree of confidence, indicating IDIF reached the CSF, became accessible to brain and with ability to exert its effects also in the brain.

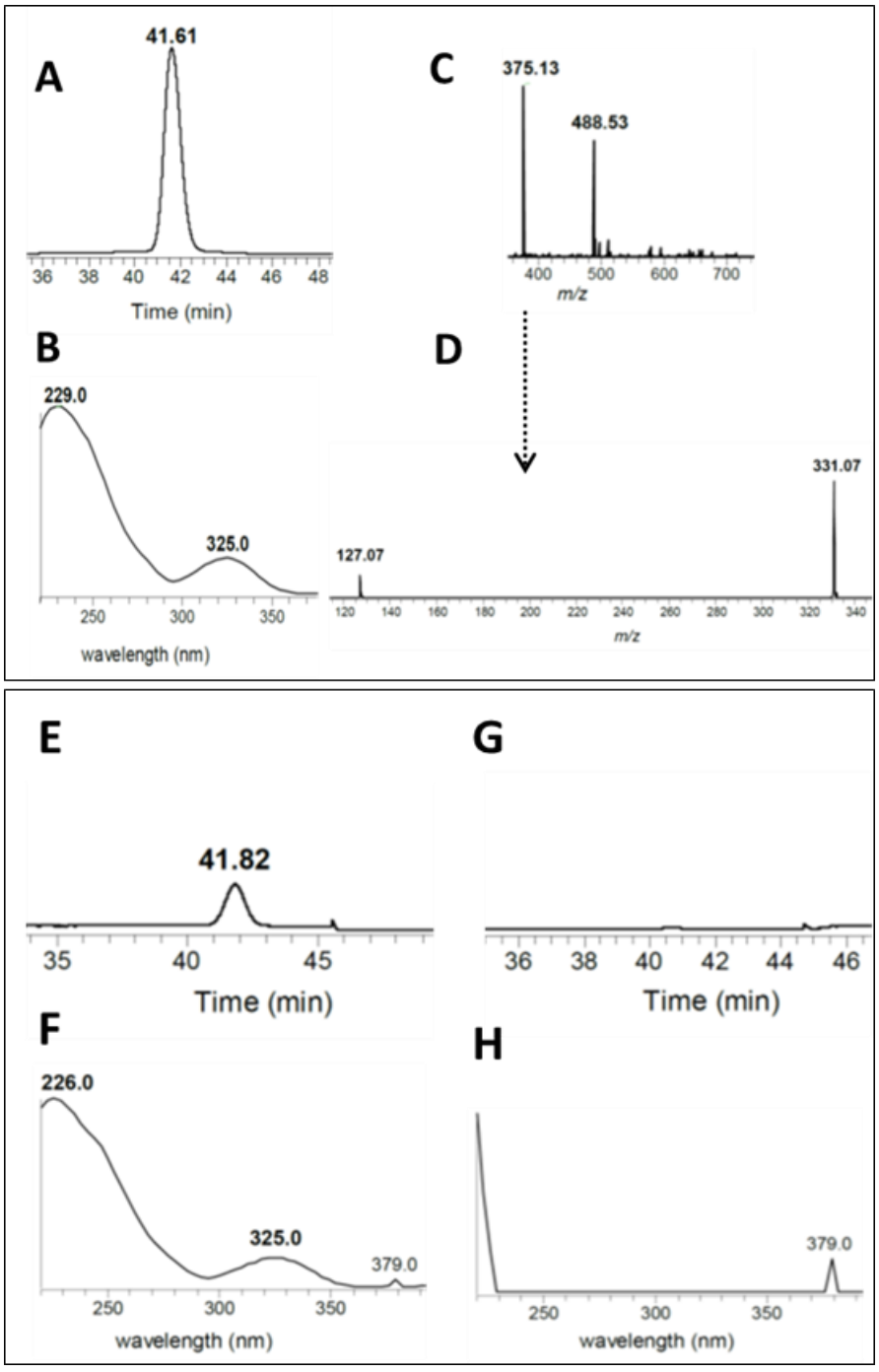

Figure 3 – Meglumine IDIF salt (0.200 mM) elutes as double peak with retention times of 2.71 minutes and 41.6 minutes. The negatively charged IDIF shows the retention time at 41.6 minutes detected by total scan PDA (Figure 3A) and exhibits two absorption bands (226 nm and 325 nm, Figure 3B). For HPLC-ESI-MS/MS analysis the deprotonated pseudomolecular ion of IDIF [M-H]- at m/z 375.13 (Figure 3C) was fragmented by collision-induced dissociation and originates the fragments with m/z 331.07 and m/z 127.07 (Figure 3D). HPLC-PDA chromatographic peak with retention time of 41.82 minutes for the treated CSF sample (Figure 3E), whereas the absence of this peak could be observed for the control CSF sample (Figure 3G). UV spectra at 41.82 minutes of treated CSF sample (Figure 3F) and control CSF sample (Figure 3H).

CSF and plasma TTR levels are not affected by IDIF administration

As expected, the quantification of TTR in both CSF and plasma evidenced the genetic reduction of TTR in AD/TTR$^{+/-}$ mice when compared to AD/TTR$^{+/+}$ animals, as we observed reduced levels of circulating TTR in both fluids in the former (Figure 4).
The impact of IDIF administration in the levels of TTR in CSF (figure 4A) and plasma (figure 4B) was also evaluated and we observed no differences in this protein levels, both in AD/TTR$^{+/+}$ and in AD/TTR$^{+/-}$ mice. Thus, IDIF did not affect TTR expression.

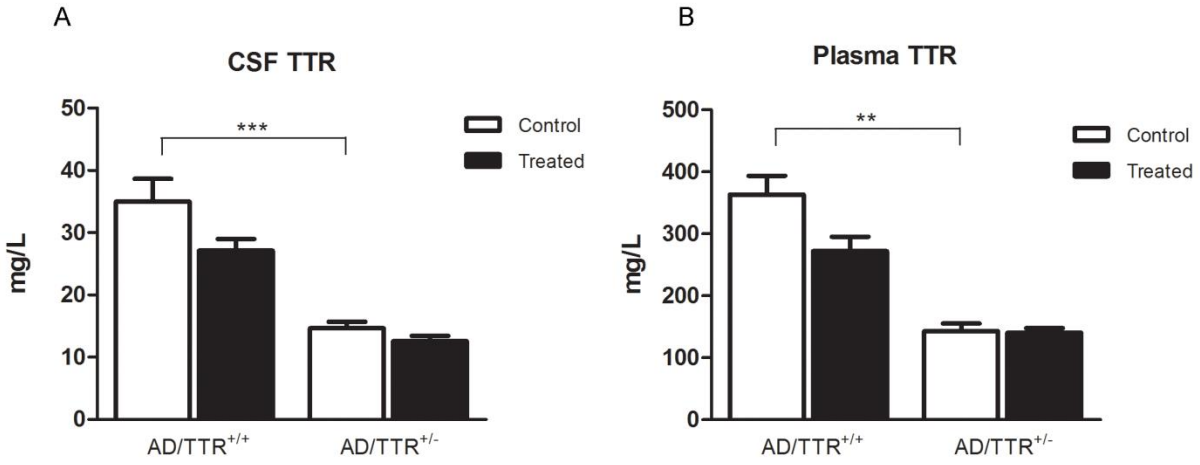

Figure 4 –TTR levels in AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ littermate mice control or treated with IDIF, in both CSF (A) and plasma (B). Significant differences were only observed between the two genetic backgrounds. Error bars represent SEM. ** $p < 0.01$; *** $p < 0.001$ (n = 7-9 animals/group).

IDIF reduces brain Aβ levels

In order to investigate the effect of IDIF in brain Aβ levels, a sandwich ELISA analyses were used to determine Aβ levels in detergent and FA extracts of hemibrains of AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ mice. In the AD/TTR$^{+/-}$ group, treated animals presented reduced levels of both FA-soluble Aβ_{40} and Aβ_{42} when compared to the age-matched non-treated controls (figure 5C and D). With respect to the detergent soluble fractions, for both Aβ_{40} (Figure 5A) and Aβ_{42} (Figure 5B) we found a trend for reduction in treated mice as compared to control animals. In the AD/TTR$^{+/+}$ group, no significant differences between control and IDIF treated mice were observed (Figure 5).
Our results suggest that TTR stabilization after IDIF administration significantly reduced brain levels of FA-soluble Aβ_{40} and Aβ_{42} in AD/TTR$^{+/-}$ mice.

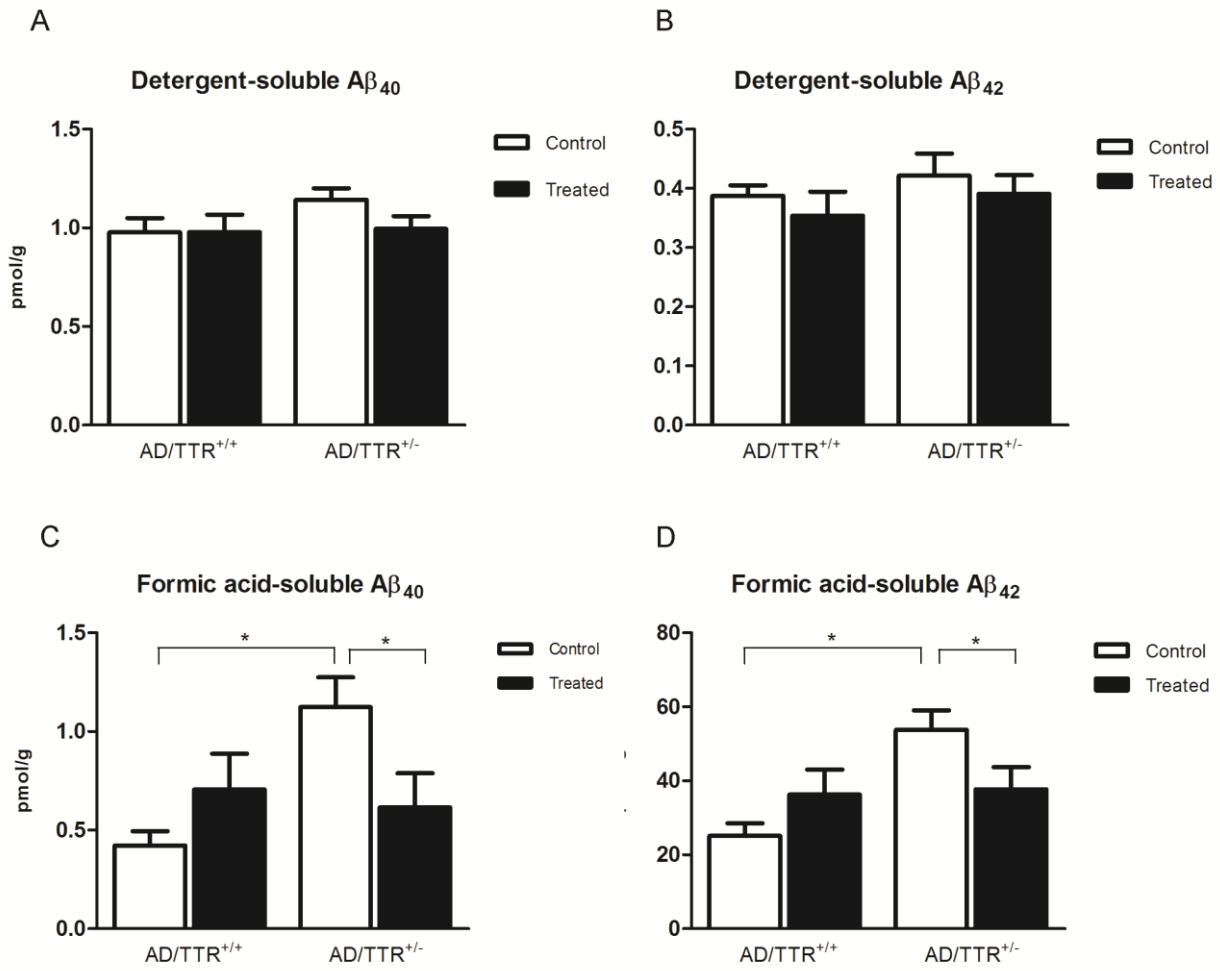

Figure 5 – Brain Aβ levels in AD/TTR+/+ and AD/TTR+/- littermate mice control or treated with IDIF, quantified by ELISA. Levels of detergent-soluble Aβ40 and Aβ42 (A and C) and of FA-soluble Aβ40 and Aβ42 (C and D) were determined. Error bars represent SEM. * $p < 0.05$. (n = 7-9 animals/group).

IDIF reduces Aβ plaque burden in AD/TTR mice

The effect of IDIF on Aβ deposition was also studied. Aβ plaque burden was estimated for both groups under investigation (AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$) and differences between control and IDIF treated animals were evaluated by means of immunohistochemical analyses using the 6E10 antibody (figure 6B) followed by quantification using the Image Pro-Plus software (figure 6A). AD/TTR$^{+/-}$ mice treated with IDIF presented decreased plaque burden compared with their control counterparts (Figure 6), corroborating the results obtained for Aβ in brain extracts. Again, no differences were found in the AD/TTR$^{+/+}$ group as Aβ plaque burden was similar between control and treated mice (Figure 6).

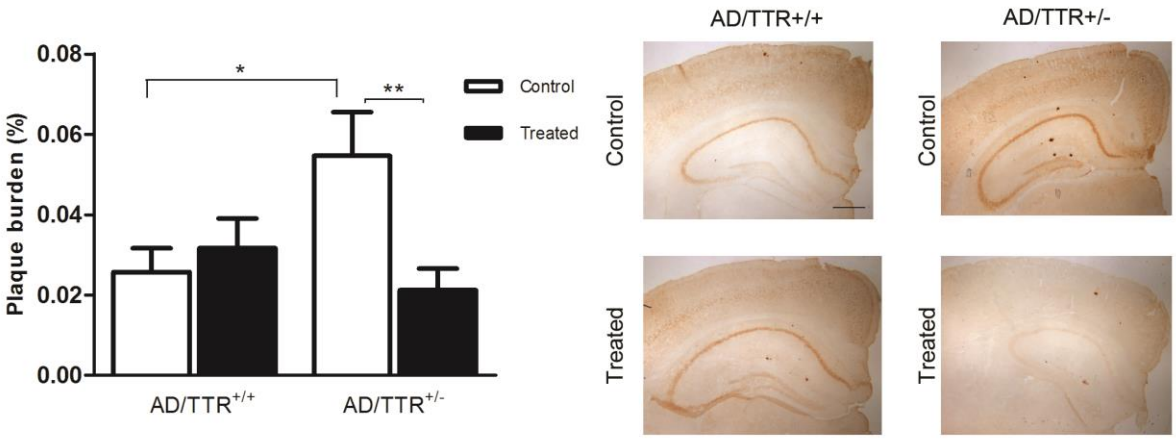

Figure 6 – Aβ plaque burden in AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ littermate mice, control or treated with IDIF. (A); Photomicrographs illustrate immunohistochemical analysis of brain Aβ plaques using the 6E10 antibody (B). Scale bar, 25 µm. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$ (n = 7-8 animals/group).

IDIF treatment reduces CSF and plasma $A\beta_{42}$ levels

In addition to quantifying the levels of Aβ in the brain, we decided it would be important to investigate Aβ levels in fluids such as CSF and plasma in order to try to gain insights into the mechanism underlying TTR protection in AD, through TTR stabilization by IDIF.

To determine Aβ levels in CSF and plasma, sandwich ELISA analyses were used. In plasma, AD/TTR$^{+/-}$ mice presented higher Aβ levels than the AD/TTR$^{+/+}$ counterparts (Figure 7), further supporting the importance of TTR in AD. IDIF administration to AD/TTR$^{+/-}$ mice lowered the peptide to levels comparable to the ones found in the

AD/TTR$^{+/+}$ littermates (Figure 7), while IDIF had no effect in this group. In CSF, no significant differences were observed, neither between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$, nor between control and treated mice (data not shown). These observations might also reflect the lower number of CSFs available; nevertheless, we detected a trend for decreased Aβ CSF levels in treated mice.

Taken together, these results suggest that IDIF might be beneficial by improving Aβ clearance, thus preventing or slowing the progression of the disease.

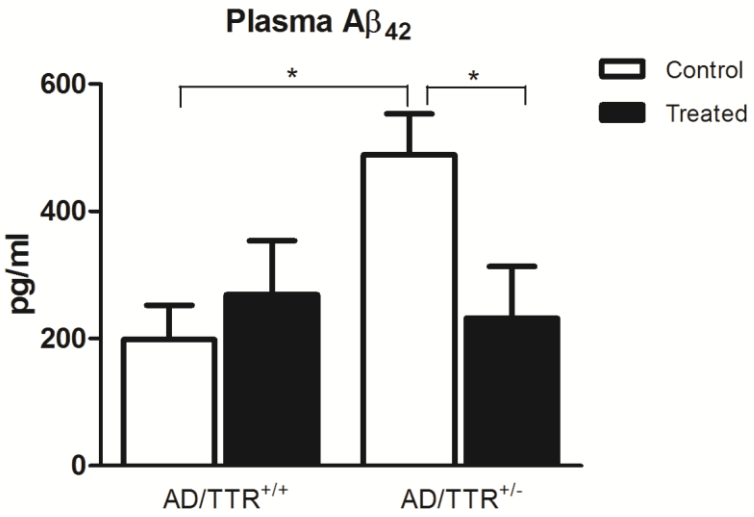

Figure 7 – Quantification of Plasma Aβ levels in AD/TTR+/+ and AD/TTR+/- littermate mice, control or treated with IDIF. AD/TTR+/- showed higher Aβ levels than their AD/TTR+/- counterparts (white bars); IDIF treatment resulted in decreased Aβ levels in AD/TTR+/- mice, remaining unaltered in AD/TTR+/- animals. Error bars represent SEM. * $p < 0.05$; (n=7-9 animals/group).

IDIF ameliorates spatial learning and memory deficits in AD/TTR mice

To assess the effect of IDIF treatment on spatial learning and memory, a MWM test was performed as described in the Materials and Methods section. The results of the hidden platform-learning phase showed that while IDIF-treated AD/TTR$^{+/-}$ mice learned to find the hidden platform —as demonstrated by the decrease in the latency along the 7-day period —, control AD/TTR$^{+/-}$ animals exhibited impaired ability to learn. In particular, significantly shorter escape latencies were found in IDIF-treated AD/TTR$^{+/-}$ mice from the fifth day of the learning phase compared to their control littermates (Figure 8B). However, no differences between these two groups were found in the probe trial (withdrawn escape platform). Regarding AD/TTR$^{+/+}$ mice, no differences were found between IDIF-treated and control animals (Figure 8A).

These results suggest that the administration of IDIF and subsequent TTR stabilization improve spatial learning skills and memory of an AD/TTR mouse model in the absence of visual and locomotor activity disturbances (data not shown).

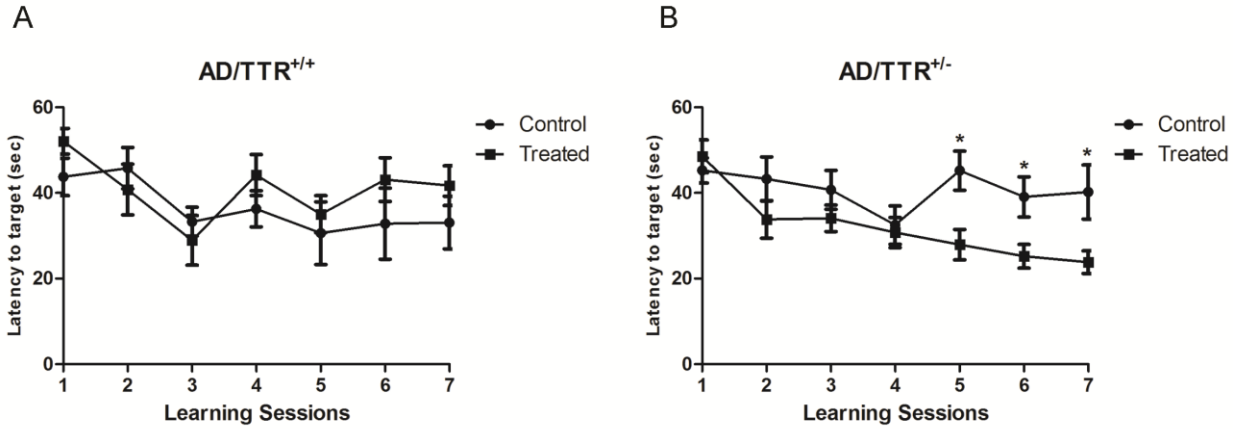

Figure 8 - Effect of IDIF on the spatial learning and memory impairments. The time spent to reached the target was observed during 7 consecutive days of training on the MWM in AD/TTR+/+ animals (control and treated) (A) and AD/TTR+/- animals (control and treated) (B). Data are expressed as means ± SEM (n = 7-9); *P<0.05, compared to the control group.

Discussion

This work presents *in vivo* evidence that TTR tetrameric stabilization by IDIF plays an important role in AD pathogenesis modulation. We showed, for the first time, that treatment with IDIF was capable of decreasing brain Aβ levels and deposition, and ameliorating cognitive deficits in an AD mouse model, through TTR stabilization.

TTR has been suggested as a protective molecule in AD but data from TTR/Aβ interaction are still controversial. Some of the studies indicated that amyloidogenic and unstable TTR mutants bind poorly to Aβ peptide (Schwarzman et al., 2004, Sciarrone et al., 2008), 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 (Hornstrup et al., 2013), further demonstrating the importance of the TTR tetramer in the protein biological activity. Other studies, however, reported that engineered monomeric TTR variants bind the peptide and arrest aggregate growth, while the tetramer promotes Aβ aggregation (Du and Murphy, 2010, Du et al., 2012, Yang et al., 2013). Very recently we showed that TTR is early decreased in plasma from MCI and AD patients (Ribeiro et al., 2012a). Additionally, plasma TTR from these patients binds less T_4 than TTR from controls (Ribeiro et al., 2012a)**.** T_4 binds TTR in a central hydrophobic channel and in order to bind, the TTR tetramer must be assembled. In this line of ideas, we suggested that TTR is destabilized in AD and its clearance accelerated, explaining the lower levels found. Importantly, we showed that the TTR/Aβ interaction can be improved, in vitro, through the use of TTR stabilizers, such as IDIF. Interestingly, epidemiological studies indicate that NSAIDs are neuroprotective, although the mechanisms underlying their beneficial effect remain largely unknown. (Ajmone-Cat et al., 2010, Côté et al., 2012).

Here we show that IDIF administered orally to AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ was able to stabilize mouse TTR *in vivo*, as deduced from its ability to displace T_4 from TTR, while TTR levels were not altered. We had hypothesized that in IDIF treated mice, after TTR stabilization, TTR clearance should normalize and thus its levels rise, compared to non-treated mice. As reported for this mouse model, TTR levels are decreased from as early as the age of 3 months, comparing to non-transgenic littermates (Oliveira et al., 2011). However, with time, as the disease continues to progress, mice are able to compensate (through a mechanism not yet unraveled), attaining TTR levels comparable to non-AD animals at age of 10 months. Thus, in this mouse model, and at ages close to the ones relevant for this study, TTR levels are already high, probably masking the expected increase in TTR levels.

Several researchers have been focusing on finding plasma markers in AD, as this fluid may also reflect disease disturbances. Similarly to what is observed in CSF, plasma TTR levels are altered in AD (Han et al., 2011, Velayudhan et al., 2011, Ribeiro et al., 2012a) leading to the assumption that peripheral TTR might also be important in the AD context. Because TTR is also synthesized in the brain, we decided it would be important to investigate if IDIF was able to enter the brain and stabilize brain TTR. In general, NSAIDs cross the blood brain barrier (BBB) efficiently, though the effective dose reaching the brain can be different under different neuropathological conditions, depending on BBB integrity (Ajmone-Cat et al., 2010). For instance, the precursor of IDIF (diflunisal) only crosses BBB in small quantities compared for example to other NSAID's, like aspirin (Macintyre et al., 2008, Yagiela. et al., 2010). Although we did not explore the mechanism of passage, we showed the presence of IDIF in the CSF, thus making it accessible to the brain. At this point we were not able to determine if the positive effects in the AD-like pathology were brought up by peripheral or central TTR, or both.

To ascertain if TTR stabilization by IDIF produced effects in AD-like neuropathology, we measured some of the available markers, such as Aβ plaque burden, brain Aβ40 and Aβ42 levels, and Aβ levels in CSF and plasma. With regard to the biochemical analyses, we evaluated the brain levels of detergent-soluble and FA-soluble $A\beta_{40}$ and $A\beta_{42}$, and revealed that AD/TTR$^{+/-}$ treated animals presented reduced levels of both FA-soluble $A\beta_{40}$ and $A\beta_{42}$ brain levels compared to age-matched controls of 7 months of age. We also analyzed Aβ plaque load by immunohistochemistry and showed it to be reduced in the AD/TTR$^{+/-}$ mice treated with IDIF. Finally, $A\beta_{42}$ levels in CSF and plasma were evaluated in order to gain insight into the mechanism by which TTR exerted its effects. The relationship between Aβ in CSF and plasma is still unclear. Burgess and co-workers, showed no alterations in plasma Aβ levels in two different AD mouse models (TgCRND8 and APP/PSI) regarding age (Burgess et al., 2006). Other authors, using another AD mouse model (Tg2576), described that Aβ levels, both in CSF and plasma, decreased with age while the non-transgenic animals showed unchanged levels (Kawarabayashi et al., 2001). In our model, we observed a trend for reduction in $A\beta_{42}$CSF levels after IDIF administration, but without statistical significance, most likely due to the limitations encountered during the collection of this fluid. On the other hand, in plasma, when we compared control AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ animals, we observed that the genetic reduction of TTR led to increase $A\beta_{42}$ levels, whereas IDIF treatment resulted in reduced plasma Aβ levels in the AD/TTR$^{+/-}$ group, suggesting that TTR promoted its clearance, leading to decreased toxicity for the organism and therefore amelioration of the AD-like neuropathology.

The importance of TTR in spatial learning and memory was firstly demonstrated by Sousa and co-workers through their studies in $TTR^{-/-}$ mice (Sousa et al., 2007). Furthermore, this role of TTR has also been described in the context of an AD mouse model (Buxbaum et al., 2008). In this study, in order to investigate whether IDIF-stabilized TTR has an impact on spatial learning and memory, we performed the MWM test. Our results clearly showed that $AD/TTR^{+/-}$ mice treated with IDIF improved their spatial learning skills and memory compared to age-matched controls. Regarding memory, the working memory seems to be especially improved since no differences were found in the probe trial which is used to assess reference memory. Our behavioral data further support a role for TTR in spatial learning and memory by providing evidence for the importance of TTR stabilization in improving cognitive deficits in an AD mouse model.

Curiously, we did not observed improvements in the IDIF treated $AD/TTR^{+/+}$ animals, in none of the assays used. Given that $AD/TTR^{+/+}$ mice exhibit a less severe AD-like neuropathology compared to $AD/TTR^{+/-}$, one might suggest that higher doses of IDIF would be necessary to result in measurable improvements. Supporting this idea is noteworthy that amelioration of IDIF treated $AD/TTR^{+/-}$ mice at both biochemical and behavioral levels did not go beyond the disease extent found in $AD/TTR^{+/+}$ animals.

In this study we decided not to use $AD/TTR^{-/-}$ animals to evaluate the importance of the TTR stabilization in this AD mouse model because it was described that 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, maybe due to the compensatory mechanisms generated by these animals as hypothesized by Oliveira and co-workers in the first characterization of this model (Oliveira et al., 2011).

In the future, it would be important to investigate whether IDIF action could be mediated through other mechanisms, namely APP processing by the modulation of γ-secretase activity (Hawkins et al., 2011) and/or action on the inflammatory process of the disease.

In conclusion, this work showed that TTR stability is critical for neuroprotection in AD and can be modulated/increased by IDIF which has the ability to enter the brain. TTR stabilization promotes Aβ peptide clearance resulting in decreased deposition and in partial reversal of cognitive deficits in the AD mouse model.

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CONCLUSIONS AND PERSPECTIVES

AD is the most common cause of dementia affecting millions of people worldwide. Characterized by a slow progressive decline in cognitive functions leading to death, AD is still an incurable neurodegenerative disorder. Researchers believe that changes that occur in the AD brain can begin 10-20 years before symptoms appear. Finding a biomarker for early detection is therefore of crucial importance for the design of therapies. Although CSF has been the main source for AD-related biochemical analysis, plasma may also reflect alterations occurring within the brain and has several obvious advantages over CSF.

In this work we found that TTR levels are decreased in plasma from both aMCI and AD patients, in particular in women. Given that AD affects more women than men (Henderson, 1997), TTR may prove to be an important AD-gender biomarker. Interestingly, 17β-estradiol is also decreased in aMCI and AD women, compared to healthy age-matched controls, providing clues on the mechanisms affected in this pathology. In fact, sex steroid hormones have been associated with the higher predisposition for AD of women and it is known that sex hormones progressively decrease with age and, in menopause, women suffer a quick decrease in hormonal production. Recently, Oliveira and colleagues reported a gender-dependent modulation of brain Aβ levels by TTR, with AD/TTR$^{+/-}$ female mice presenting increased brain Aβ42 levels compared to AD/TTR$^{+/+}$ females littermates; male mice did not present significant differences caused by the different TTR genotypes. The same work showed that TTR reduction/abolition influences brain levels of testosterone and 17β-estradiol in a gender-associated manner (Oliveira et al., 2011). Another work, reported that a treatment with 17β-estradiol in an AD transgenic mouse model results in the decrease of APP processing through β-secretase, in the increase of α-secretase activity, in the reduction of Aβ42 levels and plaque burden and also in the increased of brain TTR levels (Amtul et al., 2010). It is clear now that 17β-estradiol has an important role in AD: APP processing, Aβ levels and also in the mechanisms that contribute to Aβ clearance, such as TTR levels.

Despite the possible impact of the decrease of 17β-estradiol levels in the reduction of plasma TTR levels in women, further investigation is needed to understand why the decline of this sex hormone is more pronounced in aMCI and AD women than in healthy controls. A longitudinal study will be important to follow the behavior of estradiol levels in both groups. Moreover, it will be interesting to analyze testosterone levels in men trying to understand why men seem to be more protected than women.

Although we observed that TTR is decreased in aMCI and AD patients as compared to age-matched controls, we still do not know if this alteration is part of the causes of the disease, or if it is a consequence. Nevertheless, in an AD transgenic mice model, plasma TTR levels are early affected (3 months), suggesting an important

participation of this protein (Oliveira et al., 2011). In women, we also found a correlation with disease stage, further highlighting TTR usefulness as a biomarker. A longitudinal experiment will be important to see whether TTR predicts conversion of aMCI to AD, as it is shown by other biomarkers for AD, and also understand if TTR continues to decrease with the development of the disease, in these patients. It will be interesting to find out a possible correlation between TTR and Aβ levels. Due to the low concentration of Aβ levels in plasma, its levels in CSF should be analyzed.

We also verified that in aMCI and AD patients TTR showed decreased capacity to bind T_4. The hormone binds to TTR in the central channel and, according to TTR structural data, the tetramer must be assembled in order to the hormone to bind. Thus, it is possible that in AD, TTR destabilization results in its faster clearance and consequent decreased levels, similarly to FAP patients (Longo Alves et al., 1997). We suggested that, as a consequence of TTR decrease in plasma, and by mechanisms not fully elucidated, Aβ clearance is impaired explaining further progression of the disease. However, it was still not possible to confirm this hypothesis in this work, because in the *in vivo* approach to test the stabilization hypothesis, the drug used – IDIF - although orally administered, reached the brain and it was not possible to distinguish the effects promoted by TTR stabilization in and out the brain. Very recently, it has been reported that peripheral degradation of Aβ does not result is its reduction in the brain, thus questioning on the importance of "peripheral sink" (Walker et al., 2013). This report does not diminishes the importance of understanding the effect of peripheral TTR in brain/plasma Aβ levels as other mechanisms, in addition to the degradation of the peptide. Namely, the effects of TTR in Aβ BBB efflux should be investigated.

It was demonstrated that TTR amyloidogenic mutants present lower capacity to bind Aβ peptide, lower ability to avoid aggregation and also lower capacity to protect against Aβ toxicity than non-amyloidogenic ones (Sciarrone et al., 2008). These results support the importance of TTR stability in Aβ binding. Although TTR has been described to have the ability to interact with soluble monomeric, oligomeric and fibrillar Aβ with similar affinity, using competition binding assays (Sciarrone et al., 2008), other studies suggested that TTR binds preferably to Aβ aggregates rather than Aβ monomers (Du and Murphy, 2010), using enzyme-linked immunoassays. Our *in vivo* approach, TTR stabilization by IDIF, was applied in 5 month old females, and thus we showed that TTR is important to prevent the disease. Nevertheless, TTR might also have the ability to treat AD, and it would be interesting to apply, *in vivo*, this or similar therapies, at different stages of disease development.

We showed that TTR stabilization by IDIF potentiates TTR/Aβ binding *in vitro,* and *in vivo,* reduced plasma Aβ levels, decreased Aβ deposition in the brain and ameliorate

the cognitive deficits in an AD mouse model. The improvements in AD features were only observed in AD/TTR$^{+/-}$ treated animals. Because AD/TTR$^{+/+}$ mice exhibit a less severe AD-like neuropathology compared to AD/TTR$^{+/-}$, in the future, it would be important to analyse more sensitive AD markers that could help to see more strong effects, namely APP processing or even molecules such as kinases involved in the amyloid cascade. A new experimental design should also take into account the test of different concentrations of IDIF to evaluate a dose/response effect. Finally, since differences were not shown in plasma TTR levels between treated and non-treated animals, it would be interesting to evaluate the TTR expression in liver and also in choroid plexus.

In spite of the hypothesis on the importance of TTR stability in AD, other studies support that TTR binds Aβ peptide in its monomeric form (Yang et al., 2013) and suggest a mechanism of action for TTR in which the EF helix/loop “senses” the presence of soluble toxic Aβ oligomers causing the destabilization of TTR tetramers and the exposure of the hydrophobic inner sheet, which then sequesters these toxic oligomers (Yang et al., 2013). The difficulty in determining the quaternary structure of TTR under the conditions of the experiments is great as well as several of the techniques used may promote the dissociation into monomers. Furthermore, the experiments that suggest TTR monomer as the binding specie were performed with an engineered mutant and not with and *in vivo* existent one. The generation of a transgenic AD mouse model overexpressing an engineered monomeric TTR variant, could provide important answers in this field.

We also suggested proteolysis as a potential mechanism involved in TTR protection in AD. Only a few of the compounds that were able to improve TTR/Aβ interaction were also able to potentiate Aβ proteolysis by TTR. In this work, was also tested the impact of TTR natural ligands in its ability to bind and/or cleave Aβ peptide. It was shown that RBP abrogates TTR/Aβ interaction and prevents TTR proteolytic activity. In the future, it is important to assess the importance of Aβ proteolysis by TTR *in vivo*, and for instance to test the effects of proteolytic active and proteolytic inactive TTR variants in AD features.

This work underlines the importance of TTR stability in the design of therapeutic drugs. TTR therapeutic strategies can be used in the treatment of AD or even in the prevention of the disease. For instance, the results obtained with the IDIF treatment in an AD mouse model may make this a good starting point in the development of therapeutic strategies for the disease. Furthermore, our work highlights the importance of TTR as an early biomarker, in association with other biomarkers used in the diagnosis of AD.

APPENDIX

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ABBREVIATIONS

Å - Ångström
aa - Amino acid
AD - Alzheimer's disease
ADDLs - Aβ-derived diffusible ligands
AICD - APP Intracellular Domain
ALS - Amyotrophic Lateral Sclerosis
APL-1 - APP-like protein
APLP1 - APP-like protein 1
APLP2 - APP-like protein 2
ApoA-I - Apolipoprotein A-I
ApoB - Apolipoprotein B
ApoE - Apolipoprotein E
ApoJ - Apolipoprotein J
APP – amyloid precursor protein
APP - Amyloid β Precursor Protein
APPL - Amyloid β Precursor Peptide-like
Aβ - Amyloid β peptide
Aβ40 – amyloid derived from A-Beta peptide residues 1-40
Aβ42 – amyloid derived from A-Beta peptide residues 1-42
BBB - Blood-Brain Barrier
C. elegans - *Caenorhabditis elegans*
CNS - Central Nervous System
CSF - Cerebrospinal fluid
DCPA - 2-((3,5-Dichlorophenyl)amino)benzoic acid
DEAE - Diethylaminoethyl cellulose
Des – Diethylstilbestrol
DFPB - [4-(3, 5-difluorophenyl) benzoic acid
DIF – diflunisal
DMEM – Dulbecco's minimal essential medium
DMSO - Dimethyl Sulfoxide
DNA - deoxyribonucleic acid
DNP – Dinitrophenol
EDTA - Ethylenediamine tetraacetic acid
EGCG - Epigallocatechin-3-gallate
EOAD - Early-Onset Familial AD
FAP - Familial Amyloid Polyneuropathy
FBS - Fetal Bovine Serum

Fluf – Flufenamic Acid
HDL - High Density Lipoproteins
HFIP - 1,1,1,3,3,3-Hexafluoro-2-propanol
IDE - Insulin-Degrading Enzyme
IDIF – iodo-diflunisal
kDa - kilo Dalton
LOAD - Late-onset AD
LRP1 - Low density Lipoprotein Receptor-related Protein 1
mRNA - messenger RNA
MS - Mass spectrometry
NEP - Neprilysin
NFTs - Neurofibrillary Tangles
NPY - Neuropeptide Y
NSAIDs - Non-steroidal anti-inflammatory drugs
PNS - Peripheral Nervous System
PSEN1 - Presenilin 1
PSEN2 - Presenilin 2
RAGE - Receptor for Advanced Glycation End Products
RBP - Retinol Binding Protein
Resv – resveratrol
RNA - Ribonucleic Acid
sAPPα - Soluble APPα
sAPPβ - Soluble APPβ
SDS-PAGE - Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SSA - Senile Systemic Amyloidoses
Sw - Swedish
T_3 - Triiodothryronine
T_4 - Thyroxine
TBG - Thyroxine-binding globulin
TEM - Transmission Electron Microscopy
TFA - Trifluoroacetic acid
Th S - Thioflavin S
Th T - Thioflavin T
TTR - Transthyretin
$TTR^{-/-}$ - knockout for transthyretin
$TTR^{+/-}$ - heterozygous for transthyretin
$TTR^{+/+}$ - wild type for transthyretin

TTR L55P – leucine for proline exchange at position 55 of Transthyretin

TTR T119M – threonine for metionine exchange at position 119 of transthyretin

TTR V30M – valine for metionine exchange at position 30 of Transthyretin

TTR WT – Transthyretin wild type

TTR Y78F – tyrosine for fenilalanine exchange at position 78 of Transthyretin