Role of TTR in Aβ peptide brain efflux – Impact in Alzheimer's disease

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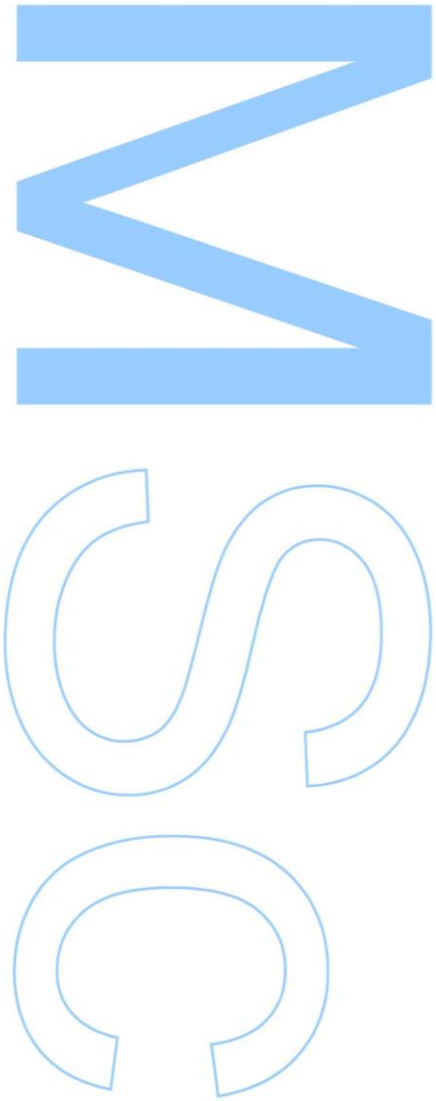

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Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder characterized by two main pathological features: neurofibrillary tangles (NFTs) composed of hyper-phosphorylated *Tau* protein and extracellular deposition of amyloid β peptide (Aβ) into plaques, found mainly in cortex and hippocampus. Brain levels of Aβ peptide, which is derived from proteolytic hydrolysis of amyloid-beta precursor protein (APP), are regulated by its production, its degradation and clearance through the brain, namely across the blood-brain barrier (BBB). Indeed, increasing evidence demonstrated that increased Aβ levels are a result of its faulty elimination across the BBB. Several molecules have been proposed as Aβ carriers and among them transthyretin (TTR) has been shown to be the major Aβ-binding protein in the cerebrospinal fluid (CSF), leading to decreased peptide aggregation and toxicity. Supporting the importance of TTR in AD is the observation of its decreased levels in CSF and plasma. It has been also suggested that TTR tetrameric stability is a key factor in TTR/Aβ interaction. Moreover, *in vivo* administration of iododiflunisal (IDIF), known to be a potent TTR tetrameric stabilizer, to an AD mice model resulted not only in decreased brain Aβ levels and deposition but also in improved cognitive function associated to the AD-like neuropathology in this AD 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 the periphery. Furthermore, TTR expression in neurons has been recently reported, as well as the co-localization of extracellular TTR with Aβ in plaques, although it is still very controversial. Taking all these data into account, we hypothesize that TTR might also be involved in Aβ clearance at BBB.

This project aims at investigating the influence of TTR in Aβ transport across the BBB, using an *in vitro* BBB cellular model and an *in vivo* study, and also at investigating the mechanisms leading to neuronal TTR expression using a neuronal cell line.

In order to achieve our main goals, we started by evaluating the effect of TTR in Aβ plasma levels in AD/TTR transgenic mice with different ages and distinct genotypes for TTR. Our results showed that, Aβ1-42/Aβ1-40 ratios were elevated in plasma of AD/TTR$^{+/-}$ mice compared with AD/TTR$^{+/+}$ animals, at the age of 6 months at which senile plaque formation starts, in this model. This indicates that TTR contributes to the maintenance of normal plasma Aβ levels, supporting the idea that TTR promotes its elimination from the brain and from the periphery.

After setting up and optimizing our cellular model, the hCMEC/D3 cell line, we performed internalization studies of labelled (FAM-Aβ1-42) or non-labelled Aβ peptide

(Aβ1-42) in the presence and absence of TTR, using flow cytometry and fluorescence microscopy. Our FACS results showed less internalization in the presence of TTR while fluorescence microscopy assays revealed no significant TTR effect in Aβ uptake. Further tests performed, using short periods of incubation, showed however, that TTR promoted Aβ internalization and possibly its degradation, as assessed by a fluorometric analysis of cellular lysates and supernatants. These results also indicated that in the initial analysis by FACS and fluorescence microscopy, and because cells were incubated for 3 hours, Aβ was internalized and then flowed out of cells, explaining the lower levels. We also conducted permeability studies to assess the effect of TTR in Aβ efflux, using cell monolayers established in transwells filters and measured Aβ transport from the basolateral (brain) to the apical (blood) side. The results showed that only TTR added to the basolateral chamber is able to promote Aβ efflux, when comparing to TTR added to the apical side. The permeability of TTR across the cell monolayers was also evaluated, and our results demonstrated that TTR was capable to cross the hCMEC/D3 cell monolayer, mostly from brain-to-blood, which was lower in the presence of Aβ. We suggest that TTR, while transporting Aβ, also crosses the monolayer or that Aβ causes impaired BBB integrity. Additionally, we performed a preliminary *in vivo* study, using mice with two copies of TTR ($TTR^{+/+}$), carrying only one copy of the TTR gene ($TTR^{+/-}$) and animals without TTR ($TTR^{-/-}$). We injected FAM-Aβ1-42 intracranially and our results indicated that, 30 minutes post injection, brains from $TTR^{+/-}$ mice retained less peptide than $TTR^{-/-}$ animals, further supporting a TTR role in Aβ brain efflux, corroborating our *in vitro* permeability results. However, we could not conclude on FAM-Aβ1-42 brain levels in $TTR^{+/+}$ mice, due to technical issues.

To further understand which mechanisms or stimulus are behind the induction of TTR gene expression in neurons, we decided to investigate if high levels of soluble, oligomeric and fibrillar forms of Aβ could induce such expression in a SH-SY5Y neuroblastoma cell line. We also studied the expression pattern of APP isoforms. Real-time polymerase chain reaction (RT-PCR) analysis showed that oligomers and fibrils were capable to induce TTR expression and to increase the expression of APP isoforms (i.e. total APP), whereas soluble Aβ did not cause a significant effect. We assessed also if 695APPswe gene produce TTR expression using the same cell line and our results showed no TTR expression. Finally, we evaluated if exogenous TTR could induce an alteration in APP isoforms expression, and no differences were observed in cells in presence or absence of TTR.

In summary, our results strengthen the importance of TTR in AD, indicating this protein as a promoter of Aβ clearance across the BBB. Our research also provided evidence that neuronal TTR production is a reaction to toxic Aβ species, further supporting TTR as a protective protein in the central nervous system (CNS).

Keywords: Alzheimer's disease, Amyloid β peptide, Blood-brain barrier, Transthyretin.

Resumo

A doença de Alzheimer (DA) é uma patologia neurodegenerativa debilitante caracterizada particularmente por duas lesões: emaranhados neurofibrilares, compostos por proteína tau híper-fosforilada, e a deposição extracelular do péptido β amiloide (βA) em placas senis ou neuríticas, encontradas principalmente no córtex e hipocampo. O péptido βA resulta da hidrólise proteolítica da proteína precursora amiloide (PPA) e os seus níveis no cérebro são regulados pela sua produção, degradação e eliminação através do cérebro, nomeadamente através da barreira hemato-encefálica (BHE). E, de facto, tem sido mostrado que os níveis de βA estão aumentados devido a uma eliminação ineficiente através da BHE. No mesmo contexto, e nos últimos anos, várias moléculas têm sido sugeridas como transportadoras do péptido βA, e entre elas, a transtirretina (TTR) tem sido descrita como a principal proteína transportadora do βA no líquido cefalorraquidiano (LCR), provocando uma diminuição da sua agregação, e consequentemente da sua toxicidade. A importância da TTR na DA é sustentada também pelo facto dos seus níveis estarem reduzidos no LCR e no plasma, e ainda a evidência de que a estabilização da sua estrutura é um fator crítico para a interação TTR/βA. Além disso, a administração *in vivo* do composto iododiflunisal (IDIF), conhecido por ser um potente estabilizador do tetrâmero da TTR, a um modelo animal de DA resultou não só num decréscimo dos níveis de βA, e na sua deposição no cérebro dos murganhos, como também na melhoria das funções cognitivas habitualmente afetadas nesta patologia. Foi ainda demonstrado que os níveis do péptido βA estavam reduzidos no plasma e apresentavam também uma tendência para redução no LCR, sugerindo que a TTR promove a eliminação do péptido βA do cérebro para a periferia. Além destas evidências, tem sido recentemente demonstrada a expressão da TTR em neurónios, como também a sua co-localização extracelular com o Aβ em placas, embora esta seja ainda uma discussão muito controversa. Tendo isto em conta, formulamos a hipótese de que a TTR poderá estar envolvida na eliminação do péptido βA através da BHE.

Este projeto tem como objetivo investigar a influência da TTR no transporte do βA através da BHE, utilizando um modelo celular da BHE e um estudo *in vivo*, e ainda procurar mecanismos que levem à expressão da TTR neuronal, usando um modelo celular neuronal.

Para alcançarmos os nossos objetivos, começamos por avaliar o efeito da TTR nos níveis de βA no plasma de murganhos transgénicos DA/TTR com diferentes idades e genótipos para a TTR. Os nossos resultados demonstraram que, o rácio βA1-42/βA1-40 apresentou-se mais elevado nos plasmas dos murganhos DA/TTR$^{+/-}$ em

comparação com os animais DA/TTR$^{+/+}$, aos seis meses de idade, altura descrita como o início da deposição cerebral do péptido βA neste modelo. Isto indica que a TTR contribui para a manutenção dos níveis fisiológicos de βA no plasma, sustentando a ideia que a TTR promove a sua eliminação do cérebro para a periferia.

Após otimização do nosso modelo celular, a linha celular hCMEC/D3, foram realizados estudos de internalização do péptido βA fluorescente e não fluorescente na presença ou ausência da TTR, utilizando citometria de fluxo e microscopia de fluorescência. Os resultados de citometria de fluxo mostraram diminuição da internalização do βA na presença da TTR, enquanto a microscopia de fluorescência não revelou diferenças significativas no efeito da TTR na internalização do βA. A análise fluorométrica dos lisados celulares e sobrenadantes com tempos de incubação mais curtos, demonstrou, no entanto, que a TTR promove a internalização do βA e que possibilita a sua degradação. Estes resultados também parecem indicar que, na análise inicial por citometria e microscopia de fluorescência, e porque as células foram incubadas durante 3 horas, o βA foi internalizado e, em seguida, começou a ser transportado para o exterior das células, explicando-se assim, os seus níveis mais baixos. Realizaram-se também estudos de permeabilidade para avaliar o efeito da TTR no efluxo do βA, usando monocamadas de células em filtros de Transwells, onde o transporte do βA foi medido a partir do transporte basolateral (cérebro) para o lado apical (sangue). Os resultados mostraram que apenas a TTR adicionada no lado basolateral é capaz de promover o efluxo do βA, comparando com a situação em que a TTR é adicionada ao lado apical. A permeabilidade da TTR através das monocamadas de células foi também avaliada, e os nossos resultados demonstraram que a TTR foi capaz de atravessar a monocamada celular, sendo mais evidente o transporte do cérebro para o sangue, mas menos acentuado na presença de βA. Sugerimos, por isso, que a TTR enquanto transporta o βA pode também atravessar a monocamada, ou então que o βA pode causar danos na BHE, afetando a sua integridade. Para além disso, foi efetuado um estudo preliminar *in vivo*, utilizando murganhos com duas cópias de TTR (TTR$^{+/+}$), com uma cópia de TTR (TTR$^{+/-}$) e sem TTR (TTR$^{-/-}$). Um Péptido βA fluorescente foi injetado intracranialmente e os resultados indicaram que, 30 minutos após a injeção, os cérebros dos murganhos TTR$^{+/-}$ apresentavam menos βA que os animais TTR$^{-/-}$, apoiando ainda mais o papel da TTR no efluxo do βA através do cérebro, corroborando os nossos resultados de permeabilidade *in vitro.* No entanto, não foi possível concluir sobre os níveis cerebrais de βA em murganhos TTR$^{+/+}$, devido a problemas inerentes à metodologia utilizada.

Para entender melhor os mecanismos ou estímulos que estão envolvidos na indução da expressão do gene da TTR em neurónios, decidimos investigar se elevados níveis de βA solúvel, oligomérico e fibrilar poderiam induzir tal expressão numa linha de celular neuroblastoma SH-SY5Y. Estudamos também a expressão do padrão de isoformas de PPA. A análise por reacção em cadeia da polimerase em tempo real (RCP-TR) mostrou que oligómeros e fibras foram capazes de induzir a expressão de TTR e de aumentar a expressão das isoformas de PPA (ou seja, o PPA total), ao passo que o βA solúvel não causou nenhum efeito significativo. Também avaliamos se o gene 695APPswe induzia a expressão de TTR utilizando a mesma linha celular, e os nossos resultados não mostraram expressão de TTR. Finalmente, avaliámos se a TTR exógena poderia induzir uma alteração na expressão das isoformas de PPA, mas não foram observadas diferenças nas células em presença ou ausência de TTR.

Em suma, os nossos resultados reforçam a importância da TTR na DA, indicando que esta proteína pode funcionar como um promotor do efluxo do βA através da BHE. O nosso estudo também forneceu evidências de que a produção de TTR neuronal é uma reação às espécies tóxicas de βA, apoiando ainda mais a TTR como uma proteína protetora no sistema nervoso central (SNC).

Palavras-chave: Doença de Alzheimer, Péptido β amiloide, Barreira hemato-encefálica, Transtirretina.

Table of Contents

List of figures

List of tables

Abbreviations

AD - Alzheimer disease

AICD - APP intracellular domain

AJ - Adherent junctions

AMT - Adsorption-mediated transcytosis

Aph – Anterior pharynx defective

APOE - Apolipoprotein E

APP – Amyloid-beta Precursor Protein

Aβ peptide - Amyloid-beta peptide

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

BBB - Blood-Brain Barrier

BCSFB - Blood-cerebrospinal fluid barrier

bFGF - Basic fibroblast growth factor

BSA - Bovine serum albumin

CNS - Central nervous system

CO_2 - Carbon dioxide

CTF - C-terminal fragment

CuBD - Copper-binding domain

DCPA - 2-((3,5Dichlorophenyl) amino) benzoic acid

DFPB - 4-(3,5-difluorophenyl)

DMP - Dimethyl pimelimidate

DNP- Dinitrophenol

EBM-2 medium - Endothelial Cell Growth Medium

ECs - Endothelial cells

EMEM - Eagle's minimum essential medium

EOAD - Early-onset AD

FAC - Familial amyloidotic cardiomyopathies

FAP - Familial amyloidotic polyneuropathy

FBS - Fetal bovine serum

FITC - Fluorescein isothiocyanate

FMRI - Functional magnetic resonance imaging

GDNF - Glial-derived neurotrophic factor

GSK3β - Glycogen synthase kinase 3β

GWAS - Genome-wide association studies

hCMEC/D3 cell line - Immortalized human cerebral microvascular endothelial cell line

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPBD1 - Heparin-binding/growth-factor-like domain 1

HPBD2 - Heparin-binding/growth-factor-like domain 2

hrec TTR - Human recombinant TTR

HSF1 - Heat shock factor 1

ICV - Intracerebroventricularly

IDIF – Iododiflunisal

IGF - Insulin-like growth factors

IP - Intraperitoneal injection

ISF - Interstitial fluid

JAMs - Junctional adhesion molecules

KPI - Kunitz-type protease inhibitor domain

LOAD - Late-onset AD

LRP1 - Low-density lipoprotein receptor-related protein 1

MCI - Mild cognitive impairment

MRI - Magnetic resonance imaging

NEAA - Non-essential amino-acids

NFT - Neurofribrillary tangles

PBS - Phosphate-buffered saline

PBS-T - Phosphate-buffered saline containing 0.05% Tween-20

PEN2 - Presenilin enhancer 2

PET - Positron emission tomography

PFA - Paraformaldehyde

PHFs - Paired helical filaments

PMSF - Phenylmethylsulphonyl fluoride

PNS - Peripheral nervous system

PP - Protein phosphatase

PSEN1 - Presenilin 1

PSEN2 - Presenilin 2

p-*tau* - Phosphorylated *tau*

RAGE - Receptor for advanced glycation end products

RBP - Retinol binding protein

RC - Random coil region

RMT - Specific receptor mediated transcytosis

rpm - Rotations *per* minute

RT - Room temperature

RT-PCR - Real-time Polymerase Chain Reaction

sAPP - Soluble secreted form of APP

SORL1 - Sortilin-related receptor

SP - Signal peptide

SPECT - Single photon emission computed tomography

SSA - Systemic amyloidosis

T4 - Thyroid hormone thyroxine

TBS - Tris-buffered-saline

TEER - Transendothelial electrical resistance

TGF-β - Transforming growth factor beta

TJ - Tight junctions

t-*tau* - Total *tau*

TTR - Transthyretin

VEGF - Vascular endothelial growth factor

ZnBD - Zinc-binding domain

Introduction

Dementia is one of the major concerns for society and is independent of social, economic, ethnic or geographical boundaries. Currently more than 44.4 million people are affected worldwide and the number of affected people is estimated to 75.6 million in 2030, and 135.5 million in 2050 (International; 2014). Epidemiologic research of dementia in Portugal revealed that approximately 153.000 people suffer from dementia (Portugal, 2009).

This mental disorder is caused by a damage of neurons in several brain regions, leading to a loss of cognitive brain functions and memory impairment (Grimm *et al.* , 2013). These, in turn, can lead to a decline in memory, thinking, reasoning, verbal communication, behavior and emotion, interfering with independence in everyday activities. Different types of dementia have been described, among them are: vascular dementia, dementia with Lewy bodies, Parkinson's disease dementia, Creutzfeldt-Jakob disease, mixed dementia, Fronto-temporal dementia and Alzheimer's disease (AD) (Fargo *et al.* , 2014).

In the next topics will be exposed several aspects related to the most common type of dementia, AD, and will be debated the role of transthyretin (TTR) in this disorder, as well as, the clearance of amyloid-beta peptide (Aβ) through the Blood-Brain Barrier (BBB).

1. Overview of Alzheimer's disease

1.1. Alois Alzheimer and Alzheimer's disease

The first description of the clinical and histological findings of the "presenile dementia" was made by Alois Alzheimer (Figure 1A), a German psychiatrist and neurophatologist, in 1907 (Alzheimer *et al.* , 1995). His famous paper entitled "Über eine eigenartige Erkranlkung der Hirnrinde" described a 51-year old woman patient, Auguste Deter (Figure 1B), who had developed progressive memory impairment, paranoid ideas, delusions, hallucinations and disorientation in time and space (Alzheimer *et al.* , 1995; Small *et al.* , 2006; Jellinger, 2006). Later, when she died, Alzheimer detected, in her atrophic brain, degenerating neurons with bundles of fibrils (neurofibrillary tangles), using a developed Bielschowsky's silver staining method, and miliary deposits (senile plaques) all over the cortex (Alzheimer *et al.* , 1995; Jellinger, 2006). However, Alois Alzheimer was not the first to describe these unusual senile aggregates. In the same year, Fischer had already published a paper where first

described senile plaques (Figure 1C) in 12 cases of senile dementia (Goedert, 2009). The “presenile dementia” was renamed into Alzheimer’s disease, in 1910 by Emil Kraepelin (Alzheimer’s mentor) and, in the same year, she included this disorder in the Textbook of Psychiatry (Jellinger, 2006; Verhey, 2009).

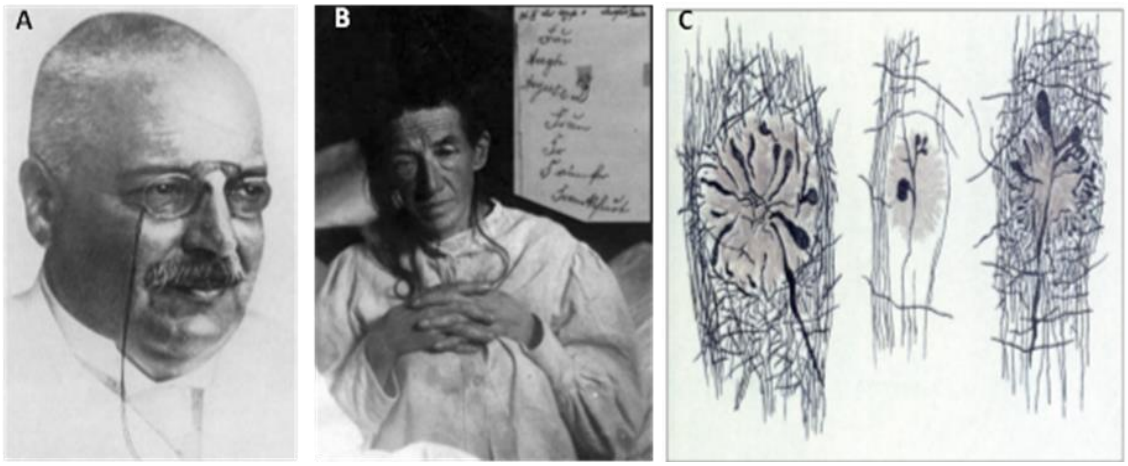

Figure 1 – History of Alzheimer’s disease. Alois Alzheimer 1864-1915 (A), its first patient, Auguste Deter, (B) and drawings of senile plaques from the illustrations of Fischer’s paper (C). Adapted from: A - Verhey, 2009; B - Jellinger, 2006; C - Goedert, 2009

After the preliminary work of Alzheimer, several scientists proceeded his clinical studies, in order to acquire the necessary knowledge to unravel the mystery around this devastating disease, currently known as the disease of the 21th century (Jellinger, 2006).

Presently, AD is considered a progressive neurodegenerative disorder of the central nervous system (CNS) with increasing prevalence in the older population, over the age 65 (Qiu *et al.* , 2009; Grimm *et al.* , 2013). The prevalence and incidence of AD increase with age comprising approximately 70% cases of dementia worldwide, with notable incidence in North America and Western Europe (Reitz *et al.* , 2011). In Portugal, this disease affects more than 90.000 people and this means that, about 1% of the total Portuguese population suffers from this condition (Portugal, 2009).

Clinically, AD is characterized by a progressive decline in cognitive functions, such as memory, language, perception, reasoning and learning, ultimately leading to death (Alzheimer *et al.* , 1995; Fargo *et al.* , 2014). According to the new diagnostic criteria and guidelines, revised in 2011 by the National Institute of Aging and the Alzheimer Association, there are three different stages of AD: preclinical AD, mild cognitive impairment (MCI) due to AD and dementia due to AD (Fargo *et al.* , 2014).

These pre-established stages aim at helping the medical entities in the diagnosis of patients presenting dementia symptoms and, thus, defining the development of the disease so that the proper monitoring is provided.

1.2. Genetics of Alzheimer's Disease

AD is classified into two forms based on its age of onset: Early-onset AD (EOAD), which typically develops before the age 65, accounting for 1-5% of all cases, and the late-onset AD (LOAD), which normally occurs late in life (>65 years) and affects more than 95% of Alzheimer's patients (Blennow *et al.* , 2006; Piaceri *et al.* , 2013; Reitz *et al.* , 2014).

Early onset form is associated with a Mendelian pattern of inheritance and it is an autosomal, dominant and familial form of disease (Blennow *et al.* , 2006). However, the majority of the patients with EOAD are sporadic cases, without family history of the disease (Piaceri *et al.* , 2013). Three different genes are involved in the pathophysiology of EOAD: Amyloid β Precursor Protein (APP) gene, presenilin 1 (PSEN1) and presenilin 2 (PSEN2) genes. Mutations in any of these three genes, which all encode proteins linked to APP, interfere with Aβ production and processing (Tanzi, 2012; Reitz *et al.* , 2014).

In contrast, LOAD onset form is sporadic and a heterogeneous form of the disease and the main risk factor is aging. Several genetics studies demonstrated that LOAD has a substantial genetic contribution (~60-80%) and is extremely influenced by environmental factors (Gatz *et al.* , 2006; Piaceri *et al.* , 2013). Thus, genetic risk factors are extremely studied, being the apolipoprotein E (APOE) one of the most investigated gene.

The relationship between *APOE* gene and LOAD is described since 1993 (Saunders *et al.* , 1993) and, lately associated as the major susceptibility gene for sporadic AD (Coon *et al.* , 2007). The *APOE* gene encodes a 299-aminoacid lipoprotein responsible for maintaining the cholesterol homeostasis, and this protein exists as three different isoforms: ε2, ε3 and ε4 (Piaceri *et al.* , 2013). Carriers of the *APOE* ε4 allele, located on chromosome 19q13, are more susceptible to development AD when compared with noncarriers, however this allele is not sufficient by itself to generate this disorder (Corder *et al.* , 1993., Schmidt *et al.* , 2014).

Sortilin-related receptor gene (*SORL1*), which is located on chromosome 11, is another gene genetically associated to LOAD, and encodes a neuronal receptor for ApoE (Rogaeva *et al.* , 2007; Reitz *et al.* , 2011). Andersen and his colleagues verified, in a study involving mice and human AD samples, that under-expression of *SORL1* leads to the over-expression of Aβ, and also that SorL1 receptor is involved in the APP processing and trafficking (Andersen *et al.* , 2005).

In addition, other genes associated with LOAD, were identified by the Genome-wide association studies (GWAS): *CLU* (clusterin or apolipoprotein J), *PICALM* (phosphatidylinositol binding clathrin assembly protein), *CR1* (complement component C3b/C4b), *BIN1* (amphiphysin II), *ABCA7*, *MS4A* cluster (MS4A6A/ MS4A4E), *CD2AP*, *CD33* and *EPHA1* (Harold et al. , 2009, Hollingworth et al. , 2011a, Hollingworth et al. , 2011b, Lambert et al. , 2009, Seshadri et al. , 2010, Naj et al. , 2011). In summary, these genes are involved in several pathways related with APP and Aβ processing, Aβ production and lipid metabolism and endocytosis (Reitz *et al.* , 2014). Nevertheless, the mechanism by which these genes are involved in the pathogenesis of AD is still unclear.

1.3. Non-genetic risk factors for Alzheimer's Disease

The vast majority of AD cases are not directly related to genetic factors. A group of factors between the environment and the lifestyle of people seems to have a significant role in AD (Alves *et al.* , 2012).

Age is the greatest risk factor for this incurable disease, but advanced age is not sufficient to trigger by itself the disease (Fargo *et al.* , 2014). In addition to age, a variety of others factors are involved with AD include a family history of the disorder, hypertension, hypercholesterolemia, diabetes, Down's syndrome, cardiovascular disease, cerebrovascular disease, gender, education, smoking, high alcohol consumption and body weight (Masters *et al.* , 1985; Hebert *et al.* , 2001; Arvanitakis *et al.* , 2004; Reitz *et al.* , 2014).

1.4. Histopathology of Alzheimer's Disease

This debilitating disorder is defined, pathologically, by the presence of extracellular amyloid-β plaques (senile plaques) and intracellular neurofibrillary tangles

(NFTs), which can lead to gliosis, neuronal and synaptic losses (Wenk, 2003; Paula VJR *et al.* , 2009; Pimplikar, 2009).

1.4.1. Senile Plaques

Neuritic or senile plaques are extracellular amyloid deposits found in the brain, mostly constituted by Aβ peptide. This distinctive lesion occurs in the CNS, more specifically in the hippocampus and the cortex (Costa *et al.* , 2008a). The amyloid plaques can be distinguished in different plaques subtypes, including neuritic, diffuse, primitive, compact, cored and cotton-wool. However, neuritic and diffuse plaques (Figure 2) are considered the most pathogenically significant subtypes in AD. In addition to the central core of Aβ peptide, neuritic plaques (also designated classical or compact plaques) are surrounded by abnormal and dystrophic neurites (axons and dendrites), and activated microglia and reactive astrocytes. Moreover, diffuse plaques are more heterogeneous than senile plaques and, can evolve to compact plaques with increasing neuritic involvement (Castellani *et al.* , 2010).

The classical amyloid plaques are not related only with AD, and are also associated with other diseases such as diffuse Lewy body, Down syndrome, dementia pugilistica and acute traumatic brain injury (Castellani *et al.* , 2010).

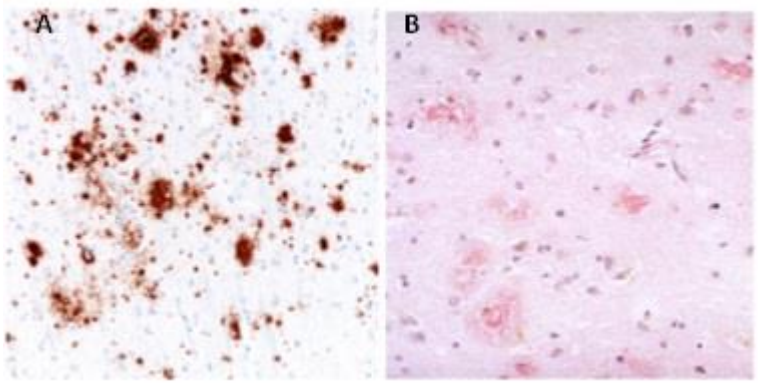

Figure 2 – Diffuse (A) and neuritic (B) plaques. Castellani et al. , 2010

1.4.2. Neurofibrillary Tangles (NFTs)

The other molecular lesion associated to AD is intracellular NFTs (Figure 3), which are bundles of numerous paired helical filaments (PHFs) composed by two

axially opposed helical filaments with a diameter of 10 nm (Pevalova *et al.* , 2006; Perl, 2010). These proteinaceous aggregates are formed by hyper-phosphorylated protein, *tau,* responsible for the assembly and stability of microtubules in the neuronal cell and for axonal transport (Mudher *et al.* , 2002; Perl, 2010). In addition to *tau*, several protein constituents are associated to this pathology, including ubiquitin, Aβ peptide and cholinesterases, although this microtubule-associated protein is considered the critical component of these filaments. Furthermore, NFTs are mainly present 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 (Perl, 2010).

These histopathological hallmark is not restricted to AD but also of other neurodegenerative diseases, such as Parkinson's disease, Parkinsonian dementia, Pick's disease, Down's syndrome, progressive supranuclear palsy, Amyotrophic lateral sclerosis and dementia pugilistica (Gong *et al.* , 2005; Castellani *et al.* , 2010; Perl, 2010).

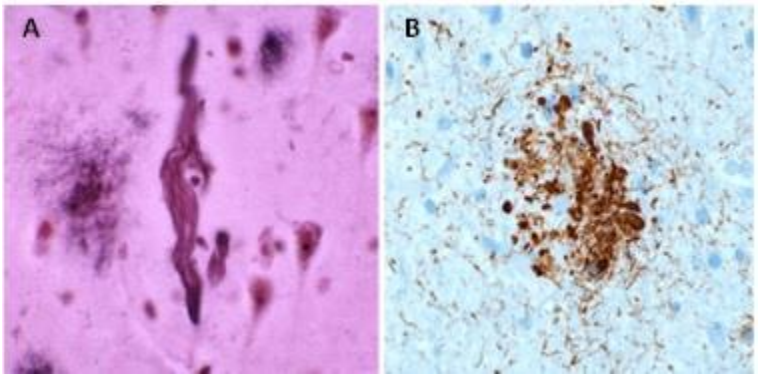

Figure 3 – Neurofibrillary tangles (A) and hyper-phosphorylated tau protein (B). Castellani et al. , 2010

1.5. The role of Amyloid β Precursor Protein (APP) and Aβ formation

As described previously, senile plaques are formed by deposition and aggregation of Aβ peptide, which is synthesized from a larger precursor protein called APP (Kim *et al.* , 2014). APP is a ubiquitously type I integral transmembrane protein and contains an extracellular N-terminal domain, a large ectodomain, one single transmembrane domain and a short intracellular C-terminal domain (Maltsev *et al.* ,

2011; Grimm *et al.* , 2013). Currently, 32 APP autosomal dominant mutations have been identified and in most cases resulting in EOAD (Cruts *et al.* , 2012).

The human APP gene is located on chromosome 21 and contains 18 exons spanning more than 170 kbp. The gene comprises several domains (Figure 4), including the region which encodes the Aβ sequence (part of exons 16 and 17), a signal peptide (SP), a heparin-binding/growth-factor-like domain 1 (HPBD1), a copper-binding domain (CuBD), a zinc-binding domain (ZnBD), a Kunitz-type protease inhibitor domain (KPI), a second heparin-binding domain 2 (HPBD2), a random coil region (RC) (Yoshikai *et al.* , 1990; Zhou *et al.* , 2011). Three different isoforms of APP are derived from the alternative splicing of exons 7 and 8, and generates APP mRNAS for APP770, APP751 and APP695 (referring to a length in amino-acids), the last one being the major neuronal isoform. These isoforms differ principally by the absence (APP695) or presence (APP770, APP751) of a KPI domain (Zhou *et al.* , 2011).

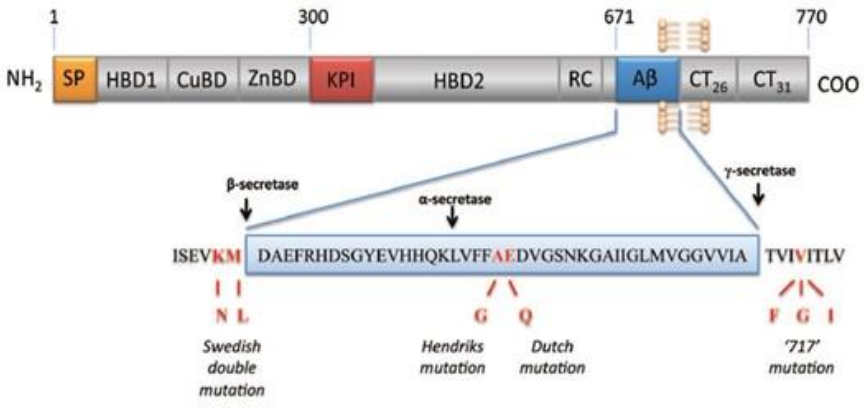

Figure 4 – Schematic representation of human APP gene. The gene is composed by a short intracellular tail, and a single transmembrane domain. Furthermore, the extracellular domain comprises SP, HBD1, CuBD, ZnBD, KPI, HBD2, RC and Aβ domains. Zhou et al. , 2011

This transmembrane protein is one of the most abundant proteins in the CNS and it is also present in peripheral tissues, including epithelium and blood cells (Paula VJR *et al.* , 2009). The proteolytic processing of APP occurs by two distinct pathways: amyloidogenic pathway, that produces Aβ peptide, and non-amyloidogenic pathway that generates a secreted form of APP, sAPPα (Mudher *et al.* , 2002).

The amyloidogenic processing pathway (Figure 5) combines the sequential actions of two enzymes: β- and γ-secretases (Kim *et al.* , 2014). The β-secretase, named β-site amyloid precursor protein-cleaving enzyme 1 (BACE-1), cleaves APP and generates N-terminal soluble secreted form of APP, sAPPβ, and a C-terminal

fragment (CTF) called 99, which contains the Aβ sequence. The fragment CTF99 is subsequently cleaved by γ-secretase, generating the APP intracellular domain (AICD) and the amyloidogenic Aβ peptide (Maltsev *et al.* , 2011; Grimm *et al.* , 2013; Yan *et al.* , 2014).

In the case of the non-amyloidogenic pathway (Figure 5), the APP is cleaved by the action of the enzyme α-secretase and is generated a soluble secreted amyloid precursor protein-α (sAPPα) and a carboxyl-terminal fragment (CTF83). Next, the CTF83 is digested by γ-secretase, liberating a non-amyloidogenic peptide (p3) and an AICD fragment (Muller *et al.* , 2012).

The γ-secretase is a complex comprising at least four distinct proteins: PSEN1 or PSEN2 as catalytic core, nicastrin, Aph (anterior pharynx defective) 1a or b, and presenilin enhancer 2 (PEN2) (Pasternak *et al.* , 2003). PSEN1 is the major constituent of this complex, and more than 180 mutations are known, which are the main causes of EOAD. Regarding the PSEN2 gene, currently 13 missense mutations causing EOAD were identified (Cruts *et al.* , 2012).

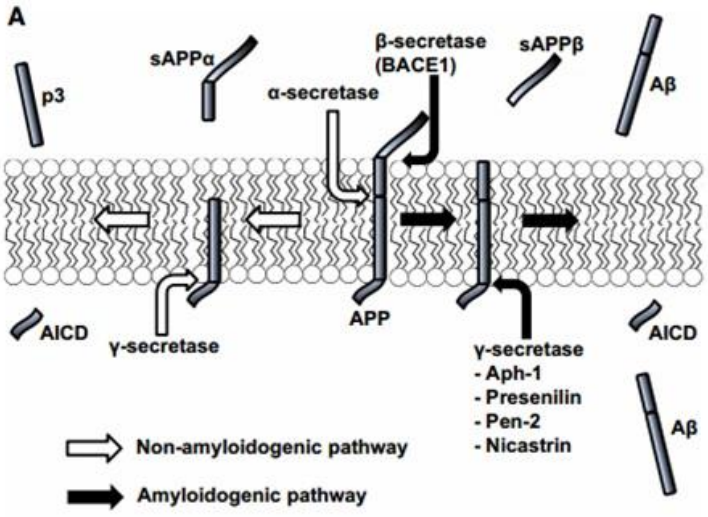

Figure 5 – Schematic representation of APP processing. The figure shows that APP protein can be processed by two different pathways: amyloidogenic (black arrow) and non-amyloidogenic pathways (white arrow). The APP is first cleaved by β- and α- secretases and generates sAPPβ/CTF99, and sAPPα/CTF83 (amyloidogenic and non-amyloidogenic pathways respectively). Then, in the amyloidogenic processing, the CTF99 is cleaved by action of γ-secretase, liberating Aβ peptide and AICD fragments. Regarding the non-amyloidogenic pathway, the CTF83 is digested by the same enzyme, γ-secretase, and resulting extracellular p3 and AICD fragments. Adapted from Kim et al. , 2014.

1.6. Aβ peptide as the cause of AD: the amyloid hypothesis

1.6.1. Aβ peptide

As described previously, Aβ peptide is derived from a sequential cleavage of APP, and was first isolated and sequenced by Glenner and Wong in 1984 (Glenner *et al.* , 1984). This peptide can be produced by several cells and found in plasma and cerebrospinal fluid (CSF).

Aβ is a 4 kDa peptide with a β-pleated sheet configuration and its length can vary at the c-terminus, according the site where γ-secretase cleaves APP, generating peptides with different lengths between 39 and 43 amino-acids (Castellani *et al.* , 2010). The 40 and 42 amino-acids species, Aβ1-40 and Aβ1-42 peptides respectively, are the most abundant isoforms in the brain, although the Aβ1-40 levels are higher than Aβ1-42 (Perl, 2010). The Aβ species (Figure 6) are released as monomers and then start to polymerizes into various structurally different forms, such as dimers, trimers, oligomers, protofibrils and fibrils, to finally deposit and aggregate into diffuse and, then neuritic plaques (Paula VJR *et al.* , 2009). Owing to its hydrophobic properties, the Aβ1-42 peptide is more prone to aggregation and fibrilization, being the most amyloidogenic specie (Perl, 2010).

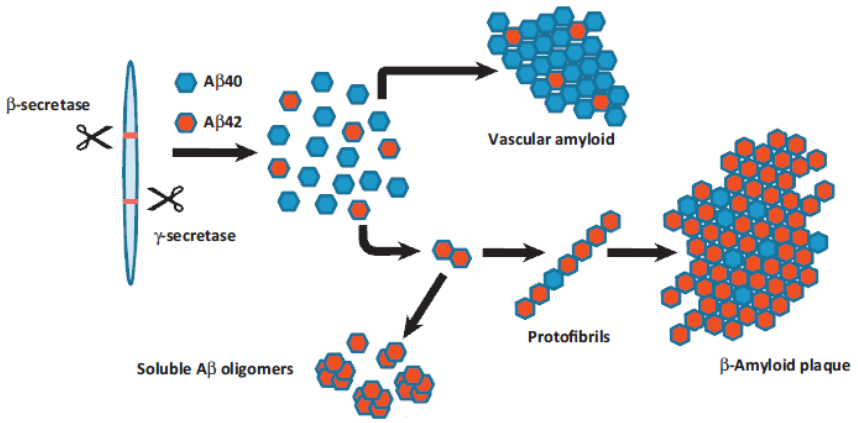

Figure 6 – Representation of distinct species of Aβ. Adapted from Hefti et al. , 2013

1.6.2. Amyloid cascade hypothesis

Currently, several hypotheses are described trying to unmask the cascade that triggers Alzheimer's disease. However, all the theories have fails and are not totally reliable.

One of the theories is “Amyloid cascade hypothesis”. This was first described in the early 1990s, and postulates that the extreme deposition of Aβ peptide, as insoluble fibrillar form, into neuritic or senile plaques, due either to their increased production or decreased clearance, is the initiating event of this pathology (Hardy *et al.* , 1992). The accumulation of Aβ triggers a cascade of neurotoxic events, including mitochondrial dysfunction, increased oxidative stress, hyper-phosphorylated tau and subsequently NFTs formation (Morley *et al.* , 2014). Ultimately, this group of events leads to disruption of calcium homeostasis and synaptic and neuronal loss in critical brain areas related with cognitive functions (Paula VJR *et al.* , 2009; Alves *et al.* , 2012). Nevertheless, the original idea that amyloid plaques are pathogenic and the main cause of AD is quite controversial. Several studies revealed, by the post-mortem observation of AD patient’s brains with severely impaired memory, absence of amyloid plaques, and another study with mouse model of AD shows that animals with amyloid plaques have normal memory function (Lesne *et al.* , 2008; Pimplikar, 2009).

Accumulating scientific data suggest that soluble Aβ oligomers represent the most toxic form of this protein that can cause AD. Three specific forms of Aβ oligomers, isolated from brains of AD patients or from transgenic mouse models of AD, were identified: Aβ dimers (≈ 8 kDa), Aβ trimers (≈ 12 kDa) and Aβ*56 (≈ 56 kDa) (Lesne *et al.* , 2006; Shankar *et al.* , 2008). Regarding this, Lesne *et al.* suggested that accumulation of Aβ*56 oligomers in the brain, causes memory deficits in a middle-aged Tg2576 mice (Lesne *et al.* , 2006), and another recent study, demonstrated that, injection of Aβ*56 oligomers intracerebroventricularly (icv) in transgenic mice brains, impairs brain function and affects mice cognitive functions (Reed *et al.* , 2011). In contrast, Aβ trimers effect is less clear, but Aβ dimers seem to have a synaptotoxic effect (Shankar *et al.* , 2008; Reed *et al.* , 2011).

Despite all the advances, several investigators have increasingly questioned the validity of amyloid hypothesis and if Aβ is or not the cause of AD. Thus, many hypotheses to explain AD pathogenesis are ongoing, including pathways that do not link to amyloid, such as the “tau and tangles hypothesis”.

1.7. Tau and tangles hypothesis

In mid 1980s, a study revealed that *tau* protein is the main constituent of NFTs, one of the hallmarks of AD (Wood *et al.* , 1986). The *tau* protein is abundant in neurons of the CNS, but is also expressed in CNS astrocytes and oligodendrocytes, and further existent in peripheral nervous system (PNS) (Paula VJR *et al.* , 2009; Kim *et al.* , 2014). This protein is responsible for stabilizing the microtubules, and belongs to the microtubule-associated proteins (MAP). A total of six different human isoforms of *tau* derived from a single gene (*MAPT* gene), located on chromosome 17 where it occupies over 100 kb and contains 16 exons, via splicing alternative (Avila *et al.* , 2004).

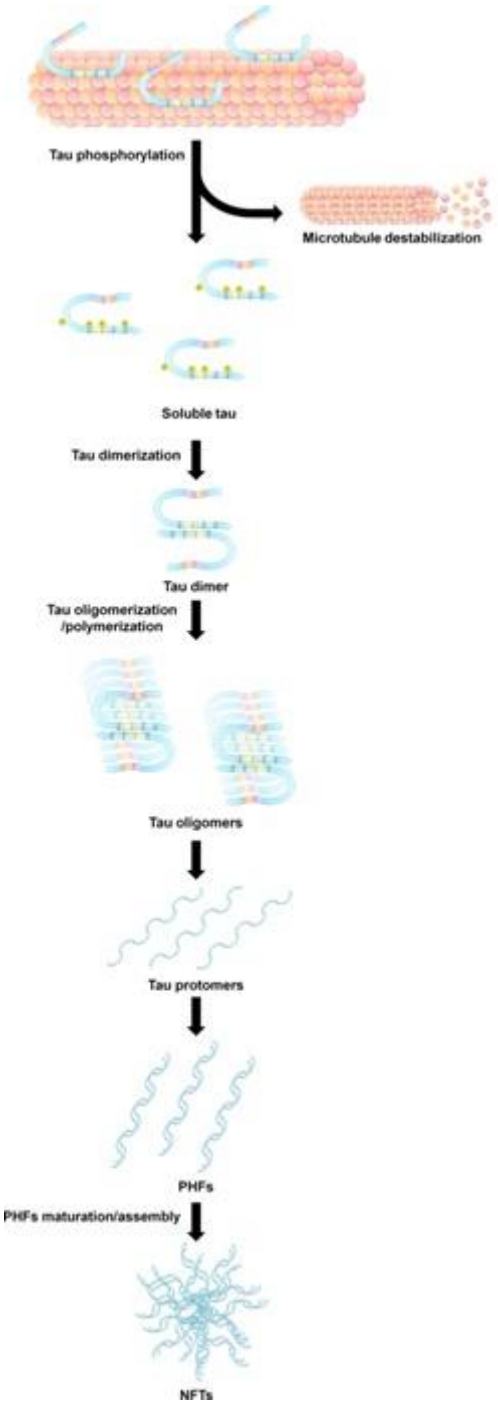

Figure 7 – Representation of NFTs formation. Hyper-phosphorylated Tau contributes to a destabilized microtubule network and NFTs formation, leading to neuronal death. Martin et al. , 2011

Structurally, *tau* protein comprises two different domains: the projection domain that contains the amino-terminal and the microtubule binding-domain containing the carboxyl-terminal. The projection domain is composed by the N-terminal rich in acidic residues and the proline-rich region, and the microtubule-biding domain has the tubulin-biding and acidic C-terminal regions (Avila *et al.* , 2004).

Under physiological conditions, tau binds to tubulin in order to promote the polymerization and inhibit the fast depolymerization of the tubulin. This process occurs by regulation of phosphorylation state of tau, and tau can be phosphorylated in 79 phosphorylation sites at serine and threonine residues. Consequently, the balance between

phosphorylation and dephosphorylation is crucial in order to promote the assembly and stabilization of the microtubules of the neurons. The most important enzyme, responsible for *tau* phosphorylation, is the enzyme glycogen synthase kinase 3β (GSK3β), but also another kinases and phosphatases, such as protein phosphatase (PP) 1 (PP1), PP2A and PP2B, are involved in this process (Paula VJR *et al.* , 2009).

On the other hand, in AD, *tau* is abnormally hyper-phosphorylated and this leads to the loss of its biological function, causing a decrease in the *tau* interaction with microtubules. This promotes the increase of soluble *tau* in the monomeric form enabling its aggregation into dimers and oligomers/protomers and then into PHFs and finally in NFTs (Figure 7) (Martin *et al.* , 2011). When NFTs accumulate in the neurons, occupy to much of the neuron, and apparently promote the neuronal death (Mudher *et al.* , 2002). Regarding this process, the “tangle hypothesis” argues that dysfunction in tau homeostasis is a primary event in AD, and that the number of NFTs increases with severity of the disease (Kim *et al.* , 2014).

Several reports have suggested a relationship between Aβ and *tau* aggregation. One of them, discovered that, after injection of Aβ1-42 into brains of mutant *tau* mice, an increase in the number of NFTs in cell bodies occurred (Gotz *et al.* , 2001).

Resuming, the explanation of the cause of AD pathogenesis is extremely debated and very controversial, but is far to be discovered.

1.8. Biomarkers of Alzheimer’s disease

The diagnosis of AD in an earlier stage of the disease, preferably before starting the first aggressive symptoms, is extremely important. However, a definitive diagnosis is only possible by examining the neuropathological hallmarks of the disease, at autopsy. Therefore, biomarkers are useful to aid in the diagnosis of AD and for the determination of disease risk. The autosomal dominantly inherited mutations are definite biomarkers of the disease, while the plasma and CSF biomarkers and neuroimaging contribute to increasing the specificity of the diagnosis (Sabbagh *et al.* , 2013).

1.8.1. Plasma biomarkers

Small and lipophilic proteins and some protein transporters able to cross the BBB are used as plasma biomarkers (Reitz *et al.* , 2014). The plasma Aβ1-42 and Aβ1-40 levels are an example, and data suggested that, in familial AD, the total Aβ levels

and Aβ1-42 levels are elevated in plasma, suggesting that this biomarker may be is useful in specific subtypes of AD (Ringman *et al.* , 2008). In addition, Schupf and his colleagues suggested that people with high Aβ1-42 levels are at increased risk of AD, and with disease progression, levels of some forms of Aβ decline in plasma (Schupf *et al.* , 2008). However, due to the large variability of person-to-person Aβ levels, variability in the timing of sample collection across studies and the use of different antibodies to detect Aβ, turns the use of this biomarker very controversial (Reitz *et al.* , 2014). Regarding this, the use of plasma Aβ1-42/Aβ1-40 ratios is more appropriate to help the diagnosis of AD. There is evidence that low plasma Aβ1-42/Aβ1-40 ratios are associated with increased risk for MCI and AD (Graff-Radford *et al.* , 2007).

Other molecules have been studied as potential plasma biomarkers, including cholesterol, homocysteine, IL-1β, TNF, IL-6 and TTR, which role in this pathogenesis will be discussed later.

1.8.2. CSF biomarkers

Due to the direct contact between brain and CSF, Aβ, total *tau* (t-*tau*), phosphorylated tau (p-*tau*) and TTR levels, in CSF, are potent biomarkers that can be used to aid the diagnosis of AD at an earlier stage of the disease.

Aβ levels in CSF have been studied by several groups, showing that Aβ1-42 levels are reduced in the CSF of patients with MCI or AD when compared to individuals with normal cognitive functions. The reduction in Aβ clearance from brain to the blood/CSF and consequently aggregation and deposition of Aβ into neuritic plaques may be an explanation to these low levels in CSF (Humpel, 2011). On the other hand, Aβ1-40 levels do not appear to be changed in AD CSF. Therefore, it has been suggested that the Aβ1-42/Aβ1-40 ratios can be used to differentiate AD patients from non-AD patients (Humpel, 2011). Contrary the Aβ1-42 levels, the t-*tau* and p-*tau* levels are increased in AD patients as compared with non-AD patients and t-*tau* seems to be a good marker with high sensitivity and specificity in predicting progression from MCI to AD (Rosen *et al.* , 2013).

Several studies reported TTR as a potential biomarker in AD. It has been suggested that TTR levels in CSF are decreased in AD patients relatively to patients without dementia (Merched *et al.* , 1998; Castano *et al.* , 2006; Gloeckner *et al.* , 2008). The relation between TTR and AD will be further discussed in this section.

Additional CSF markers, such as albumin, angiotensin cystatin C, thioredoxin and β-secretase, have been explored but the results are inconsistent leaving their predictive value as biomarkers unclear (Reitz *et al.* , 2014).

1.8.3. Neuroimaging as biomarker of AD

Currently, neuroimaging techniques have been used in early diagnosis of AD. One of the methods developed is the positron emission tomography (PET) (Sabbagh *et al.* , 2013). Briefly, the PET technique is used to identify the amyloid deposition in brain *in vivo* using FDA-approved ^{11}C Pittsburgh Compound B (^{11}C-PIB) as specific tracer. Other AD imaging modalities are magnetic resonance imaging (MRI), functional magnetic resonance imaging (fMRI) and single photon emission computed tomography (SPECT). The validity and specify of these imaging biomarkers of Aβ deposition is supported by post-mortem analysis (Reitz *et al.* , 2014).

2. Blood-Brain Barrier and Alzheimer's disease

The accumulation of Aβ in brain seems to start when an imbalance between its production and clearance occurs, leading to its aggregation and subsequent deposition into neuritic or senile plaques. Therefore, the clearance of Aβ across the BBB represents an important aspect of the AD and a therapeutic target (Nazer *et al.* , 2008). It is of crucial importance characterize BBB and understand which BBB pathways are affected in this neurodegenerative disorder.

2.1. The blood-brain barrier

The two major parts of nervous systems are CNS and PNF, and these are separated by two main biological barriers: BBB and blood-cerebrospinal fluid barrier (BCSFB) (Marques *et al.* , 2013). Alterations in morphology and functions of these barriers can disrupt the CNS homeostasis (Abbott *et al.* , 2010).

The BBB is a multicellular vascular structure and represents a problematic obstacle for a large number of biological molecules and drugs due to its impermeability. Only small lipophilic molecules, such as O_2 and CO_2, and a few drugs (barbiturates and ethanol) are capable to cross the different types of cells which comprise BBB (Abbott *et*

al. , 2006). Another physiological function of the BBB is providing essential nutrients and protects the brain from xenobiotics (Abbott *et al.* , 2006).

This barrier is composed by several distinct types of cells including endothelial cells (ECs) of cerebral capillaries and microvessels, pericytes and glial cells (astrocytes, microglia and oligodendrocytes), and also formed by tight junctions (TJ) and adherent junctions (AJ) (Figure 8). These components provide structure and functional support to the BBB (Obermeier *et al.* , 2013).

2.1.1. Cellular composition of the BBB

Cerebral endothelial cells, one of the components of BBB, have morphological characteristics of peripheral endothelial cells and epithelial cells. The ECs shows, among other features, an interconnection with TJ and AJ, very low pinocytotic and transcytotic activity, and elevated number of mitochondria signifying that these cells require a lot of energy (Wilhelm *et al.* , 2014). One of the most important functions of these cells is the creation of a paracellular barrier for ions, proteins and other molecules, and the maintenance of the vessel wall and circulatory function (Sumpio *et al.* , 2002).

Pericytes are randomly distributed along the cerebral capillaries and partially surround the endothelium. Both the ECs and the pericytes are enclosed by a local basement membrane, which contribute to form a perivascular extracellular matrix layer (basal lamina-BL1, Figure 8), used as an anchoring and signaling site for cell-cell interactions (Abbott *et al.* , 2010; Neuhaus *et al.* , 2010). The role of pericytes seems to be in BBB integrity and cerebral microcirculation, survival, migration and endothelial proliferation (Bell *et al.* , 2009; Neuhaus *et al.* , 2010). These cells secrete many molecules that influence the endothelial function, including growth factors (transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF)) and angiopoetin-1 (Wilhelm *et al.* , 2014).

The astrocytes are essential in the maintenance of the BBB due to their influence on ECs. The perivascular endfeet of astrocytes (Figure 8), which surround the endothelial cells and share the basal lamina, provide the biochemical support to ECs cells. These astrocytic feet express several molecules at their perivascular space, including glucose transporter 1 (GLUT-1), ABCB1, aquaporin-4, connexin-43, and the kir 4.1 K^+ channel (Wilhelm *et al.* , 2014). Astrocytes are able to synthesize several of

glia-derived factors, including TGF-β, glial-derived neurotrophic factor (GDNF), basic fibroblast growth factor (bFGF) and angiopoetin 1. These biologically active molecules seem to influence in endothelial cells (Abbott *et al.* , 2006).

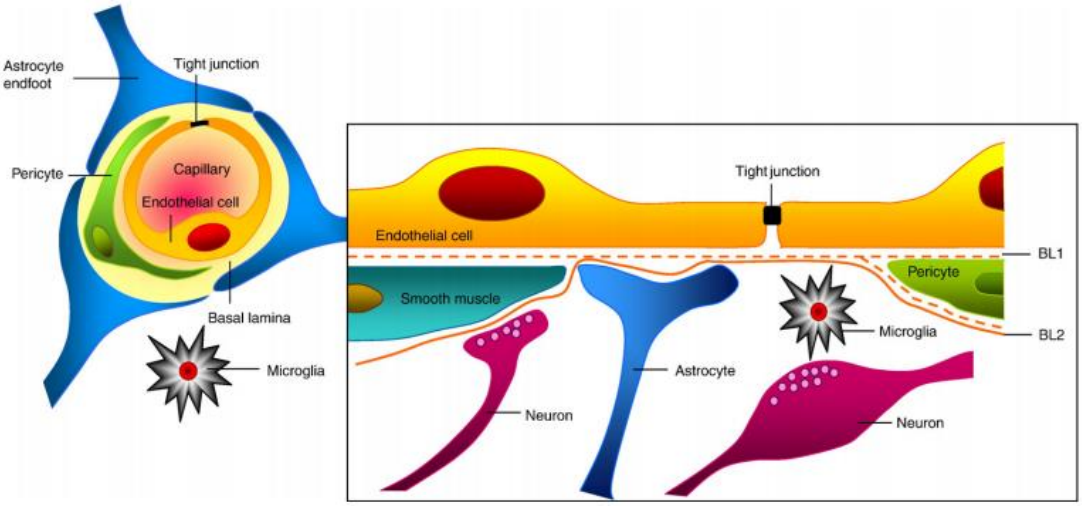

Figure 8 – Schematic representation of the cellular and molecular composition of the BBB. Abbott et al. , 2010

2.1.2. Junctional complex of the BBB

The BBB creates a “physical barrier” composed by a network of a variety of tight junctions and adherens junctions between adjacent endothelial cells (Figure 9) (Abbott *et al.* , 2006). These interendothelial junctions have a crucial role in restriction of the paracellular diffusion of the macromolecules and polar solutes between brain and endothelial cells (Luissint *et al.* , 2012).

The molecular complex of the TJ exhibits a co-interacting group of adaptor proteins, which mediates the cell-cell communications and the cytosketal tethering, such as zonula occludens (ZO-1, ZO-2 and ZO-3), 7H6, AF6, vinculin and cingulin (Cummins, 2012). The overall junctional complex comprises several transmembrane TJ proteins including occludin, MarvelD2 (tricellulin), MarvelD3, claudin and junctional adhesion molecules (JAMs) (Wilhelm *et al.* , 2014). The first integral TJ protein discovered was occludin, in 1993 (Furuse *et al.* , 1993). Occludin is a 60-65 kDa protein with multidomain tetraspan structure and two extracellular loops (Hartsock *et al.* , 2008). The C-terminal of occludin is essential to interact with ZO-1 and subsequently to help its intracellular trafficking to the plasma membranes and also to mediate its dimerization. This TJ protein also displays a MARVEL domain composed by a four-

transmembrane structural motif common among junctional proteins involved in membrane apposition and fusion events. MARVELD3 and MARVELD2 also exhibit this domain (Cummins, 2012). In addition to the occludin TJ, the claudins are other type of TJ and also belong to the tetraspan family (Wilhelm *et al.* , 2014). These proteins, with molecular weights between 20 and 27 kDa, have an important role in maintaining the paracellular barrier and in pore formation. At BBB level, claudins-1,-3,-5 and -12 have been shown to contribute to the formation of the TJs between microvascular endothelial cells. However the most important isoform is claudin-5 because its absence provokes the opening of the BBB for molecules smaller than 800 Da (Schrade *et al.* , 2012). Additionally, the JAMs proteins are other group that participate in formation and maintenance of the TJ, and belong to the immunoglobulin (Ig)-superfamily. These proteins are composed by a single transmembrane domain and two extracellular loops. There are three known JAMs (JAM-A, JAM-B and JAM-C), but the best characterized is JAM-A. This junctional adherent molecule has a crucial role in the determination of the paracellular permeability (Abbott, 2002; Wilhelm *et al.* , 2014).

The adherens junctions are the last structures which comprise the junctional complex. These proteins are responsible for cell-cell adhesion, stabilization of the actin cytoskeleton, intracellular signaling and transcriptional regulation. This group of proteins is composed by transmembrane protein E-cadherin, and members of catenin family, such as p-120-catenin, β-catenin and α-catenin. All these proteins are responsible for controlling the formation, maintenance and function of adherens junctions (Hartsock *et al.* , 2008).

Resuming, all junctional complex, composed by these several distinct proteins, is a dynamic structure responsible for the maintenance of the cellular permeability regulated by a sophisticated signaling network (Wilhelm *et al.* , 2014).

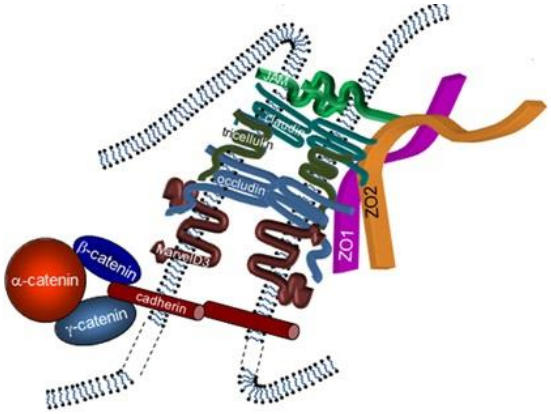

Figure 9 –Representation of composition of junctional complex of the BBB. Endotheliall cells of the BBB are interconnected by a network of tight and adherens junctions. Adapted from Wilhelm et al. , 2014.

2.2. Transport at BBB

The maintenance of the homeostasis is supported by the selective transport of the nutrients into the brain and the exclusion of xenobiotics out of the brain. The transport across the BBB is made by specific transport systems (Figure 10) present on the luminal (apical membrane or blood) and the abluminal (or basolateral membrane or brain) membranes. Generally, most of the transporters are members of the solute carrier family (SLC) or members of active and energy-dependent ATP binding cassette (ABC) transporter family (Neuhaus *et al.* , 2010).

One of the main types of transport is the passive diffusion, where solutes can diffuse through the membranes of the endothelium and enter in the brain passively (Abbott *et al.* , 2010). The lipophilicity favors this process and lipidic molecules, in general, show higher permeability (Neuhaus *et al.* , 2010). Thus, molecules from blood gases such as O_2 and CO_2, and non-polar molecules perform this type of transport at the BBB.

In addition, the removal of metabolites and toxic molecules is made by active efflux (with consumption of ATP) from the brain and the BBB by members of the ABC transporters family (Neuhaus *et al.* , 2010). These transporters are a superfamily of proteins and the main ABC transporters are P-glycoprotein (P-gp, Multidrug resistance Protein, ABCB1/MRDR1), the multi-drug resistance-associated proteins (MRPs, ABCC1, 2, 4, 5 and possibly 3 and 6) and ABCG2/BCRP (Breast cancer resistance protein). Regarding this, the first discovered export pump at the BBB was P-gp, localized in the luminal brain membranes, and its major role is protection of the brain from neurotoxic endogenous molecules (Cordon-Cardo *et al.* , 1989; Abbott *et al.* , 2010). In general, the function of these ABC transporters is the neuroprotection by detoxifying the brain (Abbott *et al.* , 2010).

Due to its polarity, several nutrients and biological molecules cannot across the BBB freely. However, these nutrients are extremely necessary for metabolism. Therefore, the BBB endothelium shows a number of specific solute carriers to supply the CNS with these substances (Abbott *et al.* , 2010). Many of these transporters belong to the SLC superfamily, which comprises 55 gene families with at least 362 putatively functional protein-coding genes. These several genes can express distinct types of carriers such as passive transporters, symporters and antiporters, as well as mitochondrial and vesicular transporters (He et al. , 2009). The most abundantly expressed SLC carriers are SLC2A1, SLC7A5, SLC16A1, SLC1A3 and SLC1A2

(Shawahna *et al.* , 2011). Specifically, the SLC2A1 (GLUT-1 transporter) is the glucose transporter, which is transported by facilitated diffusion. However, other substrates are transported by this group of SLC including amino-acids and oligopeptides, cations and anions, bile salts, essential metals, neurotransmitters, vitamins, fatty acids and lipids, nucleosides, ammonium, choline, thyroid hormone and urea (He *et al.* , 2009).

At last, some substances are transported across the BBB endothelium by endocytosis, although the brain endothelium presents lower levels of this process compared with peripheral capillaries. However, the transport of too large molecules - peptides and proteins – is made via a vesicular route, either by a specific receptor mediated transcytosis (RMT) or by non-specific adsorption-mediated transcytosis (AMT), and in most cases, molecules can be transcytosed by adsorption to the clathrin-coated or caveolar vesicle membrane. Thus, the RMT is active by a specific interaction with RMT receptors expressed on brain ECs, and the main ligands are insulin, transferrin, LDL-cholesterol, leptin and insulin-like growth factors (IGF-I and IGF-II). By contrast, the AMT is triggered by an electrostatic interaction between a cationic substance and negatively charged luminal membrane of the ECs. (Herve *et al.* , 2008; Neuhaus *et al.* , 2010).

Several of these types of transporters are involved in the flux of Aβ peptide across the BBB being the receptor-mediated transport, the main responsible for transport of Aβ.

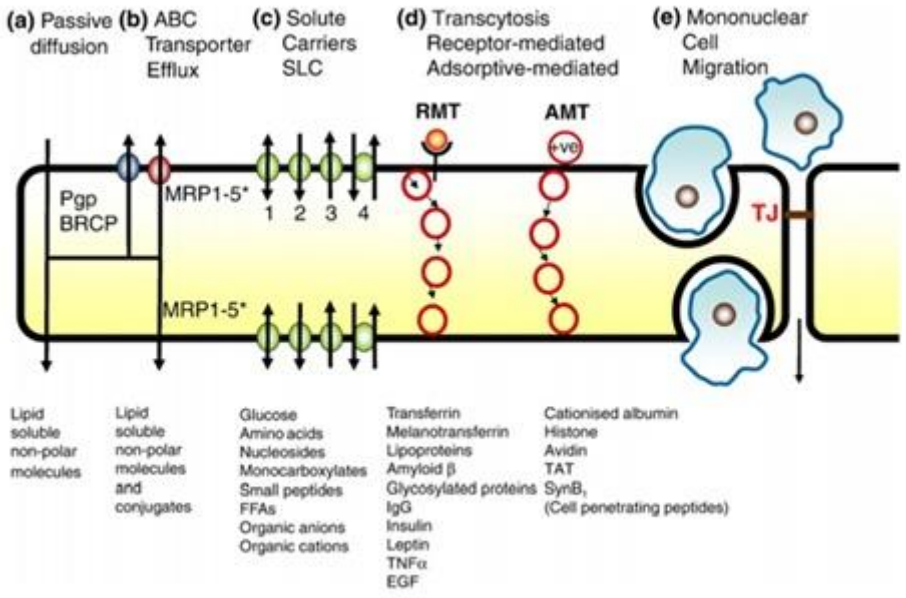

Figure 10 – Scheme of several routes of transport across the BBB. Abbott et al. , 2010

2.3. Aβ clearance across the BBB

The clearance of Aβ from the brain takes place by three distinct pathways: transport across the BBB, proteolytic degradation (by action of Neprilysin (NEP) and insulin-degrading enzyme (IDE)) in the brain and bulk flow of CSF (Bell *et al.* , 2009; Grimm *et al.* , 2013; Qosa *et al.* , 2014). In this topic will be discussed the mechanism that occurs at the BBB.

The efficiency of Aβ clearance from brain interstitial fluid (ISF) across the BBB is influenced by several Aβ binding transport proteins such as ApoE and ApoJ (clusterin), TTR and α-2-macroglobulin (α2M) and BBB receptors, including LRP1 and receptor for advanced glycation end products (RAGE) and Aβ degrading enzymes (Deane *et al.* , 2009).

2.3.1. Receptor-mediated transport

Aβ is transported bidirectionally across the BBB (Figure 11): brain-to-blood and blood-to-brain directions (Banks *et al.* , 2005).

The main receptor of the BBB responsible for the efflux of Aβ is LRP1. This receptor mediates Aβ transcytosis across the BBB and the process initiates at the abluminal side of the endothelium and, then is directly eliminated from brain into bloodstream (Shibata *et al.* , 2000; Zlokovic, 2004). LRP1 is a ~600 kDa receptor, a member of the low-density lipoprotein (LDL) receptor family (Donahue *et al.* , 2006). It is composed by an extracellular domain (heavy α-chain, 515 kDa), a transmembrane domain (85 kDa) and cytoplasmatic light β-chain domain). The α-chain has four ligand-binding domains (clusters I-IV), consisting of 2, 8, 10 and 11 cysteine-rich complement repeats, respectively. In addition to Aβ, the domains II and IV bind over forty ligands and include the next classes: lipoproteins, coagulation factors, growth factors, extracellular matrix proteins, chaperones and bacterial/viral proteins (Nazer *et al.* , 2008). The cytoplasmatic chain is composed by two NPXY motifs, one YXXL motifs and two di-leucine motifs (Li *et al.* , 2001; Deane *et al.* , 2009). This shorter cytoplasmitic tail is phosphorylated on serine and/or tyrosine residues, which interacts with distinct adaptor proteins such as disabled-1 (Dab-1), FE65 and postsynaptic density protein 95 (PSD95) (Kanekiyo *et al.* , 2014). Thus, in the CNS, LRP1 has a critical role as scavenger and transporter and a transmembrane cell signaling receptor.

Briefly, the removal of Aβ from the brain initiates when Aβ binds to the LRP1 at the endothelial membrane and crosses the BBB to the blood. Then, in bloodstream the free Aβ is sequestered by soluble LRP (sLRP), which is the truncated extracellular domain of LRP1, and recognized as major endogenous peripheral “sink” agent of Aβ. Finally, the circulating Aβ is degraded in the liver and also in the kidney (systemic clearance). (Bell *et al.* , 2009).

Additionally, Aβ peptide seems to have other transporters such as P-gp and LRP2. It was suggested that LRP2, also called megalin/gp330 receptor, is involved in efflux of Aβ, when this peptide forms a stable complex with the ApoJ (Bell *et al.* , 2007). Relatively to the P-gp, an ATP-dependent efflux pump expressed on the luminal surface of brain endothelium, one *in vivo* study demonstrated that when [^{125}I]Aβ1-40 and [^{125}I]Aβ1-42 was injected into the CNS of P-gp-null mice, the rate of clearance was half of that in controls mice. Furthermore, in the same study, APP transgenic mice treated with P-gp inhibitor demonstrated increased levels of Aβ within brain ISF compared with non-treated ones (Cirrito *et al.* , 2005). Thus, P-gp seems to cooperate with LRP in transport of Aβ out of the brain (Jeynes *et al.* , 2013). However, the mechanisms of efflux by P-gp and megalin remains unclear (Sagare *et al.* , 2013).

On the other hand, RAGE receptor is implicated in the Aβ influx into the brain from the periphery (Deane *et al.* , 2003). RAGE is a multiligand receptor of the immunoglobulin superfamily, located on the luminal membrane of the brain endothelium (Deane *et al.* , 2009). Furthermore, RAGE is a 45 kDa receptor comprising three immunoglobulin domains (one V-type and two C-type) followed by a single transmembrane region, and a short C-terminal cytoplasmatic tail (Park *et al.* , 2010; Sagare *et al.* , 2013). The extracellular V-domain is necessary to the interaction with several ligands such as AGE proteins, S100/calgrulins, monomeric and oligomeric Aβ, amphoterin, and the family of crossed β-sheet macromolecules (Yan *et al.* , 2010).

Summarizing, the balance between RAGE and LRP1 receptors, the main Aβ transporters across the BBB, is extremely important to maintain Aβ physiological levels. Any alterations in these receptors or proteins related to them can provoke BBB dysfunction and impaired clearance of Aβ from the brain

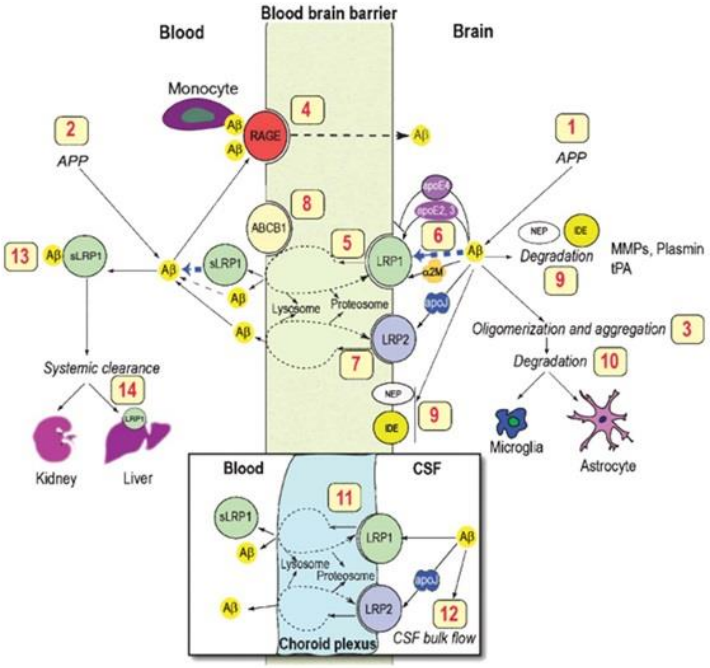

Figure 11 – Schematic representation of the mechanism of the Aβ clearance across the BBB. Aβ transport is mediated by several pathways: (1) central and (2) systemic production of Aβ from its precursor APP; (3) oligomerization and aggregation; (4) influx of Aβ mediated by RAGE; (5) efflux of Aβ across BBB by LRP1; (6) interaction between ApoJ /ApoE and Aβ; (7) efflux of Aβ mediated by megalin (LRP2); (8) efflux of Aβ mediated by P-gp receptor; (9)enzymatic degradation of Aβ by NEP and IDE; (10) cellula degradation by glial cells; (11) Aβ transport in choroid plexus; (12) Aβ removal via ISF-CSF bulk flow; (13) Sequestration of Aβ by sLRP1; (14) Aβ systemic clearance at liver and kidney. Bell et al. , 2009

2.4. BBB dysfunction and AD

In AD, one of the possible reasons for the accumulation of Aβ peptide into amyloid plaques is an impaired clearance of this peptide across the BBB (Mawuenyega *et al.* , 2010). It has been suggested that imbalance of Aβ clearance may be caused by alterations in Aβ BBB receptors, such as LRP1, RAGE and P-gp (Figure 12).

As mentioned above, LRP1 mediates the efflux of Aβ peptide out of the brain. Clearly, failure in this transport leads to an increase of Aβ deposition in the brain. Several studies demonstrated that LRP1 expression in brain endothelium decreases with aging in rodents, as well as in AD models and AD patients (Shibata *et al.* , 2000; Deane *et al.* , 2004; Donahue *et al.* , 2006; Bell *et al.* , 2009). Thus, one of the possibilities for Aβ accumulation is down-regulation of LRP1. Moreover literature

reports that the expression of LRP is negatively regulated by Aβ levels (Wang *et al.* , 2006).

Other important evidence in AD is a decreased expression of the P-gp Aβ transporter with increased Aβ levels in AD brain, which seems to progressively contribute to Aβ accumulation (Vogelgesang *et al.* , 2002).

By contrast, when Aβ accumulates in AD brain, RAGE expression increases in microvessels, neurons or microglia (Yan *et al.* , 1996). The up-regulation of RAGE leads to excessive amounts of Aβ in AD brain through a positive-feedback mechanism (Shibata *et al.* , 2000). The interaction Aβ/RAGE in AD seems to mediate a relevant neuronal dysfunction, inflammatory responses in the endothelium, NF-κB-dependent apoptosis and finally neuronal death (Deane *et al.* , 2009).

Thus, decreased expression of Aβ efflux transporters at the BBB (LRP1 and P-gp) and increased expression of Aβ influx transporter RAGE seems to contribute to Aβ accumulation in the brain by imbalance in the clearance pathway at the BBB.

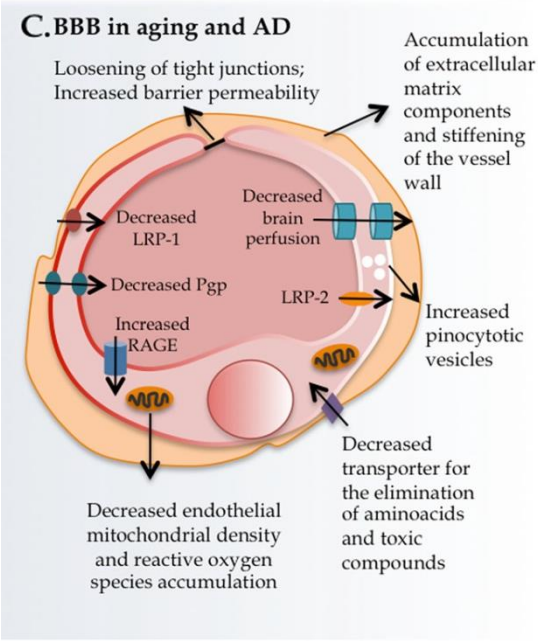

Figure 12 – Alterations at the BBB in aging and AD. Adapted from Marques et al. , 2013

3. Transthyretin

Several molecules have been suggested as Aβ carriers and among them TTR has received special attention (Schwarzman *et al.* , 1994). TTR was first described by Seibert and Nelson, in 1942, as an X component with a slightly mobility greater than albumin (Seibert; *et al.* , 1942). TTR is mainly synthetized in the liver and in the choroid plexus of the brain, being secreted into the plasma (3-7 μM) and CSF (0.1-0.4 μM), respectively (Soprano *et al.* , 1985; Yang *et al.* , 2013).

3.1. TTR structure and functions

Initially, TTR was designated as prealbumin, but its current name is derived from its mains physiological functions: transport of the thyroid hormone thyroxine (T_4) and retinol (vitamin A), through the binding of retinol binding protein (RBP) (Power *et al.* , 2000; Costa *et al.* , 2008a). Besides its well-known transport capabilities, another TTR function was described by Liz et al. proposing that TTR might be a protease able to cleave apolipoprotein A1 (ApoA1) *in vitro*, and later suggested that TTR is also capable to cleave lipidated ApoA1 and that this cleavage decreases its ability to promote the efflux of cholesterol (Liz *et al.* , 2004; Liz *et al.* , 2007). Moreover, Costa and co-workers showed for the first time that Aβ peptide is cleaved by TTR (Costa *et al.* , 2008a). Newly, Liz et al. also characterized TTR as a metallopeptidase, and more recent FRET study confirmed this recently described inducible mechanism proposed for TTR based on 3D structure in presence of Zinc and a series of point mutations (Liz *et al.* , 2012; Gouvea *et al.* , 2013).

Structurally, TTR is a 55 kDa homotetrameric transport protein and each monomer has two four-strand β-sheets, composed by an inner sheet of strands D, A, G and H, and an outer sheet of strands C, B, E and F, as well as a small α-helix and a loop that connects strands E and F. The assembly of the monomer into dimers is stabilized by an extensive hydrogen bonding and subsequently assembly of two dimers linked by hydrogen bonds and hydrophobic bonds between loop AB from one dimer and strand H from the other dimer. Consequently, tetramer assembly (Figure 13) forms a hydrophobic channel where T_4 binds, while retinol binds at surface of the protein and does not interfere with T_4 binding (Morais-de-Sa *et al.* , 2004; Yang *et al.* , 2013). The specific domains on TTR involved in Aβ/TTR binding were probed by Du et al. in 2012. This study demonstrate by an array method that Aβ binds strongly to TIAALLSPYSYS

(residues 106-117) sequence that correspond to strand G on the inner β-sheet of TTR, but a weakly bound was also detected in residues 59-83, which includes strand E through the E/F helix and loop (Du *et al.* , 2012).

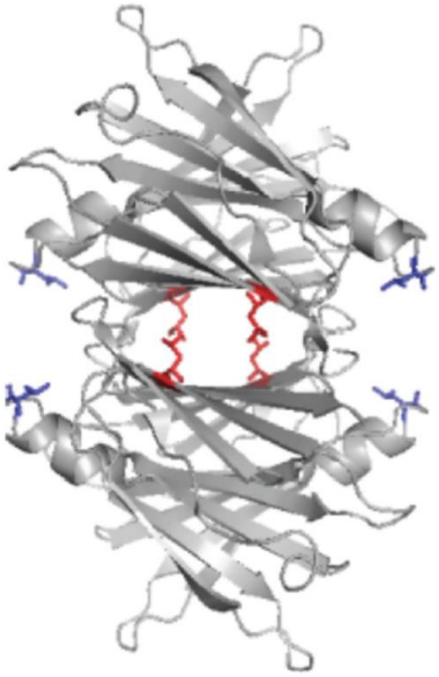

Figure 13 – Structure of homotetrameric TTR. The colour red shows Leucine 82 on end of EF strand and blue shows leucine 110 on strand G. Yang et al. , 2013

3.2. TTR as cause of disease

Besides its protective role in AD, which will be debated forward, TTR is also associated with disease, in particular with amyloidosis, a group of disorders defined by the formation and aggregation of misfolded proteins which leads to its accumulation in distinct organs (Westermark *et al.* , 2007). The main syndromes associated to TTR aggregation are senile systemic amyloidosis (SSA), familial amyloidotic polyneuropathy (FAP) and cardiomyopathies (FAC) (Buxbaum *et al.* , 2009).

The main amyloidotic disorder associated to TTR is FAP. This disorder was described more than 60 years ago by the Portuguese professor Corino de Andrade (Andrade, 1952) and TTR was identified as principal component present in deposits of FAP patients in 1978 (Costa *et al.* , 1978). Thus, FAP is a hereditary autosomal dominant disease characterized by deposition of fibrils especially in PNS, and the most common mutation that cause FAP is the substitution of a valine residue for a

methionine at position 30 (V30M), however more than 100 mutations are currently known (Saraiva *et al.* , 1984; Fleming *et al.* , 2009b).

3.3. TTR in the nervous system

It has been demonstrated that TTR has several neuroprotective properties in numerous contexts. Studies with TTR null mice revealed 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). Additionally, increased levels of NPY, known as an 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), supporting the importance of TTR in the modulation of depressive behavior. Furthermore, Sousa and co-workers also described that $TTR^{-/-}$ mice showed memory impairment compared with $TTR^{+/+}$ animals, indicating that the absence of TTR accelerate cognitive deficits usually associated with aging (Sousa *et al.* , 2007).

In addition, TTR was associated to nerve regeneration. Fleming et al. revealed, for the first time, that TTR can act as a nerve regeneration enhancer by the observation that $TTR^{-/-}$ mice presented a decrease in regeneration of a sciatic crushed nerve (Fleming *et al.* , 2007). Later, the same authors showed that absence of TTR leads to impaired retrograde transport and decreased axonal growth, and also that the effect of TTR in neurite outgrowth and nerve regeneration is mediated by megalin-dependent internalization (Fleming *et al.* , 2009a).

It was also established a relationship between TTR and ischemia, one of the major causes of brain injuries in world. Santos and co-workers proposed 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).

More recently, a new role for TTR was proposed in CNS, as a transcription inducer of insulin-like growth factor receptor I (IGF-IR), known as a protective receptor against apoptosis (Kooijman, 2006). Vieira and colleagues described, for the first time, that TTR induces increased levels of IGF-IR, and they also showed, using a GFP-IGF-IR fusion protein, that TTR triggers IGF-IR nuclear translocation in cultured neurons, unveiling a new neuroprotective role for TTR (Vieira *et al.* , 2014).

3.4. TTR and its protective role in AD

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 *et al.* , 1996). The importance of TTR in AD is supported by the observation that TTR is decreased in CSF of AD patients (Hansson *et al.* , 2009); Ribeiro et al. and others showed that TTR is also decreased in plasma/sera from AD patients (Han *et al.* , 2011; Ribeiro *et al.* , 2012a; Velayudhan *et al.* , 2012). Importantly, this decrease is observed early in disease development as indicated by the lower plasma TTR levels measured not only in AD patients but also in patients with MCI; it is also reported that TTR levels correlate with disease state, prompting TTR as an attractive early biomarker (Ribeiro *et al.* , 2012a). Very recently, researchers have identified 10 plasma proteins, including TTR, strongly associated with disease severity and disease progression (Hye *et al.* , 2014). In addition, data from transgenic mice has shown 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; Oliveira *et al.* , 2011). Oliveira and co-workers provided evidence for gender-associated modulation of brain Aβ levels by TTR and suggested that female transgenic AD/TTR mice with one copy of TTR gene has elevated Aβ levels in the brain when compared to female transgenic AD/TTR mice with two copies of the TTR gene. This report demonstrated also that TTR reduction influences brain levels of 17β-estradiol in a gender-associated manner (Oliveira *et al.* , 2011). Interestingly, Ribeiro et al. evaluated TTR levels in plasma of MCI/AD patients and showed that AD women presented lower TTR levels compared to women control. However, MCI and AD men did not present significant differences compared to control men, further supporting a gender-associated modulation by TTR in AD (Ribeiro *et al.* , 2012a).

Regarding the nature of TTR/Aβ interaction, several researchers confirmed the binding between TTR and Aβ as well as the possibility of binding to the distinct Aβ species. Buxbaum and co-workers demonstrated that TTR has the capacity to bind toxic Aβ aggregates in extracellular and intracellular environment in a chaperone-like manner (Buxbaum *et al.* , 2008). Liu and Murphy also described binding of TTR to aggregated forms of Aβ (Liu *et al.* , 2006). Other reports confirmed the role of TTR as

an Aβ binding protein and also showed, using competition binding assays that TTR binds to different Aβ species including soluble, oligomers and fibrils. Additionally, Costa et al. demonstrated that TTR is capable of interfering with Aβ fibrillization by inhibiting and disrupting fibril, suggesting the inhibition/disruption of Aβ fibrils as a possible protective mechanism underlying the protective role of TTR in AD (Costa *et al.* , 2008b). Supporting the hypothesis that TTR binds to Aβ, in another report, Costa et al. demonstrated that TTR (recombinant and isolated from human sera) is capable to cleave Aβ in multiple positions (Costa *et al.* , 2008a).

Understanding which TTR conformation binds to Aβ is an important discussion between investigators. Some authors argued that Aβ monomers bind preferentially to the TTR in monomer form (Du *et al.* , 2010; Yang *et al.* , 2013) while other investigators claim the opposite (Schwarzman *et al.* , 2004; Ribeiro *et al.* , 2012b). In fact, Yang and his co-workers proposed one novel mechanism of action for TTR: when EF helix/loop of the TTR "feels" the presence of soluble toxic Aβ oligomers triggers an destabilization of the TTR tetramer and subsequently exposes the hydrophobic inner sheet, which leads to the scavenging of the oligomers (Yang *et al.* , 2013). Differently, other investigators claim that the tetramer is essential for Aβ binding and that unstable TTR mutations (in which dissociation of the tetramer occurs) do not bind or bind poorly to Aβ (Schwarzman *et al.* , 2004; Ribeiro *et al.* , 2012b).

As mentioned previously, TTR mutations are associated with FAP, a systemic amyloidosis with a special involvement of the peripheral nerve. It is believed that the amyloidogenic potential of the TTR variants is related to a decrease in tetrameric stability and it is thought that dissociation of the tetramer into monomers is the basis of a series of events that leads to the formation of TTR amyloid (Cardoso *et al.* , 2002). 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). 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 (Almeida *et al.* , 2004; Morais-de-Sa *et al.* , 2004).

To further gain insights into the factors affecting TTR decrease in AD, plasma TTR binding to T_4 was assessed and authors found a decreased ability of the protein to carry T_4 in AD patients, indicating that this function of TTR is impaired (Ribeiro *et al.* , 2012a). This observation indicates that TTR is destabilized and its clearance accelerated, explaining the lower levels found in AD. It was then hypothesized that TTR stabilization may be a key factor in the TTR/Aβ interaction as also previous works

showed that TTR amyloidogenic variants bind less to Aβ peptide (Costa *et al.* , 2008b). Authors then proposed 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: drugs such as iododiflunisal (IDIF), resveratrol, dinitrophenol (DNP), 2-((3,5Dichlorophenyl) amino) benzoic acid (DCPA) and 4-(3,5-difluorophenyl) (DFPB), were tested and found to strengthen TTR/Aβ interaction (Ribeiro *et al.* , 2012b). In addition, the effects of one of such drugs – IDIF – were studied *in vivo* in an AD mouse model (Ribeiro *et al.* , 2014). The results showed that IDIF administered orally bound TTR in plasma and stabilized the protein, and was able to enter the brain. 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 (Ribeiro *et al.* , 2014).

Very recently, it has been shown TTR expression in neurons and some hints on its regulation have already been advanced. Kerridge and colleagues showed that TTR is expressed in SH-SY5Y neuroblastoma cell line, and that it is up-regulated by the AICD fragment of APP, specifically derived from the APP695 isoform (Kerridge *et al.* , 2014). Induced accumulation of functional AICD resulted in TTR up-regulation and Aβ decreased levels. Wang and colleagues reported that TTR expression in SH-SY5Y cells, primary hippocampal neurons and the hippocampus of APP23 mice is significantly enhanced by heat shock factor 1 (HSF1) (Wang *et al.* , 2014).

The expression of TTR by neurons, together with the observation of Aβ reduced levels in plasma and CSF upon IDIF treatment (Ribeiro *et al.* , 2014), lead us to hypothesize that TTR might also be involved in Aβ efflux at the BBB.

Objectives

The main goals of this project were to study how TTR exerts its protective role in AD, by investigating the influence of TTR in Aβ transport across the BBB, using a cellular BBB model and an *in vivo* study in mice. We also aimed at finding stimulus that can induce TTR neuronal synthesis, using a neuronal cell model. The two chapters of this project had the following objectives:

1. To investigate the influence of TTR in Aβ transport across the BBB we will:

    a. ascertain the effect of TTR in Aβ plasma levels by measuring Aβ1-40 and Aβ1-42 concentrations in plasma of AD/TTR$^{+/+}$, AD/TTR$^{+/-}$ and AD/TTR$^{-/-}$ female mice, at different ages;
    b. optimize a cellular model for the BBB – Immortalized human cerebral microvascular endothelial cell line (hCMEC/D3);
    c. evaluate the influence of TTR in Aβ internalization and efflux by hCMEC/D3 cells, using different approaches;
    d. investigate the effect of TTR in hCMEC/D3 basolateral-to-apical permeability to Aβ1-42, using a transwell system;
    e. assess the effect of TTR in Aβ clearance *in vivo*, by injecting FAM-Aβ1-42 intracranial in mice with different TTR genotypes.

2. To find stimulus that lead to neuronal TTR synthesis, we will:

    a. investigate the effect of different Aβ species in TTR and APP genes expression using a SH-SY5Y neuroblastoma cell line;
    b. ascertain the influence of 695APPswe isoform in TTR gene expression versus the effect of exogenous TTR in expression of APP gene, using SH-SY5Y cell line.

Chapter I: Exploring the protection of Transthyretin in Alzheimer's disease: effects on Aβ peptide clearance at brain-blood barrier

Material and methods – chapter I

1. Animals

The AD mouse model APPswe/PS1A246E/TTR was used for the quantification of Aβ levels in plasma at different ages. The colony was generated by crossing APPswe/PS1A246E transgenic mice (Borchelt *et al.* , 1997) (B6/C3H background) purchased from The Jackson Laboratoy with TTR-knockout mice ($TTR^{-/-}$) (SV129 background) (Episkopou *et al.* , 1993) as previously described (Oliveira *et al.* , 2011). In this study, we used cohorts of littermates APPswe/PS1A246E/$TTR^{+/+}$ (carrying 2 copies of the TTR gene), APPswe/PS1A246E/$TTR^{+/-}$ (carrying 1 copy of the TTR gene) and APPswe/PS1A246E/$TTR^{-/-}$ (without TTR gene) female mice aged 3, 6 and 10 months (n=6 for each genotype and age).

TTR-wild type (+/+), TTR-heterozygous (+/-) and TTR-knockout (-/-) mice in a SV129 background (Episkopou *et al.* , 1993) were obtained from the littermate offspring of heterozygous breeding pairs. These animals were utilized for the i*n vivo* brain Aβ1-42 efflux assay and the study was performed using male mice with 3 months of age with different TTR genotypes (n=3). The TTR-wild type (+/+) and TTR-knockout (-/-) mice were also used to obtain sera or plasma with different TTR genotypes.

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

2. Blood collection

Mice were profoundly anesthetized with an anesthetic combination of ketamine (75 mg/Kg) and medetomidine (1 mg/Kg) by intraperitoneal injection (IP). Blood was collected from the inferior vena cava with syringes with and without EDTA (as anticoagulant) to obtained plasma or sera, respectively, followed by centrifugation at

1000 rpm for 15 minutes at room temperature (RT). Plasma and sera samples were collect and frozen at -80 °C until used.

All efforts were made to minimize pain and distress; all animal experiments were carried out in accordance with the European Communities Council Directive.

3. Aβ levels quantification

Plasma Aβ1-42 and Aβ1-40 levels were quantified using Aβ1-42 Human Ultrasensitive ELISA Kit and Aβ1-40 Human ELISA Kit (both Invitrogen), respectively, according to the manufacturer's instructions.

The same ELISA analysis was also used to determine the Aβ1-42 levels in upper chamber (or apical side) and lower chamber (or basolateral side) of transwells in BBB transport studies.

4. Preparation of Aβ1-42 peptides

The Aβ1-42 peptide was purchased from Genscript, dissolved in hexafluoro-2-propanol (HFIP) and kept at room temperature overnight. The HFIP was removed under a stream of nitrogen and the residue was then dissolved in DMSO at 2mM.

Aβ1-42 labeled with 5-(and-6)-Carboxyfluorescenin (FAM) – FAM-Aβ1-42 – was purchased from Anaspec and dissolved in PBS with 1% NH_4OH. For *in vitro* experiments, FAM-Aβ1-42 was diluted in Endothelial Cell Growth Medium (EBM-2 medium) and for *in vivo* experiments FAM-Aβ1-42 was diluted in Phosphate Buffered Saline (PBS).

5. Recombinant TTR production and purification

Human recombinant TTR (hrec TTR) was 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 Bradford Method (Bio-Rad), using bovine serum albumin (BSA) as standard.

6. Cell culture

Immortalized human cerebral microvascular endothelial cell line (the hCMEC/D3 cell line) was kindly provided by Dr. P.O. Couraud (Institute Cochin, Paris, France). The original brain endothelial cells used for generation of the cell line were isolated from the temporal lobe of brain of an adult female with epilepsy. The hCMEC/D3 cell line had been immortalized by lentiviral transduction using large SV40 T antigen (Weksler *et al.* , 2005).

Cells were used between passages 25 and 35. Cell culture surfaces were coated with rat tail collagen type I solution (Corning life sciences) at a concentration of 150 µg/mL and incubated for 1 hour at 37^{o}C. For culturing, cells were grown in EBM-2 medium (Lonza) supplemented with 5% of fetal bovine serum (FBS) (Gibco), 1% of penicillin-streptomycin (Lonza), 1,4 µM of hydrocortisone (Sigma), 5 µg/mL of Ascorbic acid (Sigma), 1% of chemically defined lipid concentrate (Gibco), 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) and 1 ng/mL of human basic fibroblast growth factor (bFGF) (Sigma). Cells were cultured in an incubator at 37^{o}C in a humidified atmosphere of 95% and 5% of carbon dioxide (CO_2). Cell culture medium was changed every 2-3 days. During experiments cells were maintained in 0% FBS medium.

7. Protein extraction

Proteins from washed hCMEC/D3 cells were extracted in lysis buffer (20 mM MOPS pH 7.0; 2 mM EGTA; 5 mM EDTA; 30 mM sodium fluoride; 60 mM β-glycerophosphate pH 7.2; 20 mM sodium pyrophosphate; 1 mM sodium orthovanadate; 1% triton X-100), 1 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitors (GE healthcare) by scraping the cells, followed by 20 minutes on ice. Extracts were then centrifuged at 14 000 rpm at 4^{o}C for 20 minutes, and supernatants were used for protein analysis. Total protein concentration was quantified in all extracts by the Bradford Method (Bio-Rad), using BSA as standard.

8. TTR depletion from human sera

Human sera, available in the laboratory from volunteer's donors, were depleted to remove TTR using column affinity chromatography. The first step consisted in the coupling of the protein G sepharose beads (GE healthcare) with the polyclonal rabbit

anti-human prealbumin (Dako) (2 mg of antibody per mL of beads) during 3 hours with shaking. After incubation, the beads were washed and incubated 1 hour with a fresh crosslinking solution (20 mM dimethyl pimelimidate (DMP) in 100 mM sodium borate pH 9.0) on a shaking plataform. Then, the beads linked to antibody were transferred to a column and further incubated with 1 mL of human serum for 2 hours at RT. After column packing, the TTR depleted serum was collected followed by elution of TTR protein with a suitable Gentle Ag/Ab elution buffer (Thermo Scientific). The TTR depletion from human serum was confirmed by 15% SDS-PAGE followed by transference to a nitrocellulose membrane as described above.

9. Western-Blot analysis

The presence of TTR in human serum after depletion, as well as the expression of tight junctions proteins, such as claudin-3 and occludin, and LRP1 by hCMEC/D3 cells was studied by western blot analysis.

Protein extract denatured samples (30 µg) were separated (constant amperage of 25 mA) by acrylamide/bisacrylamide gel (10% for LRP1 and 15% for claudin-3, occludin and TTR) (Bio-rad). After SDS-PAGE, the proteins were transferred (2 hours at a constant voltage of 100 V) to nitrocellulose membrane (Whatman™ Ge healthcare – Protan BA 83), using a wet system (Bio-rad Criterion Blotter). The membranes were blocked 1 hour at RT with 5% powered skimmed milk in PBS containing 0,05% Tween-20 (PBS-T). After blocking, membranes were then incubated with primary antibodies in 3% powered skimmed milk/PBS-T against the proteins under study (table 1). Then, washed membranes were incubated for 1 hour at RT with sheep anti-rabbit immunoglobulins conjugated with horseradish peroxidase (The binding site; 1:10000) in 3% powered skimmed milk/PBS-T. The blots were developed using Clarity™ Western ECL substrate (Bio-rad) and proteins were detected and visualized using a chemiluminescence detection system (ChemiDoc, Bio-rad). When necessary, membranes were stripped for 10 minutes at RT with gentle shaking using a commercial stripping buffer (Restore™ Western Blot stripping buffer; Thermo scientific), for re-utilization of the membranes, according to the manufacturer's instructions.

Table 1. List of antibodies used in Western Blot and immunofluorescence analysis.

		Western Blot	Immunofluorescence
Protein	**Primary antibody**	**Dilution**	**Dilution**
Human TTR	Polyclonal rabbit anti-human prealbumin (Dako)	1:2000	-
LRP1	Polyclonal rabbit antibody anti-LRP1 (H:80) (Santa Cruz Biotechnology)	1:200	1:100
Claudin-3	Polyclonal rabbit antibody anti-claudin-3 (Invitrogen)	1:250	1:12.5
Occludin	Polyclonal rabbit antibody anti-occludin (Invitrogen)	1:250	1:12.5
Aβ peptide	Rabbit anti-β-amyloid antibody (Sigma-Aldrich)	-	1:200

10. Immunofluorescence

hCMEC/D3 were grown to confluence on glass coverslips (Thermo Scientific) coated with rat tail collagen type I solution. Cells were washed in PBS and fixed with ice-cold acetone 10 minutes on ice or PFA for 20 minutes at RT. Fixed cells were permeabilized and blocked with 3% BSA and 0,25% Triton X-100 in PBS followed by incubation with primary antibodies against the proteins under study (table 1). After washing, cells were incubated with Alexa Fluor-488 donkey anti-rabbit IgG antibody (Invitrogen, 1:1000). Then, coverslips were mounted with Fluoroshield™ with DAPI (Sigma-Aldrich) and visualized and photographed with Zeiss Axio Imager Z1 equipped with an Axiocam MR3.0 camera and Axiovision 4.7 software.

11. *In vitro* Aβ1-42 uptake assays

11.1. Flow cytometry analysis

For quantification of Aβ1-42 uptake by flow cytometry, stable hCMEC/D3 cells were plated into a 6-well plate coated with collagen type I and grown to confluence. Cells were washed with PBS and incubated for 3 hours at 37°C with 500 ng/mL of FAM-Aβ1-42 in presence or absence of TTR, through the addition of 2.5% human serum or 2.5% TTR depleted serum (obtained as previously described), respectively, or using 2.5% recombinant TTR. After incubation, cells were washed twice with PBS and enzymatically detached using trypsin (Gibco), and then, suspension cells were centrifuged at 1000 rpm at RT for 5 minutes. The pellet was washed with PBS and cells centrifuged as described above. Then, the pellet was resuspended with PBS, and if necessary fixed with 4% PFA. Finally, cells were analyzed in FACS Canto II equipment (BD Biosciences) using Blue laser excitation of 488 nm. The flow cytometry data were analysed using Flowjo workstation available at IBMC.

11.2. Fluorescence microscopy

Imaging experiments of Aβ1-42 uptake by hCMEC/D3, cells were grown to confluence on coated glass coverslips. Hereafter, cells were exposed to 500 ng/mL of FAM-Aβ1-42 during 3 hours at 37°C in presence or absence of TTR (2.5% human sera and 2.5% TTR depleted sera, respectively). After incubation, cells were washed with PBS and then fixed with ice-cold acetone for 10 minutes on ice, and posteriorly mounted with Fluoroshield with DAPI.

Aβ1-42 uptake assay was also performed using mice sera obtained from mice of different genotypes for TTR. Thus, cells were incubated with 500 pg/mL Aβ1-42 for 3 hours at 37°C with $TTR^{(-/-)}$ and $TTR^{(+/+)}$ sera (2.5% for both sera). For this assay, the primary antibody used against Aβ peptide was rabbit anti-β-amyloid antibody (table 1), and the immunostaining protocol was done as described above in immunofluorescence topic and all the coverslips were visualized with Zeiss Axio Imager Z1.

12. Uptake and efflux of Aβ1-42 – Fluorometric assay

Fluorescence assay was performed using hCMEC/D3 cells cultured onto collagen type I coated 24-wells.

Briefly, for the uptake assay, cells were washed twice with PBS and then, incubated at 37°C with 500 ng/mL FAM-Aβ1-42, in presence or absence of 2.5% hrecTTR, for 3, 5, 10, 15 and 30 minutes. After this, incubation media (supernatants) were collected and the cells were washed twice with PBS followed by incubation with 1% Triton x-100 (Sigma-Aldrich) in PBS for 1 hour at 65°C to cause cells lysis. Fluorescence of cell lysates was measured using a SynergyMx microplate reader (excitation: 495 nm; emission: 521 nm; BioTek; Gen5™ data analysis software).

In the efflux assay, cells were first incubated with 500 ng/mL of FAM-Aβ1-42 for 15 minutes (first incubation), in presence or absence of 2.5% hrecTTR. After incubation, the media were replaced with fresh media, and cells were further incubated at 37°C for a different time periods (5, 10, 15, 20 and 30 minutes) to allow efflux of FAM-Aβ1-42 (second incubation). Then, the supernatants were collected at each time point and fluorescence was quantified using fluorometer, as described previously.

13. Basolateral-to-apical permeability assay

For BBB transport experiments, hCMEC/D3 cells were seeded on type I collagen pre-coated transwells filters (polyester 12 well, pore size 0.4 µm; Costar). Culture medium was changed every 3 days and assays were performed 10 days after seeding.

To identify filters in which apical medium leaked into the basolateral medium, apical media were supplemented with 0.25 mg/mL Fluorescein isothiocyanate (FITC)-labeled dextran (molecular mass of 70 KDa; Sigma-Aldrich) before transport studies. The concentration of FITC-labeled dextran was determined fluorometrically (excitation: 492 nm; emission: 518 nm) in the collected basolateral media using the same microplate reader as described previously. Wells, in which the basolateral FITC-labeled dextran concentration exceeded 125 ng/mL, indicating that the cell layer had been disrupted, were excluded from analysis. The FITC-labeled dextran concentration was calculated from a standard curve ranging from 0.25 mg/mL to 62.5 ng/mL.

Thus, transport studies were initiated by treatment of hCMEC/D3 monolayers with different Aβ1-42 preparations added to the basolateral chamber of the Transwell.

Thus, in the basolateral compartment (Brain side) was added 0% FBS medium with 500pg/mL Aβ1-42 supplemented or not with TTR (by addition of 2.5% human sera or 2.5% TTR depleted serum, respectively). The apical compartment (Blood Side) was filled with 0% FBS medium enriched with 2.5% human sera or 2.5% TTR depleted serum. Experiments were also performed using cells incubated only with human serum or TTR depleted serum in apical and basolateral sides, in order to evaluate the passage of TTR across hCMEC/D3 cells in absence of Aβ1-42. Then, filters were incubated at 37°C, and after 3, 24 and 48 hours samples were drawn from both sides and were replaced with the same amount of 0% FBS medium.

At the end of experiment, Aβ1-42 levels, in both compartments, were quantified by a commercially available kit as described above and following manufacture's recommendations. The percentages of passage of Aβ1-42 to the blood side in presence or absence of TTR were calculated using the next equation:

$$\%\ \text{passage of A}\beta 1-42\ \text{to blood side} = \frac{\text{Concentration of A}\beta 1-42\ \text{Blood side}}{\text{Concentration of A}\beta 1-42\ \text{lood side+ Concentration of A}\beta 1-42\ \text{brain side}} \times 100 \quad (1)$$

On the other hand, TTR levels, in both sides of transwells, were measured by ELISA as described below. The percentage of TTR that moved on to the opposite side to that on which it was initially placed was calculated using the following expressions:

$$\%\ \text{passage of TTR to blood side} = \frac{\text{Concentration of TTR Blood side}}{\text{Concentration of TTR blood side+ Concentration TTR brain side}} \times 100 \quad (2)$$

$$\%\ \text{passage of TTR to brain side} = \frac{\text{Concentration of TTR Brain side}}{\text{Concentration of TTR blood side+ Concentration TTR brain side}} \times 100 \quad (3)$$

14. Human TTR ELISA

Sandwich ELISA was used to determine the TTR concentration in apical side and basolateral side of transwells in BBB transport studies. 96-well-plates (Maxisorp-Nunc) were coated overnight at 4°C with a polyclonal rabbit anti-human TTR antibody (1:200 dilution) in 50 mM carbonate/bicarbonate buffer. After washing with PBS-T, 96-wells were blocked with a commercial blocking reagent, PowerBlock (Biogenex), for 2 hours at RT, and after this, samples and standards were applied to the wells during 2 hours at RT. Then, 96-Wells were incubated, 1 hour at RT, with a sheep anti-human TTR antibody (1:2500; Abcam) followed by incubation with anti-sheep IgG alkaline phosphatase antibody produced in donkey (3:10000; Sigma-Aldrich) for 1 hour at RT. Development was performed with SigmaFAST™ p-nitrophenyl phosphate (Sigma-Aldrich). The TTR concentration was calculated from a standard curve ranging from 1.79 to 86 ng/ml.

15. Transmission Electron Microscopy

hCMEC/D3 cells were grown to confluence on rat tail collagen type I coated transwells filters. After BBB transport experiment, cells on filters were fixed with 2% glutaraldehyde and 4% Paraformaldehyde (PFA) buffered in 0.1 M sodium cacodylate (pH 7.2). Cells were then washed repeatedly with 0.1 M cacodylate buffer, and post fixed overnight with 2% osmium in cacodylate buffer at 4^{o}C. Then, washed specimens were dehydrated in graded ethanol solutions and embedded in epon (Electron Microscopy Science). The ultrathin sections were mounted on 400-mesh copper or nickel grids (Electron Microscopy Science). The Copper grids were used for ultrastructure visualization of hCMEC/D3 cells while the nickel grids were used for immunolabeling, as described next.

For immuno staining, nickel grids were incubated 1 hour at RT with 14.4 % sodium metaperiodate followed by blocking with 2% BSA in Tris-buffered-saline (TBS) for 30 minutes. Then, the ultrathin sections were incubated, overnight at 4 ^{o}C, with a monoclonal anti-β-Amyloid antibody produced in mouse (1:200, Sigma-Aldrich). After washing with 0.1% BSA in TBS, grids were incubated 1 hour at RT with immunogold conjugate anti-mouse IgG produced in goat (1:20, British-Biocell International).

Finally, copper and nickel grids were double negatively stained with 2% uranyl acetate in ethanol and lead citrate. All grids were exhaustively observed on a JEOL JEM-1400 transmission electron microscope equipped with an Orious Sc1000 digital camera.

16. Brain Aβ efflux – *In vivo* preliminary study

Aβ1-42 clearance from the brain was studied *in vivo* in mice with different TTR genotypes (n=3 for each genotype), using the intracranial microinjection technique. In brief, immediately before surgery mice were weighed and anesthetized with intraperitoneal injection of an anesthetic combination of ketamine and medetomidine (7.5 mg/Kg and 0.1 mg/Kg, respectively) and placed in a stereotaxic apparatus (Stoelting Co.). The cranium was exposed using an incision in the skin and one hole was drilled through the cranium over the right lateral ventricle injection site to the following coordinates: mediolateral -1.0 mm, anterior-posterior -0.22 mm and dorsal-ventral -1.88 mm. Then, 2 µg of FAM-Aβ1-42 in PBS were injected into the brain using a 10 µL microsyringe (Hamilton Co.) connected to 26 gauge needle (RN Needle 6 pK,

Hamilton Co.) at a rate of 0.75 µL/min (4 µL final volume). After injection, the microsyringe was left in place for 3 minutes to minimize any backflow, and then, the incision was closed with sutures (Surgicryl), and the wound was cleaned with 70% ethanol. After surgery, the animals were kept warm, using a warming pad, and blood samples were collected by the tail vein, periodically in a capillary tube (previously coated with EDTA) at 15 and 30 minutes. At the time of sacrifice (after 30 minutes), the mice were re-anesthetized with 75 mg/Kg ketamine and 1 mg/Kg medetomidine, and, after total absence of reflexes in the paw and tail, mice were perfused through the injection of sterile PBS pH 7.4 via the inferior vena cava until the liver becomes blanched. Then, liver and brain were rapidly collected, and the blood samples were centrifuged at 1000 rpm for 15 minutes at RT. Plasma, brain and liver samples were frozen at -80 ^{o}C until used.

At the end of intracranial injection in all mice, liver and half of brain were weighted and homogenized in 750 µL of 50 mM TBS pH 7.4 containing protease inhibitor cocktail. After this, brain and liver homogenates were centrifuged for 20 minutes at 14000 rpm at 4^{o}C. Following centrifugation, supernatants were collected and then, fluorescence of brain and liver supernatants was measured using fluorometer (excitation: 495 nm; emission: 521 nm). Aβ1-42 levels in brain, plasma and liver samples were also quantified using human Aβ1-42 ELISA Kit (Invitrogen) as previously mentioned.

17. Statistical analysis

All quantitative data were expressed as mean±SEM. First of all, data was assessed whether it followed a Gaussian distribution. When found to follow Gaussian distribution, differences among conditions or groups were analyzed by one-way ANOVA with a Bonferroni's Multiple Comparison Test. In the cases of non-Gaussian distribution, differences among conditions were analyzed by non-parametric Kruskal-Wallis test and comparisons between two groups were made by Student t-test with a Mann Whitney test. p values lower than 0.05 were considered statistically significant. Statistically analysis were carried out using Graphpad Prism 5 software for windows.

Results – chapter I

A very recent work in our laboratory, using an AD/TTR mice colony, showed that Aβ1-42 levels were increased in plasma of AD/TTR$^{+/-}$ 7 month old female mice, compared with plasma of AD/TTR$^{+/+}$ (Ribeiro *et al.* , 2014). These results suggested that TTR has a fundamental role in sequestration of Aβ followed by its clearance from the periphery, thus preventing or slowing the progression of the disease.

1. Effect of TTR in plasma Aβ levels

In this sense, and to obtain a better knowledge on the effect of the TTR in plasma Aβ levels, our approach consisted in extend the study, thus evaluating plasma Aβ1-42 and Aβ1-40 levels in AD/TTR female mice 3-, 6- and 10- month-old with two different genotypes for TTR: AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$. Aβ1-42 and Aβ1-40 levels in plasma were determined using sandwich ELISA. Results are shown in the form of Aβ1-42/Aβ1-40 ratio, which was established to constitute a much more specific biomarker for AD, when compared to Aβ1-42 and/or Aβ1-40 levels.

As shown in figure 14, Aβ1-42/Aβ1-40 ratio is similar between mice of the two genotypes studied at the age of 3 months, althought we can not speculate if these are already high ratios or not. This is a transgenic model with overexpression of APP and therefore of Aβ, and we have no comparison with a non-disease situation. Differently, at the age of 6 months Aβ1-42/Aβ1-40 ratio is higher for AD/TTR$^{+/-}$ mice than for AD/TTR$^{+/+}$, indicating a higher degree of the disease in mice with TTR genetic reduction, at this age.

It is also possible to observe that between 3 and 6 months of age, Aβ1-42/Aβ1-40 ratio decreases for AD/TTR$^{+/+}$ mice, contrarily to AD/TTR$^{+/-}$ animals whose AD/TTR$^{+/-}$ ratios continued to increase. We can hypothesize that AD/TTR$^{+/+}$ mice tried to counteract the disease and Aβ accumulation by eliminating the peptides. AD/TTR$^{+/-}$ mice, however, due to their TTR reduction, are unable to prevent Aβ accumulation and disease progression.

At advanced disease stages such as at 10 month of age, no differences were detected between AD/TTR$^{+/+}$ and AD/TTR$^{+/-}$ animals. According to our results, Aβ1-42/Aβ1-40 ratios were maintained in AD/TTR$^{+/-}$ mice (compared to 6 months), whereas

in AD/TTR$^{+/+}$ animals, ratios were increased again, probably due to massive Aβ accumulation which could no longer be counteracted by TTR.

Taken together, our results suggest that TTR influences plasma Aβ by reducing its levels, correlating with disease burden, i.e., Aβ1-42/Aβ1-40 ratios are elevated in plasma of AD/TTR$^{+/-}$ mice, and these animals present more signs of AD disease. Interestingly, statistical differences between the genotypes under study were only observed at 6 months, the age previously reported as the beginning of Aβ deposition in this model.

Given our results and knowing that Aβ transport is made via BBB and BCSFB, we decided to explore the effect of TTR in Aβ brain efflux across the BBB.

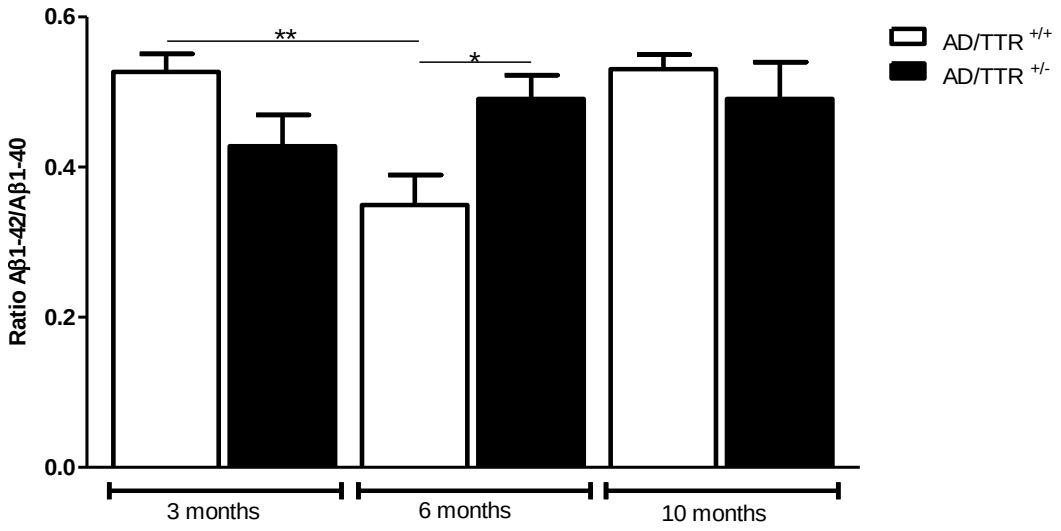

Figure 14 – Effect of TTR genetic reduction in plasma Aβ1-42/ Aβ1-40 ratios. Results are shown for 3-, 6- and 10-month-old female mice with two distinct genotypes for TTR: AD/TTR$^{+/+}$ (white bars) and AD/TTR$^{+/-}$ (black bars). Total number of animals analyzed: by age, n=6 per group and by genotype, n=6 per group. Data are expressed as mean±SEM. Error bars represent SEM. *p<0.05 and **p<0.01 in a one-way ANOVA with Bonferroni's multiple comparison test.

2. Effect of TTR in Aβ efflux across the BBB – *in vitro* studies

2.1. Characterization of the BBB model

Currently, four immortalized human brain capillary endothelial cell lines are available to establish an improved human in vitro BBB model – hCMEC/ D3, human brain microvascular endothelial cell line (hBMEC), TY10 and BB19 (Eigenmann *et al.* , 2013). Regarding these cell lines, the hCMEC/D3 cell line represents a valid and powerful model in constructing *in vitro* a BBB model, and presents a less expensive and more logistically feasible alternative to primary hBMEC cells (Daniels *et al.* , 2013).

Thus, the hCMEC/D3 cells were used throughout this study, and firstly we decided to validate the model by characterizing this cell line regarding several features critical to the BBB integrity and structure, as well as to the Aβ efflux: expression of junctional proteins and expression of the main Aβ transporter at the BBB, the LRP1 receptor.

2.1.1. Expression of junctional proteins

In the context of endothelial cell junctions, hCMEC/D3 cells were tested to claudin-3 and occludin by immunofluorescence and western blot analysis. As shown in figures 15 and 16, hCMEC/D3 cells are positive for TJ structural proteins, claudin-3 and occludin. These results indicate that, the integrity, tightness and structure as well as the paracellular contact between endothelial cells are guaranteed by these TJ proteins. Along with others TJ proteins expressed by hCMEC/D3, the claudin-3 and occludin ensure, with high efficiency, the control of transport across the cells monolayer.

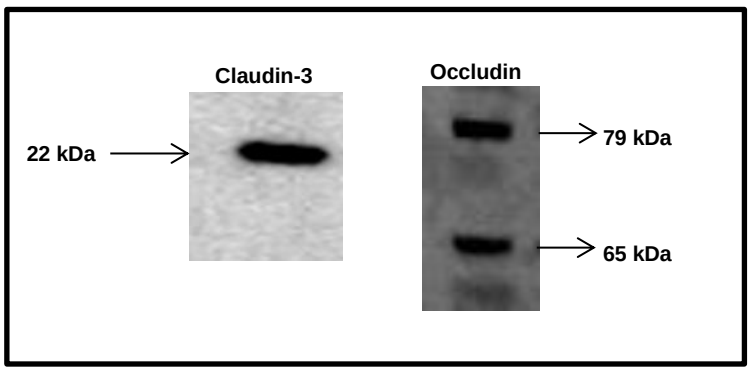

Figure 15 – Western blot analysis of TJ proteins from cell lysates of hCMEC/D3 cell line. 30µg of total protein was subjected to SDS-PAGE and western blotting for individual markers. hCMEC/D3 cells expressed TJ proteins claudin-3 (22 kDa) and occludin (79 kDa and 65 kDa).

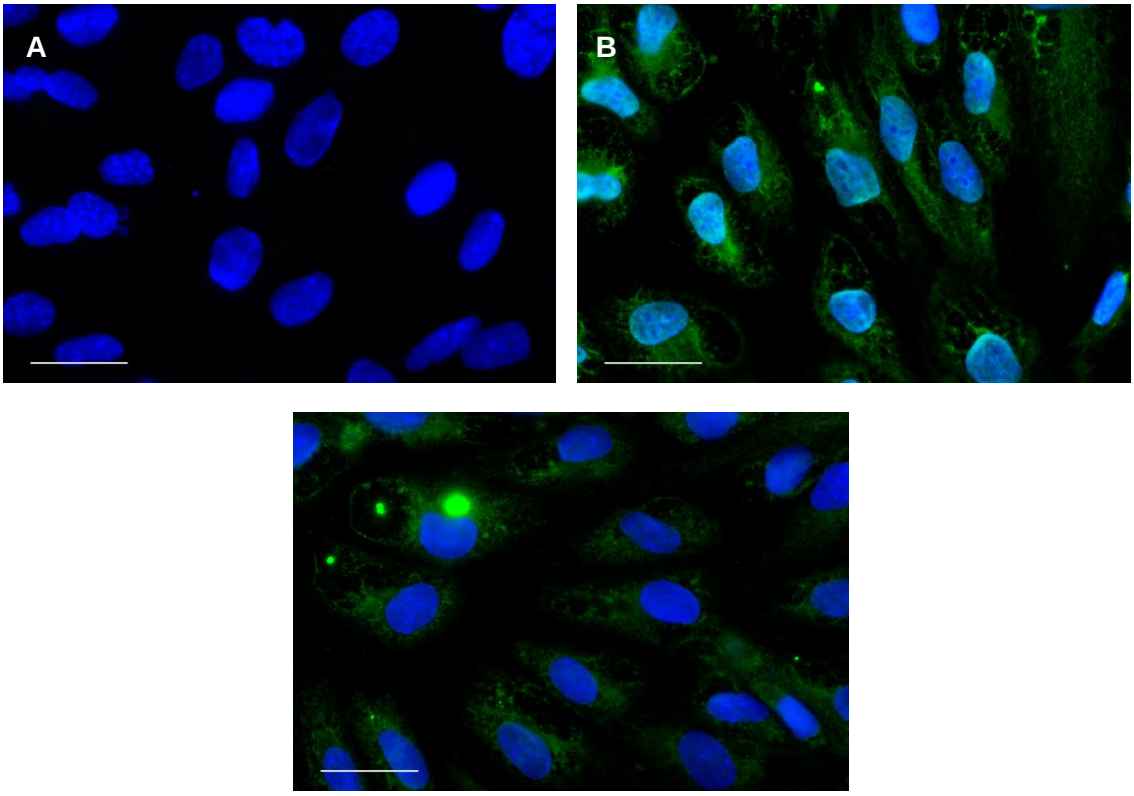

Figure 16 – Immunofluorescence staining of TJ proteins in hCMEC/D3 cell line. (A) Control of hCMEC/D3 cells incubated with secondary antibody (anti-rabbit Alexa 488); (B) - Immunostaining of confluent hCMEC/D3 with antibody against claudin-3 (green); C- Immunostaining of confluent hCMEC/D3 with antibody against occludin (green). Nuclei of hCMEC/cells stained with DAPI (blue). Scale bars: 25 µM

2.1.2. Expression of LRP1 receptor

The expression of the efflux transport receptor – LRP1 – by the hCMEC/D3 cell line is a key factor when validating this model both for BBB studies purposes as for Aβ transport research. Similar to TJ proteins, we performed immunofluorescence and western blot analysis to verify if LRP1 exists in the hCMEC/D3 cells. Our results show that LRP1 is significantly expressed in these cells, ensuring the Aβ transport through the cells monolayer (Figure 17).

Faced with these results, hCMEC/D3 cell line appears particularly appropriated for Aβ efflux studies across the cell monolayer. Thus, next we will present and discuss the experiments undertaken to investigate whether or not TTR interferes with Aβ uptake and efflux across the BBB.

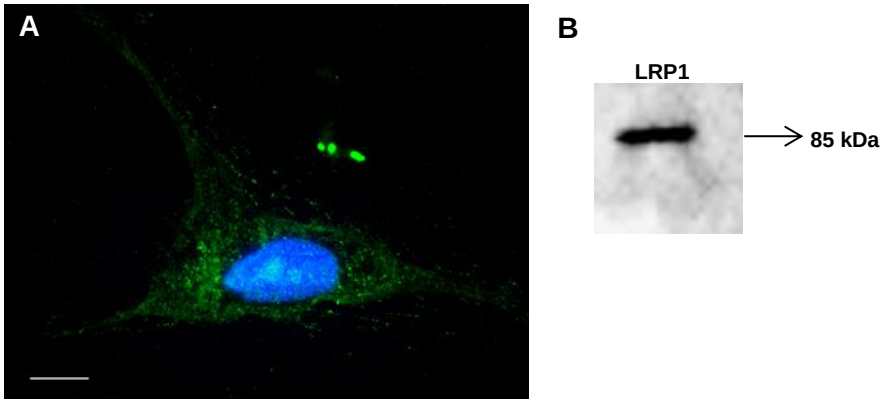

Figure 17 – Expression of LRP1 receptor in hCMEC/D3 cell line. (A) Immunofluorescence analysis of LRP1 expression in hCMEC/D3 cells stained with an antibody against LRP1 (green); Nucleus of cells stained with DAPI (blue); Scale bar: 10µM; (B) Western blot analysis of LRP1 expression. The band corresponds to the c-terminal subunit (85 kDa), which contains the transmembrane domain and cytoplasmic tail of LRP1.

2.2. Influence of TTR in Aβ internalization by hCMEC/D3 cells

To test the hypothesis of TTR participation in Aβ brain uptake, hCMEC/D3 cells were incubated with labelled (FAM-Aβ1-42) or non-labelled (Aβ1-42) Aβ in presence or absence of TTR. Then, Aβ internalization was analyzed by FACS and fluorescence techniques. In these *in vitro* experiments, we used three different TTR sources: human and WT mice sera and hrec TTR protein. In the cases where hrec TTR were used, we utilized cell media to mimic the absence of the protein, whereas for mice and human sera TTR, we used $TTR^{-/-}$ mice sera and TTR-depleted sera, respectively.

2.2.1. Depletion of TTR from human sera

In order to obtain human serum without TTR, serum available in the laboratory was subjected to a column affinity chromatography technique to remove TTR, using an antibody against TTR. Then, to confirm the removal of TTR from serum, we performed western blot analysis. Our results (Figure 18) show no TTR in human TTR-depleted serum (third row) compared with initial human serum TTR (first row). Furthermore, as shown in figure 18 we were able to recover TTR from the beads as confirmed by the absence of TTR in beads after elution (second row), and also by the presence of TTR in eluate (fourth row).

Therefore, TTR was efficiently removed from human serum, making this serum a good negative control to the presence of TTR in serum form to be used in next *in vitro* experiments.

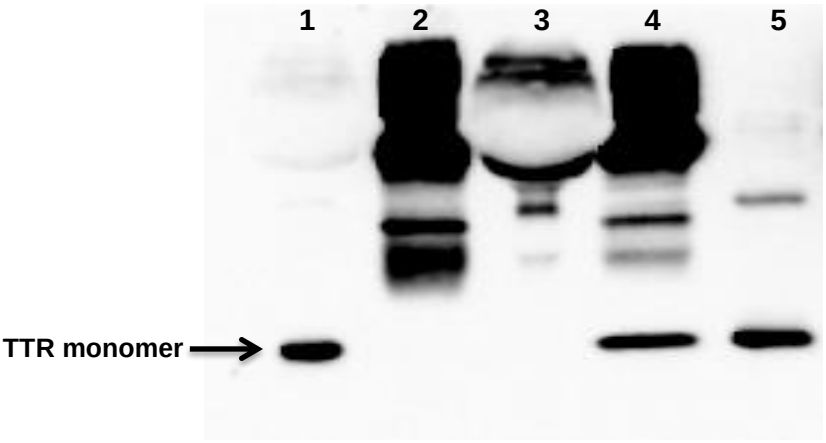

Figure 18 – Western blot analysis of TTR depletion from human sera. TTR were removed from human serum using a column affinity chromatography technique, where beads of protein G were linked to an antibody against TTR. After TTR linking to the beads, serum was applied and then collected in order to obtain TTR-free serum. Finally, TTR was eluted and collected.1-human serum; 2- beads of protein G after TTR elution; 3- human TTR-depleted serum; 4- Eluted TTR; 5- hrec TTR.

2.2.2. Flow cytometry analysis

FACS results (Figure 19) reveal that, in presence of TTR FAM-Aβ1-42 uptake by the hCMEC/D3 cells is significantly lower than in absence of TTR. As shown in figure 19, cells incubated with cell media alone internalized about 90% of the FAM-Aβ1-42 added, whereas cells incubated with hrec TTR internalized about 72% of the peptide. However, human TTR-depleted serum also produced lower levels of peptide internalization (about 66%), suggesting that serum interferes with process. Nevertheless, in presence of TTR serum, the internalization levels are even lower (30%), indicating an effect of this protein.

Altogether, these observations indicate that TTR produces an effect in Aβ internalization by the hCMEC/D3 cells, although we could not clearly ascertain the role of this TTR in this process.

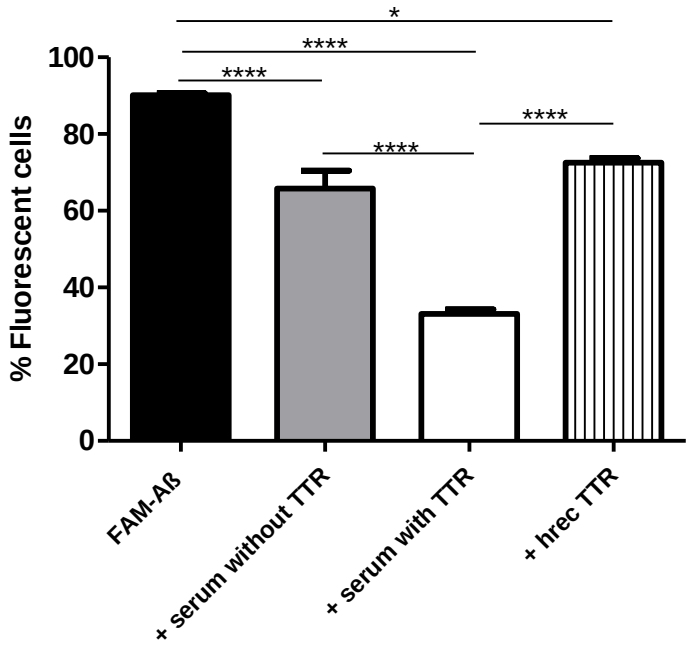

Figure 19 – FACS-based analysis of FAM-Aβ1-42 internalization. hCMEC/D3 cells were incubated with 500 ng/mL of the FAM-Aβ1-42 in presence (human sera with TTR and hrec TTR, white and stripped bars, respectively) and absence (human TTR-depleted sera, gray bar) of TTR, during 3 hours. Data are expressed as mean±SEM. *p<0.05 and ****p<0.0001 in one-way ANOVA, with a Bonferroni's multiple comparison test.

2.2.3. Fluorescence microscopy analysis

To further confirm if TTR is or not involved in Aβ uptake by hCMEC/D3 cells, we performed fluorescence analysis. We used FAM-Aβ1-42 incubated with human sera or hrec TTR. Alternatively we utilized Aβ1-42 incubated with $TTR^{+/+}$ mice sera. Both cases were compared to peptide incubated in the absence of TTR (human TTR-depleted sera, cell media and $TTR^{-/-}$ mice sera, respectively).

Fluorescence results of FAM-Aβ1-42 uptake (figure 20) demonstrated that this peptide incubated alone in cell media is modestly internalized by the hCMEC/D3 cells after 3 hours of treatment (Figure 20 B). Interestingly, cells incubated with hrec TTR shows higher internalization of the FAM-Aβ1-42 (Figure 20 E), indicating that TTR promotes Aβ uptake. However, cells treated with TTR present in human sera (Figure 20 D), although presenting higher amounts of internalized Aβ than cells incubated with FAM-Aβ1-42 in cell media, evidenced a similar behavior to cells incubated with human TTR-depleted sera (Figure 20 C), again questioning on the role of TTR in Aβ uptake by hCMEC/D3 cells.

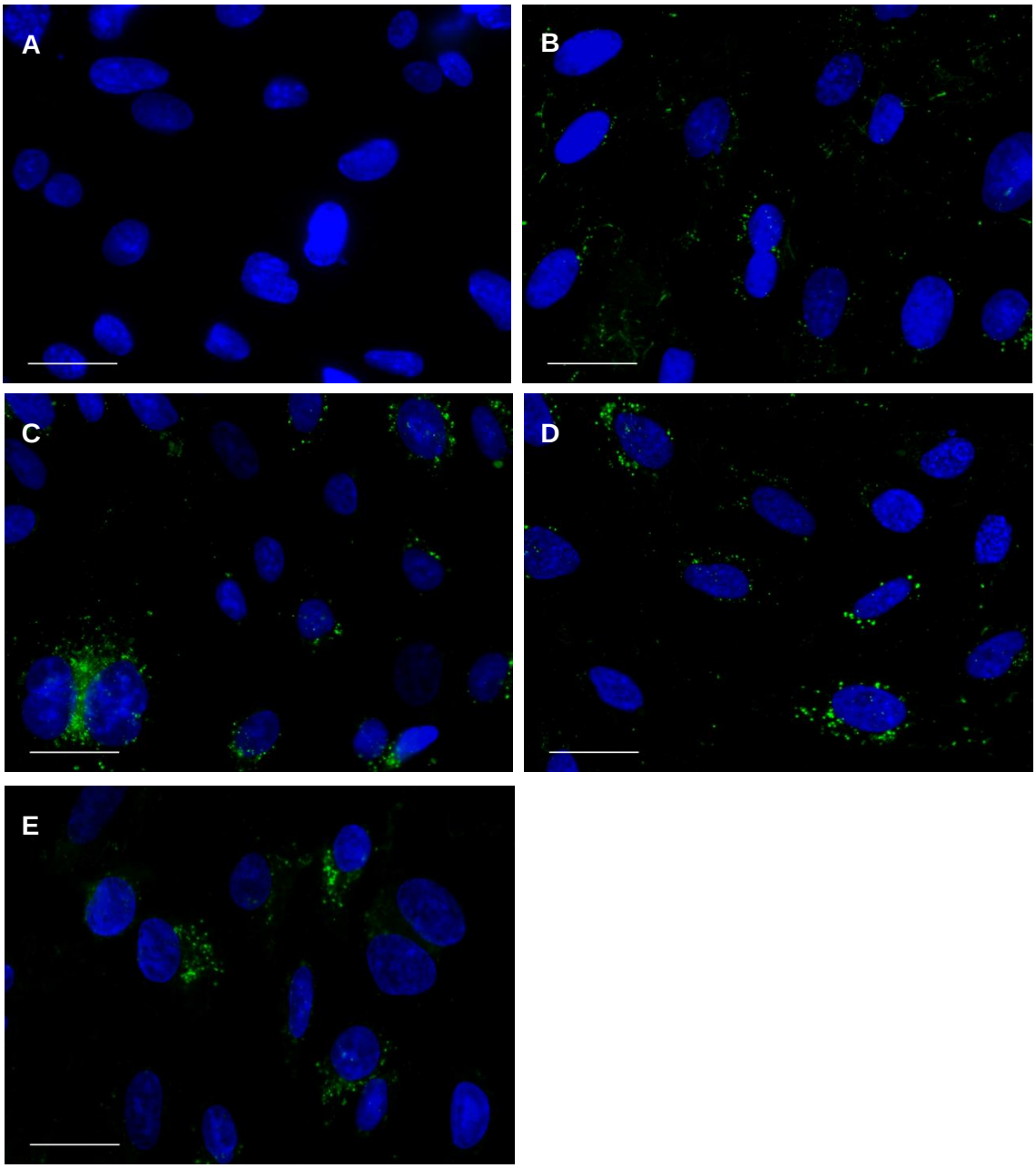

Figure 20 – Fluorescence analysis of the FAM-Aβ1-42 uptake by the hCMEC/D3 cells. Endothelial cells were incubated with 500 ng/mL of the FAM-Aβ1-42 (green), in presence (human serum TTR or hrec TTR) and absence (human TTR-depleted serum) of TTR, during 3 hours. (A) Control of hCMEC/D3 cells; (B) hCMEC/D3 cells incubated with FAM-Aβ1-42; (C) hCMEC/D3 cells incubated with FAM-Aβ1-42 and hrec TTR; (D) cells treated with FAM-Aβ1-42 and human TTR-depleted serum; (E) cells treated with FAM-Aβ1-42 and human serum TTR; nuclei stained with DAPI (blue); Scale bars = 25 µM

In similar assays performed with sera from $TTR^{+/+}$ mice, and with sera from $TTR^{-/-}$ animals to mimic the absence of the protein, we used non-labelled Aβ (Aβ1-42) and thus we proceeded to immunofluorescence analysis using an anti-amyloid antibody.

As it can be seen in figure 21, internalization of Aβ1-42 occurs (Figure 21 B) abundantly under all situations analyzed, and no significant differences are observed in the presence of $TTR^{+/+}$ and $TTR^{-/-}$ sera (Figure 21 C and D) when compared to the control (Figure 21 A).

Altogether, fluorescence results show that Aβ1-42 is internalized by the hCMEC/D3 cells, but suggest that TTR does not exert a significant influence in Aβ1-42 uptake after 3 hours of incubation. Nevertheless, it is necessary take in account that, the immunofluorescence may not be the best approach to investigate the influence of TTR in Aβ uptake, because it is not a quantitative technique and therefore may fail to detect the differences. Indeed, FACS analysis which represents a quantitative technique showed significant differences in Aβ internalization by hCMEC/D3 cells, revealing less internalization in the presence of TTR (incubation with cell media alone versus with hrec TTR; incubation with human sera TTR-depleted versus with human sera). Nevertheless, these experiments were important to show that there are no significant differences in behavior between FAM-Aβ1-42 and Aβ1-42.

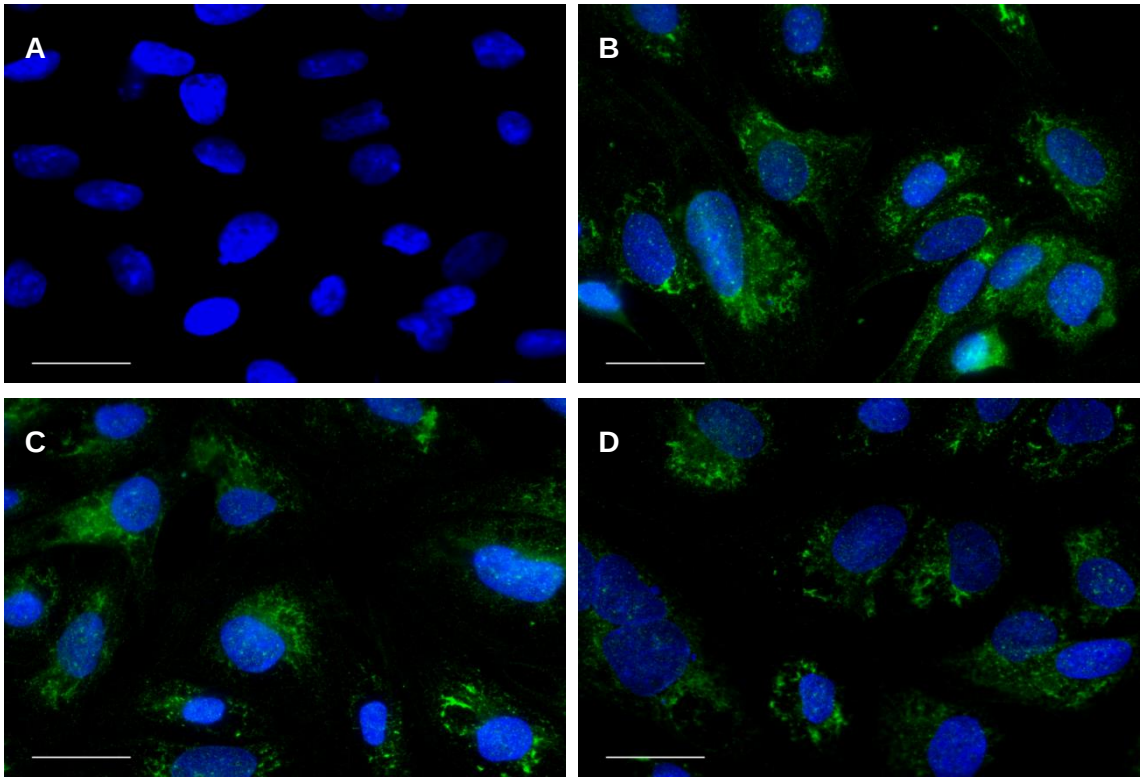

Figure 21 – Immunofluorescence analysis of the Aβ1-42 internalization by the hCMEC/D3 cells. Cells were incubated with 500 pg/mL of the Aβ1-42, with TTR ($^{-/-}$) and TTR ($^{+/+}$) mice sera. Immunostaining of cells with antibody against Aβ1-42 (green); (A) Control of hCMEC/D3 cells; (B) hCMEC/D3 cells incubated with Aβ1-42; (C) cells treated with Aβ1-42 and with TTR ($^{-/-}$) mice serum; (D) cells treated with Aβ1-42 and with TTR ($^{+/+}$) mice serum; Nuclei stained with DAPI (blue); Scale bars = 25 µM.

2.3. Fluorometric analysis

In the light of the previous results, and because we expected a positive effect of TTR in Aβ transport, we wonder if three hours of incubation would be too long to observe differences in Aβ internalization, thus we decided to test short periods of time. At this point, and also aiming at establishing a faster and cheaper assay, we performed fluorometric analysis of cell lysates and cell conditioned media of hCMEC/D3 cells. Using this assay we were also able to measure Aβ efflux.

We first evaluated FAM-Aβ1-42 uptake by hCMEC/D3 cells, in the presence or absence of hrecTTR. Cells were incubated for 3, 5, 10, 15 and 30 minutes, and then, fluorescence of cell lysates was measured in a fluorometer. As shown in figure 22, fluorescence levels increases until 15 minutes of incubation in both conditions, and at this time point an appreciable difference is noted between the two conditions, indicating that in the presence of hrec TTR Aβ uptake is increased. However, differences are not statistically different, probably due to the number of replicas used.

Interestingly, at 30 min of incubation, and in the presence of TTR, fluorescence levels were already lower than at 15 min, and very similar to the ones measured in the absence of TTR (which continued to raise from 15 to 30 minutes).This observation might imply that at 30 min and in the presence of TTR, FAM-Aβ1-42 is already flowing out of cells, whereas in the absence of TTR the peptide is still entering. Importantly, these experiments shed some light on our previous FACS and fluorescence microscopy results, since for these periods of incubation, the peptide must have already gone out of cells, masking the potential effect of TTR.

In fact, these results seems to contradict the results shown in figures 19-21, indicating that TTR might be an important intervening in Aβ uptake by a yet unknown mechanism. Despite this fact, it is important to refer that the number of replicas has to increase, in order to obtain results with statistical significance, and in this way be able to demonstrate the possible role of TTR in Aβ internalization.

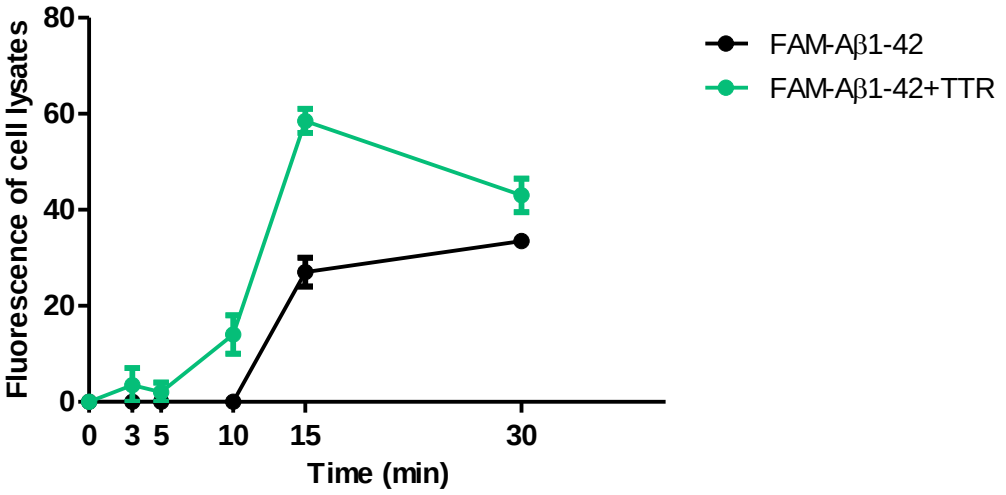

Figure 22 –Fluorescent analysis of FAM-Aβ1-42 uptake by hCMEC/D3 cells. Cells were incubated with FAM-Aβ1-42 in presence (green line) or absence (black line) of hrec TTR, during 3, 5, 10, 15 and 30 minutes. After this, cells were lysate and fluorescence was measured in a fluorometer (excitation: 495 nm; emission: 521 nm). Data are expressed as mean±SEM.

Given our evidence that after 15 minutes FAM-Aβ1-42 levels inside cells achieve a maximum, and after that starts to going out of the hCMEC/D3 cells, we decided to focus now in Aβ efflux across the hCMEC/D3, to ascertain the role of TTR in this process, using the same technique.

For this, cells were firstly incubated with FAM-Aβ1-42 for 15 minutes (first incubation), in presence or absence of hrecTTR, and then media replaced by fresh media. To allow efflux of FAM-Aβ1-42 cells were further incubated during 5, 10, 15, 20 and 30 minutes (second incubation).

As it can be seen in figure 23, there seems to be a tendency to faster efflux in absence of hrec TTR, until 10 minutes of incubation. This might imply that TTR contributes to maintain Aβ inside cells and/or for its degradation. However, no statistically significant results are observed, perhaps because the low number of replicas. Thus, similarly to the uptake assays, it is essential to perform these studies with more replicas to unravel the role of TTR in Aβ uptake and efflux.

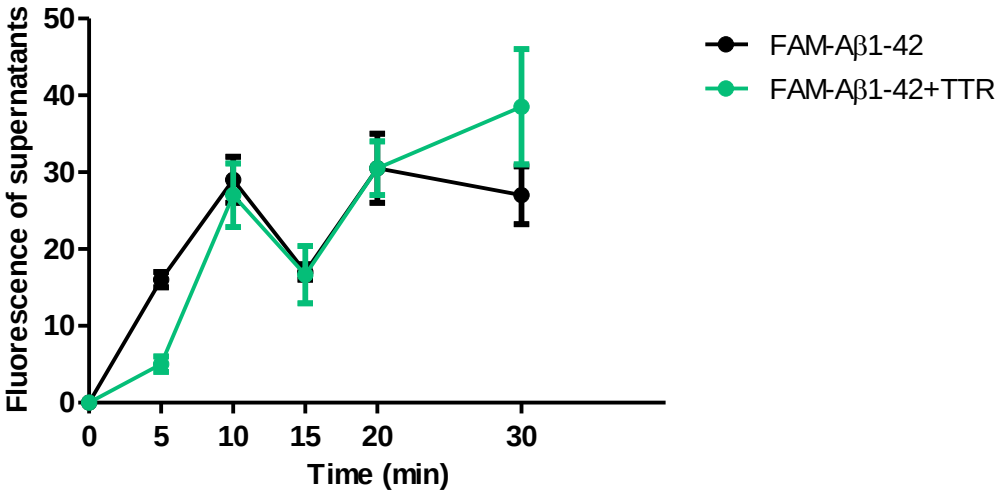

Figure 23 – Fluorescent analysis of FAM-Aβ1-42 efflux across hCMEC/D3 cells. Cells were incubated with FAM-Aβ1-42 in presence (green line) or absence (black line) of hrec TTR, during 15 minutes (first incubation). Then, new media were replaced and cells were incubated for 5, 10, 15, 20 and 30 minutes (second incubation). After this, supernatants were collected and fluorescence of supernatants was measured fluorometrically (excitation: 495 nm; emission: 521 nm). Data are expressed as mean±SEM.

2.4. Role of TTR in hCMEC/D3 basolateral-to-apical permeability to Aβ1-42

For permeability studies, hCMEC/D3 cells were seeded on transwell filter inserts, as shown in figure 24 A, during 10 days until reaching maximal confluence and permitting TJ formation. Thus, at this point, the cell monolayers should show restricted paracellular permeability, and its confirmation was done using FITC-labeled dextran as a low molecular weight paracellular diffusion marker. In this approach, FITC-labeled dextran 0.25 mg/ml was added to the apical chamber, and then incubated for 1 hour. Wells in which FITC-labeled dextran exceeded 125 ng/mL on basolateral chamber were considered to have the monolayer disrupted and thus were excluded from the experiment (an example of this test is shown in annexes section).

After this, cells were incubated with Aβ1-42 added to the basolateral compartment (Brain side) in cell media without FBS supplemented or not with TTR (by addition of 2.5% human sera or 2.5% TTR depleted serum, respectively).The apical compartment (Blood Side) was filled with 0% FBS medium enriched with 2.5% human sera or 2.5% TTR depleted serum. Aβ peptide was also added to non-seeded filters to show free passage of the peptide when compared to cell seeded ones, in which Aβ is

transported in a specific manner using known receptors, namely LRP1. In addition, we can also infer on the integrity of the monolayer. At the end, Aβ peptide transported to the apical chamber was determined at 3, 24 and 48 hours.

Our data (Figure 24 B) indicates that, in presence of TTR in brain side, the passage of Aβ1-42 to the blood side is significantly higher compared to the situation where TTR was added to the blood side, in all time points evaluated. However, we still need to evaluate Aβ permeability in total absence of TTR and in sera-free conditions, to further ascertain the role of TTR in Aβ transport.

Given our evidences, we decided to investigate if TTR crosses the hCMEC/D3 monolayer, during Aβ1-42 efflux. To assess this, we quantified TTR in the supernatants of the above experiment, and also evaluated TTR apical-to-basolateral and basolateral-to-apical passage in transwells in the absence of Aβ peptide.

As shown in figure 24 C, TTR crosses the hCMEC/D3 cell monolayers, in both directions: brain-to-blood and blood-to-brain. In addition, we observe that TTR transport is faster from brain-to-blood than blood-to-brain. On the other hand, in presence of Aβ1-42 the percentages of TTR that crossed the monolayer are always lower relatively to the conditions without Aβ1-42, for both directions, perhaps because TTR/Aβ lessens its availability to cross cell monolayer. At this point we can not rule out the hypothesis that TTR/Aβ binding affects TTR recognition by the antibody used for the ELISA measurements.

Altogether, we suggest that TTR can bind to Aβ1-42 and promote its clearance across the BBB, maintaining the Aβ1-42 levels balanced in brain. However, it is still unclear if TTR crosses the BBB together with Aβ1-42, as well as if the mechanism involved uses the receptors already described.

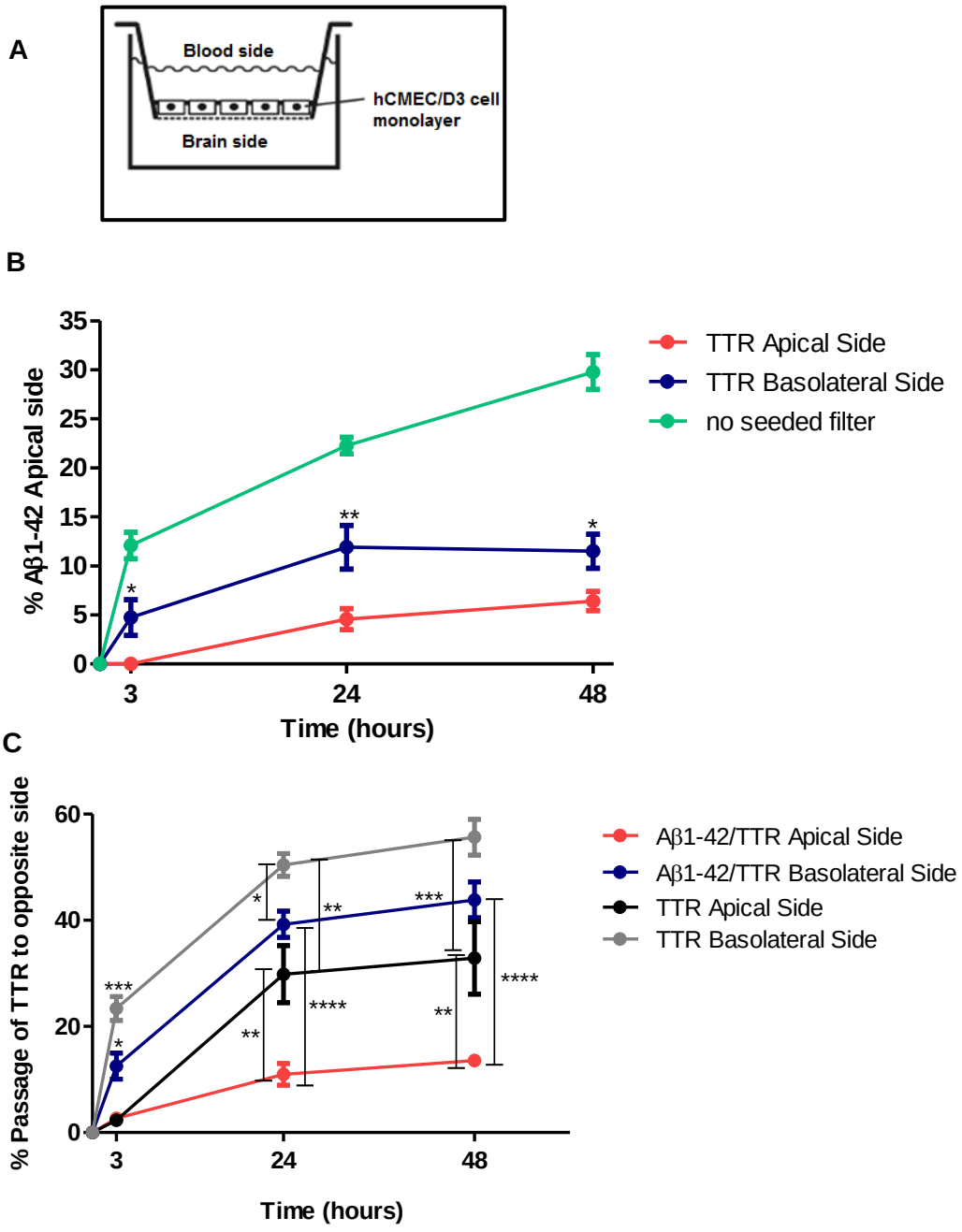

Figure 24 – BBB transport study across the hCMEC/D3 cell monolayers. (A) hCMEC/D3 cells were grown during 10 days on transwells filter inserts separates an apical and basolateral compartments representing the blood and brain sides, respectively; (B) In efflux assay, cells were treated with Aβ1-42 (on brain side) in presence (human sera TTR) or absence (human TTR-depleted sera) of TTR for 3, 24 and 48 hours. Two different conditions were performed: human serum TTR on brain side and human TTR-depleted serum on blood side (blue line) and human TTR-depleted serum on brain side and human serum TTR on blood side (pink line). As a control, on no seeded transwell filter insert, Aβ1-42 was placed on brain side (green line). (C) Bi-directional transport of TTR across the hCMEC/D3 monolayer. Cells were incubated in absence of Aβ1-42, with human serum with TTR on brain side and TTR-depleted serum on blood side (gray line) and human serum with TTR on blood side and TTR depleted serum on brain side (black line). TTR and Aβ1-42 concentration were quantified by ELISA; Data are expressed as mean±SEM; *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 in one-way ANOVA, with a Bonferroni's multiple comparison test.

We next assessed the localization of Aβ1-42 in hCMEC/D3 cell monolayers, as well as the ultrastructure of these cells by TEM, after the transport assays. Visualization of structure of cells by TEM revealed intact and confluent cell monolayers, without cell disruption, indicating that the integrity and paracellular contact between cells were guaranteed (Figure 25 A). In addition, immunogold staining against Aβ1-42 shows that, Aβ1-42 is internalized by hCMEC/D3 cells (Figure 25 B), and that vacuoles are the organelle where Aβ1-42 accumulates (Figure 25 C).

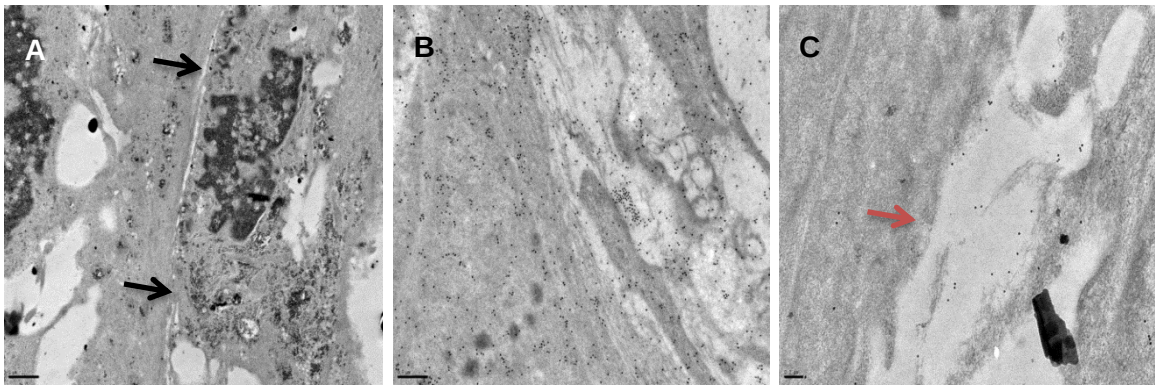

Figure 25 – Electron microscopic analysis of hCMEC/D3 cell line after BBB transport assay. (A) Ultrastructure image of hCMEC/D3 cells. Black arrows represent interface between 2 different cells. Scale bar: 1µM; (B) and (C) Immunogold staining with an antibody against Aβ1-42. Black dots represent Aβ1-42 and red row shows a vacuole where this peptide has a tendency to accumulate. Scale bars = 0.5 µM and 0.2µM, respectively.

3. Effect of TTR in brain Aβ clearance – an *in vivo* preliminary study

In order to investigate *in vivo*, the effect of TTR in Aβ clearance out of the brain, mice with different genotypes for TTR ($TTR^{+/+}$, $TTR^{+/-}$ and $TTR^{-/-}$) were injected with 2 µg of FAM-Aβ1-42 on the right lateral ventricle. After 30 minutes post-injection, Aβ levels in brain and liver were quantified using a sandwich ELISA analysis and a fluorometer assay, while Aβ plasma levels, at 15 and 30 minutes, were measured only by ELISA.

Our data (Figure 26 A) noticeably show that, mice with one copy of TTR have less Aβ in the brain compared with $TTR^{-/-}$ mice, after 30 minutes of injection. Thus, this result suggests that TTR plays a role in Aβ clearance trough the brain. However, no significant differences, among mice with different TTR genotypes, were observed in Aβ plasma levels, both for 15 and 30 minutes after injection (Figure 26 B). The measuring of Aβ in plasma was problematical, because the volume of blood collected was very low, which prevented to measure Aβ in plasma in duplicates. Furthermore, it was not

possible to quantify Aβ levels in livers, perhaps due to the interference of the tissue by its autofluorescence. In addition, it is essential refer that, data on $TTR^{+/+}$ mice were not shown because we believe that FAM-Aβ1-42 injection failed in two of the three mice available for this genotype, and thus Aβ was not detected in brain, plasma and liver samples.

Nevertheless, it is necessary to take in account that, the number of animals was low, and it is crucial to repeat this experiment with more animals, in order to verify the possible effect of TTR in Aβ clearance. At this moment, we can suggest that TTR promotes Aβ1-42 efflux across the BBB, by increasing its transport to the periphery, contributing to the maintenance of Aβ1-42 levels in the brain.

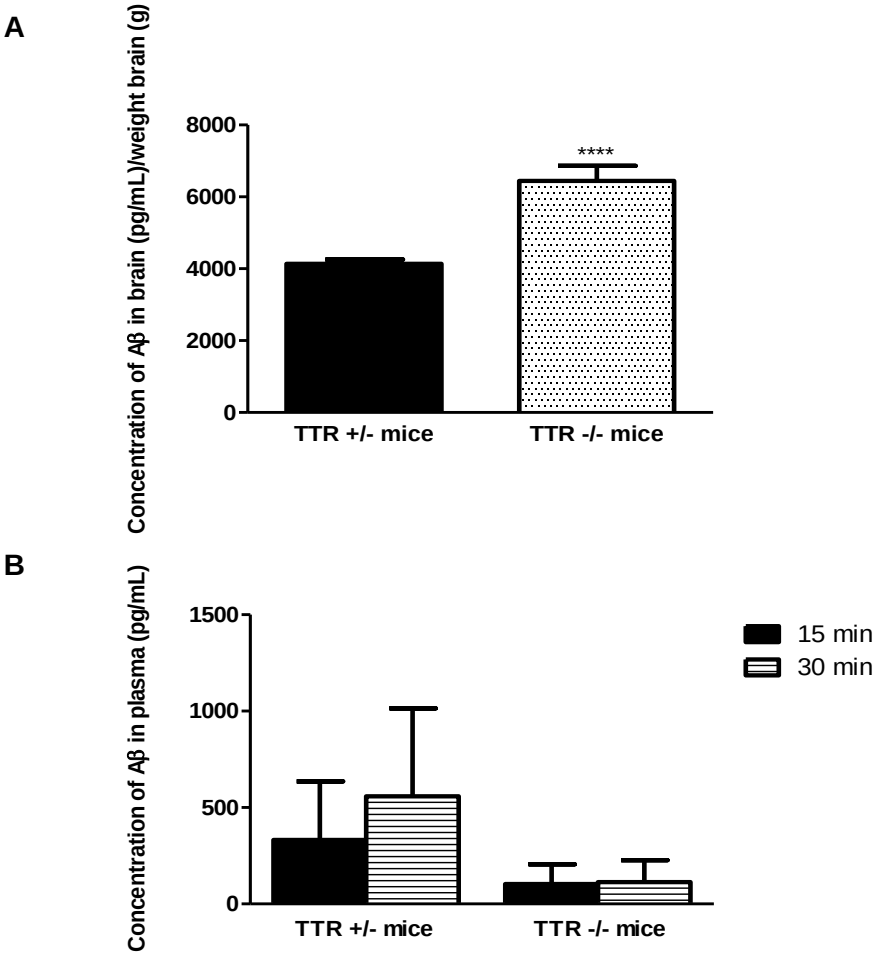

Figure 26 – Aβ1-42 clearance across the BBB. Mice with different genotypes for TTR ($TTR^{+/+}$, $TTR^{+/-}$ and $TTR^{-/-}$) were injected with 2 µg of FAM-Aβ1-42 on right lateral ventricle, and after this, concentration of Aβ in plasma, brain and liver were measured by ELISA and fluorometrically (excitation: 495 nm; emission: 521 nm). (A) Concentration of Aβ in brain after 30 minutes of injection, in $TTR^{+/-}$ and $TTR^{-/-}$ mice, quantified by ELISA kit (n=2 for each genotype); (B) Concentration of Aβ in plasma after 15 and 30 minutes of injection, in $TTR^{+/-}$ and $TTR^{-/-}$ mice, measured by ELISA kit (n=3 for each genotype); Data are expressed as mean±SEM; ****p<0.0001 in Student t-test with a Mann Whitney test.

Discussion – chapter I

AD is the most prevalent form of dementia, worldwide, and its complexity makes this neurodegenerative disorder one of the most investigated pathologies in the last years. The molecular mechanisms responsible for the known pathological features are very controversial, and still remain unclear. Therefore, understand how this neurological disease really operates and expose factors or molecules that are involved in AD, are two crucial aspects in deciphering AD. Thus, it has been suggested an association between TTR and AD, and this protein has been shown to be important in AD protection.

Based on previous results of our group showing that AD mice with TTR genetic reduction have increased levels of brain and plasma Aβ, our working hypothesis has been that TTR participates in Aβ clearance from the brain.

In this view, we investigated whether TTR had any influence in Aβ plasma levels in samples of AD/TTR transgenic mice, and if this effect was modified with aging. Our results indicated that TTR influences plasma Aβ by decreasing its levels, supporting the idea that TTR promotes its elimination from the brain and from the periphery, although, unfortunately, we did not have the opportunity to measure Aβ levels in the CSF of the same animals. In young mice (3 months) in which signs of disease are still absent, no differences were detected among the two genotypes studied. As disease progressed, AD/TTR$^{+/+}$ were able to counteract the accumulation of Aβ, whereas AD/TTR$^{+/-}$ had their Aβ plasma levels further increased. Curiously, these significant differences between the two genotypes analysed were observed at 6 months, the age which was indicated as the starting of Aβ deposition in this model (Oliveira *et al.* , 2011). At advanced stages, differences faded, and Aβ amounts increased in AD/TTR$^{+/+}$ animals, attainting levels equivalent to the AD/TTR$^{+/-}$ counterparts. It is important to refer that the degree of disease in this model is not only (and probably not even mostly) produced by the higher or lower TTR concentrations. This is a transgenic APP and PSEN model, and thus Aβ production is highly increased and consequently many other pathways are affected.

Since Aβ can be transported bidirectionally across the BBB, it has been hypothesized that there might be a balance between CSF and plasma Aβ levels (Ghersi-Egea *et al.* , 1996). Although Aβ peptides can be eliminated via non-specific bulk flow of brain ISF and BCSFB, BBB clearance seems to be responsible for the

mostly elimination of Aβ under physiological conditions (Shibata *et al.* , 2000). Moreover, it has been proposed that Aβ generated in the brain binds to cholesterol/ApoE and, in this way crosses the BBB, to be further delivery in the liver and kidney for its degradation (Sparks, 2007). Both at the BBB and at the liver, Aβ uses LRP1 to enter cells and interestingly TTR is internalized in the liver by a RAP-sensitive receptor and thus it is possible that TTR binds Aβ and crossing the BBB together.

In light of this, we used the hCMEC/D3 cell line as a representative model for the BBB to study the influence of TTR in Aβ brain-to-blood transport.

Our FACS and fluorescence microscopy results showed lower levels of internalized Aβ in the presence of TTR or no differences, respectively, contrarily to our expectations. We further explored this issue by changing the conditions of the assay, mainly by decreasing the incubations time to 15 minutes and using a fluorimetric measurement in cell lysates, and results indicated that TTR promoted Aβ internalization. Thus, we believe incubations of 3 hours (as performed for FACS and fluorescence microscopy) resulted in Aβ internalization followed by its efflux. Yamada and his co-workers using TR-BBB cells, another BBB cell line model, also showed that Aβ is rapidly internalized through a LRP1-mediated mechanism, approximately during 10 minutes peptides are internalized, and then its efflux begins (Yamada *et al.* , 2008). Furthermore, it is also possible that the apparent lower Aβ inside cells resulted from its degradation, partially favoured by TTR. Supporting this hypothesis is our observation that in the fluorometric assay, during the second incubation without FAM-Aβ1-42, the release of peptide from cells was apparently faster in the absence than in the presence of TTR, after 10 min. Indeed, TTR has been characterized as a metalloprotease (Liz *et al.* , 2012) and uses Aβ as substrate, in addition to ApoA-1 and NPY peptide (Liz *et al.* , 2007) and proteolysis has been suggested as a mechanism underlying TTR protection in AD (Costa *et al.* , 2008a). However, we could not clearly ascertain the role of this TTR in these processes, and it is necessary to repeat this experiment with a higher number of replicas, in order to confirm the effective role of TTR, including in Aβ degradation.

It also should be noted that, in presence of serum Aβ internalization levels were even lower relatively to serum-free conditions, revealing its interference. Serum is a fluid enriched by several proteins, including proteases, which can degrade Aβ peptides decreasing its levels, and thus reduced its availability to be internalized by the hCMEC/D3 cells. By contrast, serum is also composed by apoE and α2-macroglobulin, known as Aβ carriers, and for this reason Aβ should form a complex with apoE or/and

α2-macroglobulin facilitating its internalization by cells (Qiu *et al.* , 1999; Kang *et al.* , 2000; Gylys *et al.* , 2003). Again, experiments with sera were performed using incubations of 3 hours and thus, the effect of other Aβ transport might have been masked, similarly to TTR effect. Thus, further experiments using all TTR sources available must be undertaken using appropriate incubation times.

Our basolateral-to-apical permeability results demonstrated a significant effect of the TTR at increasing Aβ efflux from brain-to-blood, suggesting a crucial role of this protein in Aβ clearance out the brain. Regarding this, we evaluated TTR transport across the hCMEC/D3 cells, and our results showed that by itself this protein crossed the monolayer of hCMEC/D3 cells and in absence of Aβ, its transport was even higher from brain-to-blood. In fact, some studies even suggest the passage of CSF TTR to blood as a biomarker for BBB disruption (Marchi *et al.* , 2003).

In this view, we believed that in AD pathogenesis, when Aβ production starts to increase, TTR can rescue this peptide in order to promote its elimination across the BBB, preventing its deposition in neuritic plaques. At this point, remains the doubt if TTR crosses or not BBB linked to Aβ, and if this really occurs, what is the mechanism by which it happens. Hereupon, two endocytic TTR-related receptors have been described, namely megalin (also known as LRP2), known for renal uptake of TTR, and an unidenfied receptor-associated protein (RAP)-sensitive receptor (Sousa *et al.* , 2000; Sousa *et al.* , 2001). Regarding nervous system, megalin is expressed by several cell types, including oligodendrocytes (Wicher *et al.* , 2006), astrocytes (Bento-Abreu *et al.* , 2008), cortical neurons (Chung *et al.* , 2008) and DRG neurons (Fleming *et al.* , 2009a). One hypothesis would be TTR/Aβ complex bind to megalin, and this receptor triggers its transport from brain-to-blood. Supporting this proposal, Bell et al. demonstrated that ApoJ/Aβ complex is able to bind to the LRP2, promoting transcytosis of this complex and subsequent brain-to-blood efflux of Aβ (Bell *et al.* , 2007). These data support our initial hypothesis, however some reports suggested that Aβ can play a central role in mediating increased BBB permeability, and potentially disrupt CNS homeostasis. A study with Tg2576 AD model, with overexpression of APP and therefore of Aβ, demonstrated increased BBB permeability after 4 months of age, prior to plaque deposition and disease onset (Ujiie *et al.* , 2003). Another report detected that Aβ increases hCMEC/D3 cell paracellular permeability, maybe as a consequence of reduced occludin levels at cell junctions (Tai *et al.* , 2010). Regarding these reports, during our experiment with 48 hours of incubation, Aβ could affect permeability of cells monolayer, allowing a higher passage of Aβ and TTR.

Despite our findings, these experiments were made with a BBB cell line, which although displaying excellent characteristics such as transporters, metabolizing enzymes and TJ proteins, is not enough to ascertain with reliability the role of TTR in Aβ efflux. It is possible ameliorate the transwell transport system by increasing barrier tightness. It has been reported that co-culturing of hCMEC/D3 cells with human astrocyte cell lines improves transendothelial electrical resistance (TEER) values, an indicator of paracellular permeability (Hatherell *et al.* , 2011). Nevertheless, in future reported that attempt to improve the model and at the same time we will assess the effect of other TTR sources, namely hrec TTR and mouse rec TTR. In addition, we will reduce the incubation times, since it has been reported that *in vivo* Aβ is effluxed from the brain in 30 minutes (Shibata *et al.* , 2000), although other works concerning Aβ transport using transwells have using long incubation times, similarly to our experiments (Nazer *et al.* , 2008).

Finally, our in vitro results were corroborated by our preliminary *in vivo* study, which demonstrated that, 30 minutes after Aβ injection, brain Aβ levels of $TTR^{-/-}$ mice were higher than in $TTR^{+/-}$ mice, indicating that TTR enhances Aβ elimination out of the brain, supporting our initial theory.

On the other hand, we could not quantify Aβ in the liver, 30 minutes post-injection, and also due to the small volume of plasma, we could not accurately assess whether or not the peptide was transported out of the brain or simply degraded. Moreover, this *in vivo* study has to be repeated with more animals per group and also with the three genotypes for TTR, in order to elucidate the contribution of TTR in Aβ efflux, and its possible implication in AD pathogenesis.

In summary, our study showed differences in plasma Aβ levels of AD/TTR mice suggesting that TTR is implicated in Aβ brain efflux. Using different approaches, including uptake/efflux assays as well as permeability studies across a monolayer of cells mimicking the BBB, we showed that TTR promotes Aβ internalization by and transport across cerebral microvascular endothelial cells, highlighting the role of TTR as a transporter protein, a role that has long been known for TTR.

We thus propose that TTR exerts its protective function in AD by binding Aβ peptide and delivering the peptide at the BBB for elimination, avoiding its accumulation in the brain and deposition in senile plaques. Future work should address the exact mechanism underlying TTR/Aβ interaction at the BBB, the association of Aβ degradation as well as the receptors involved.

Chapter II: Investigating stimulus triggering TTR synthesis in neuronal cells

Material and Methods – chapter II

1. Cell culture

Human neuroblastoma cell line (SH-SY5Y cell line) were cultured in 25-cm^2 flasks and maintained at 37^oC in a 95% humidified atmosphere and 5% CO_2. Cells were grown in Eagle's minimum essential medium (EMEM) and Ham's F12 medium (1:1; Gibco) supplemented with 10% FBS (Gibco), 1% non-essential amino-acids (NEAA) (Sigma-Aldrich), 2 mM L-Glutamine (Gibco) and 1% of penicillin-streptomycin (Lonza). Cell culture medium was changed every 3 days.

SH-SY5Y cells stably transfected with the plasmid carrying the human APP Swedish mutation K595N/M596L (isoform 695) cDNA (APPswe), were previously prepared (Rita costa, PhD thesis) and were available in the laboratory. The plasmid had been kindly provided by the Professor Sam Sisodia. This transfected cells were grown in 25-cm^2 flasks in the same medium that SH-SY5Y cells but supplemented with 1 mg/ml G418 (Amresco). In the next sections, the APPswe transfected SH-SY5Y cell line will be referred to as SH-SY5Y/APPswe cell line. During experiments all the cell lines were maintained in 1% FBS medium.

2. Preparation and production of Aβ1-42 species

The Aβ1-42 peptide was prepared as described in the chapter I. Three Aβ1-42 species were produced: soluble, oligomers and fibers. Soluble Aβ1-42 was obtained by instantly diluting the peptide in the appropriate medium, whereas to prepare oligomers, Aβ1-42 was diluted to 100 μM in Ham's F12 medium and incubated for 48 hours at 4^oC. For fibril formation, Aβ1-42 100 μM in Ham's F12 medium was incubated at 37^oC for 10 days. Samples were then visualized by transmission electron microscopy to confirm the presence of the different Aβ1-42 species.

3. Transmission electron microscopy

For visualization by TEM, sample aliquots were absorbed to carbon-coated collodion film supported on 400-mesh copper grids, and negatively stained with 1%

uranyl acetate. The grids were visualized with a JEOL JEM-1400 transmission electron microscope equipped with an Orious Sc1000 digital camera.

4. Effect of Aβ species in expression of human APP and TTR genes

This experiment was performed using SH-SY5Y cells grown in 25-cm^2 flasks to 90% of confluence. Then, cells were incubated for 24 hours at 37^oC with 2.5 ng/mL Aβ1-42 and with 10 μM of each Aβ1-42 species obtained as described above (soluble, oligomers and fibers). Additionally, SH-SY5Y and SH-SY5Y/APPswe cells were incubated with 0% FBS media during 24 hours. At the end of treatment period, cells were harvested and RNA was extracted for RT-PCR analyses, as mentioned below.

5. Influence of TTR in expression of human APP gene

SH-SY5Y cells were cultured in 25-cm^2 flasks until 90% of confluence. After culturing, cells were incubated with 1 μM hrecTTR for 24 hours at 37^oC, and then RNA was extracted and subjected to real-time PCR (RT-PCR), as described below in gene expression analysis topic.

6. Gene expression analysis

For RT-PCR analysis, total RNA was isolated from confluent cells using Trizol reagent (Invitrogen) following the instructions of the manufacturers and was quantified by reading the absorbance at 260 nm with a NanoDrop photometer. RNA purity was determined using the A260/A280 ratio (average >1.8). Then, 5 μg of RNA was first-reversed transcribed into cDNA using SuperScript R First-Strand kit (Invitrogen). The cDNA was then used for RT-PCR, to observe the expression of human APP (total APP and APP isoforms) and WT TTR genes. Human β-actin was used as an internal control for normalization. PCR amplification was performed using a commercial KAPA HiFi PCR kit (KAPAbiosystems), according to the manufacture's protocol. The PCR primers and the conditions used for PCR are shown in table 2.

Samples were further analysed on a 1% (Total APP and Human β-actin) and 3% agarose gel (WT TTR and APP isoforms) and visualized by gel imaging system (ChemiDoc, Bio-Rad) in the presence of ethidium bromide.

Table 2. List of PCR primers sequences and PCR conditions.

Gene	Primer sequences	PCR conditions
β-Actin	Reverse: 5' CAC CAT CAG GCC CTG GTC CC 3' Forward: 5' ACA GAG CCT CGC CTT TGC CG 3'	Initial denaturation: 95°C, 5 minutes Denaturation, annealing and extension (35 cycles): 95°C, 45 seconds 55°C, 1 minute 72°C, 1.30 minutes Final extension: 72°C, 5 minutes
WT TTR	Sense: 5' ATG GCT TCT CAT GGT CTG CT 3' Anti-sense: 5' GAA GTC CCT CAT TCC TTG GG 3'	
Total APP	Reverse: 5' TCT GTG GTG GTG GTG GTG 3' Forward: 5' GAT GCG GAG GAG GAT GAC 3'	
APP isoforms	Reverse: 5' CTG GAA ATG GGC ATG 3' Forward: 5' GTA GTA GAA GTA GCA G 3'	Initial denaturation: 95°C, 5 minutes Denaturation, annealing and extension (35 cycles): 95°C, 45 seconds 45°C, 45 seconds 72°C, 1.30 minutes Final extension: 72°C, 5 minutes

Results – chapter II

It has been reported that TTR levels in CSF are reduced in several AD patients, but the meaning of this decrease is still not clear (Serot *et al.* , 1997; Gloeckner *et al.* , 2008; Hansson *et al.* , 2009). In the last years, it has been proposed that TTR sequesters Aβ in CSF, but other sites of sequestration were not been identified. Further it was assumed that, in CNS, TTR was only produced in choroid plexus and not in neurons (Li *et al.* , 2011a). However, Li and co-workers demonstrated that neurons transcribe TTR mRNA, which is subsequently translated to produce TTR protein. They also suggested the possibility of the CSF TTR concentration being determined in part by its synthesis in neurons, and that reduced TTR levels are associated with neuronal loss (Li *et al.* , 2011b). In addition, an *in vitro* study showed that TTR is expressed in SH-SY5Y neuroblastoma cell line, and that it is up-regulated by the AICD fragment of APP, specifically derived from the APP695 isoform (Kerridge *et al.* , 2014). This group also showed that, accumulation of functional AICD caused TTR up-regulation and subsequent a decrease of Aβ levels. Furthermore, other group demonstrated in SH-SY5Y cells, primary hippocampal neurons and the hippocampus of APP23 mice, that TTR expression is significantly improved by HSF1 (Wang *et al.* , 2014). Thus, one can propose that, TTR protects through sequestration of Aβ in neuritic plaques and consequently its reduction by clearance out of the brain.

In light of these discoveries, we decided to investigate if certain stimulus, namely different Aβ species (soluble, oligomers and fibers), can induce TTR gene expression and alteration in APP isoforms gene profile using SH-SY5Y cell line. Additionally, we also evaluated if the overexpression of the 695APPswe isoform affects TTR expression versus the influence of TTR in APP gene expression.

1. Effect of Aβ species in the expression of TTR and human APP genes

1.1. Influence of soluble Aβ1-42 in TTR and APP genes expression

The SH-SY5Y cell line does not express TTR and to explore the potential effect of Aβ species in induction of the TTR gene expression and also in the alteration of APP

gene expression (including total APP gene and APP isoforms), SH-SY5Y cells were incubated firstly with a physiological concentration (500 pg/ml) of soluble Aβ1-42.

Analysis by RT-PCR showed no TTR expression (data not shown) and even after using Aβ1-42 at 2.5 ng/ml, no TTR was observed (Figure 27).

Since it has been reported that, in AD, there is a shift in the expression of APP isoforms with APP-KPI isoforms being more expressed, we decided to investigate if Aβ1-42 could induce such alteration. However, we did not detect any variation in total APP and APP isoforms expression in the presence or absence of the peptide (Aβ 2.5 ng/ml).

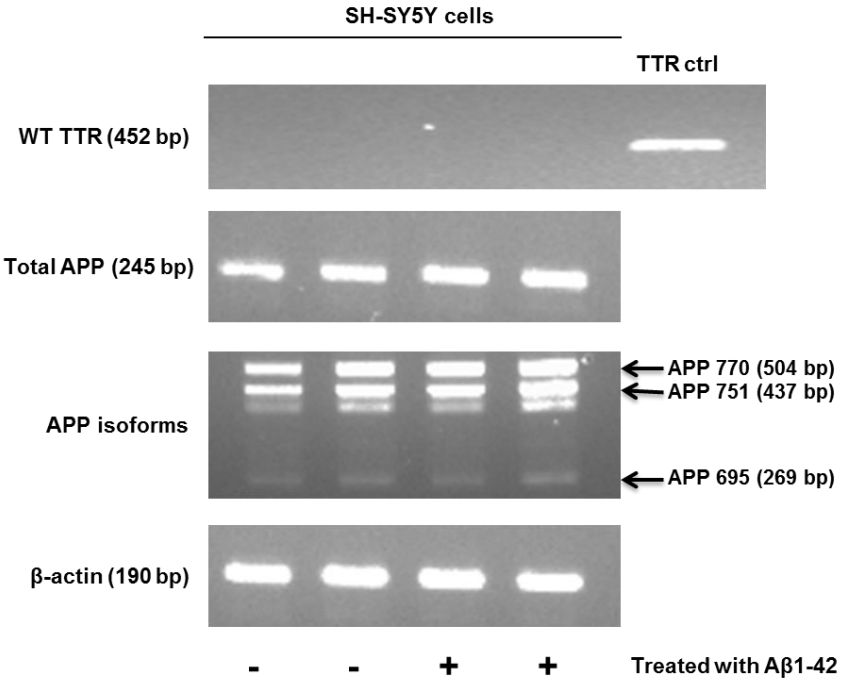

Figure 27 – RT-PCR analysis of TTR and APP genes expression in SH-SY5Y cell line after treatment with Aβ1-42. SH-SY5Y cells were incubated with 2.5 ng/mL of Aβ1-42 (+) and without this peptide (-) in 0% FBS media, during 24 hours. Total RNA was extracted and subjected to RT-PCR, using primers for APP isoforms (APP 770 (504 bp), APP 751 (437 bp) and APP 695 (269 bp)), Total APP (245 bp), WT TTR (452 bp) and β-Actin (190 bp). Plasmid which expresses TTR was used as a control (ctrl).

1.2. Effect of Aβ aggregated forms in TTR and APP isoforms genes expression

Since Aβ peptide induces toxicity in oligomerized and aggregated forms, we next evaluated TTR and APP isoforms gene expression in SH-SY5Y cells incubated

with 10 µM of soluble Aβ, oligomers and fibers, for 24 hours. The presence of different Aβ species was confirmed by ultrastructure by TEM (Figure 28). The use of synthetic Aβ1-42 is highly challenging and achieving a preparation containing 100% of soluble monomeric peptide is nearly impossible. Thus, visualization of the soluble Aβ preparations revealed mostly individual particles but also some small aggregates (Figure 28 A, arrows and arrowheads, respectively); quite different, the oligomeric preparations were abundantly populated by aggregates and oligomers of different sizes (Figure 28 B); also very distinct from the above samples, the fibril preparations, although heterogenous in nature, presented as main species fibrillar structures with the characteristic diameter of amyloid fibrils (approximately 10 nm wide) (Figure 28 C, D).

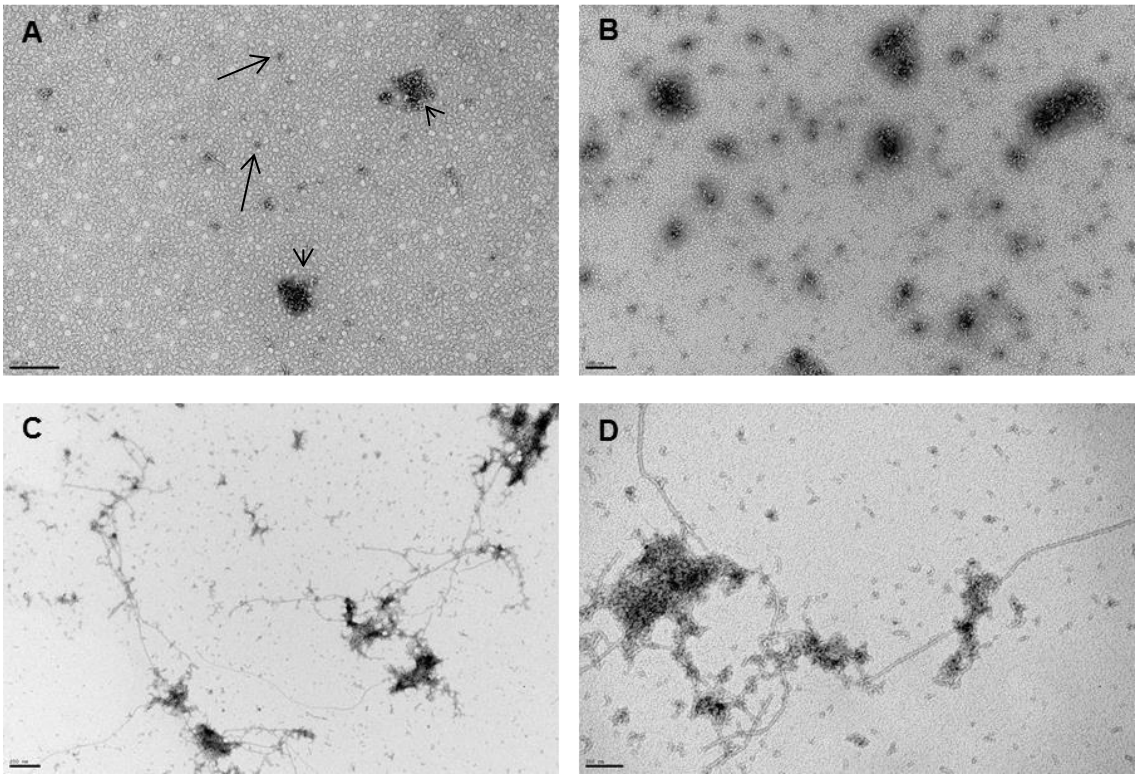

Figure 28 – Morphological characterization of Aβ species by TEM analysis. (A) Soluble Aβ1-42 (obtained by instantly diluting the peptide in the appropriate medium); (B) Oligomers (Aβ1-42 was diluted to 100 µM in Ham's F12 medium and incubated for 48 hours at 4oC); (C) and (D) Fibrils (100 µM Aβ1-42 in Ham's F12 medium was incubated at 37oC for 10 days); Scale bars: A, B and C – 200 nm; D – 100 nm.

RT-PCR results (Figure 29) reveal that, the presence of Aβ oligomers and fibers induces TTR expression in SH-SY5Y cells, and also causes an increase in the expression of all APP isoforms.

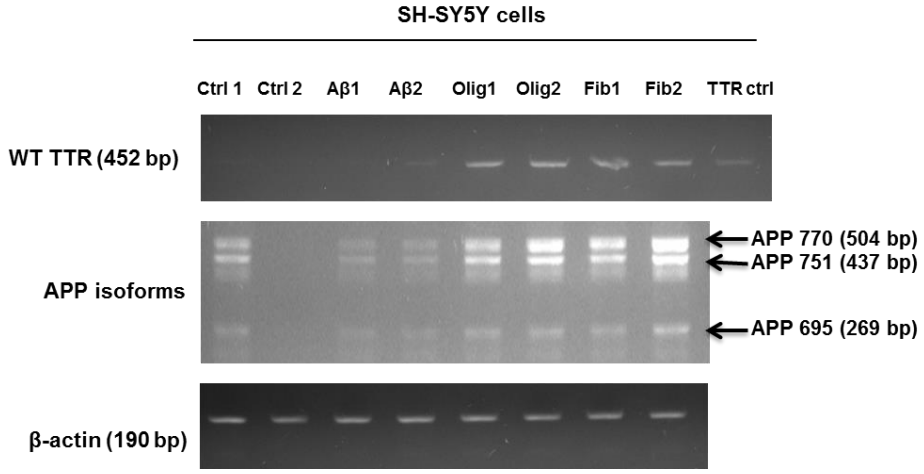

Figure 29 – RT-PCR analysis of TTR and APP genes expression in SH-SY5Y cells, after treatment with different forms of Aβ1-42. Cells were incubated with 10 µM of soluble Aβ (Aβ1 and Aβ2), oligomers (olig1 and olig2) and fibers (Fib1 and Fib2), and as control (Ctrl1 and Ctrl2), cells were incubated only with 0% FBS media, during 24 hours. Total RNA was extracted and subjected to RT-PCR, using primers for APP isoforms (APP 770 (504 bp), APP 751 (437 bp) and APP 695 (269 bp)), WT TTR (452 bp) and β-Actin (190 bp). Plasmid which expresses TTR was used as a control (TTR ctrl).

1.3. TTR expression in APPswe transfected cells

Finally, we assessed TTR gene expression in cells overexpressing the human APPswe gene to further investigate the mechanisms leading to the presence of TTR in neuronal cells and also in an attempt to confirm the results obtained with exogenous aggregated Aβ. However, we do not observe any TTR expression, similarly to non-transfected cells (Figure 30). It is possible that the levels of peptide secreted by transfected cells were not sufficient to induce TTR gene expression or that secreted Aβ was not in the form of oligomers and/or aggregates.

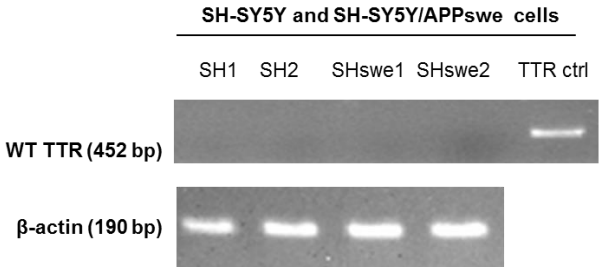

Figure 30 – RT-PCR analysis of TTR gene expression in SH-SY5Y cells transfected with APPswe gene. SH-SY5Y (SH1 and SH2) and SH-SY5Y/APPswe (SHswe1 and SHswe2) cells were incubated with 0% FBS media during 24 hours and RNA were extracted and subjected to RT-PCR, using WT TTR primer (452 bp).

Taken together, these findings suggest that the presence of neurotoxic species of Aβ, such as oligomers and fibers, induces neuronal TTR mRNA expression and might result in TTR protein neuronal synthesis, which in turn binds to oligomers and fibers of Aβ. Consequently, this interaction will interfere with Aβ oligomerization and fibrillization by disrupting oligomers and fibril, and will result in increased Aβ clearance, preventing the toxic damage in neurons.

2. Influence of TTR in expression of human APP gene

As already mentioned, brains from AD and non-AD subjects present different patterns of APP isoforms expression, suggesting that some of them contribute to disease development.

We next evaluated if the presence of TTR induces changes in such profile. As depicted in figure 31, no differences are observed in cells incubated with or without TTR, suggesting that the mechanism of TTR protection in AD does not affect APP expression.

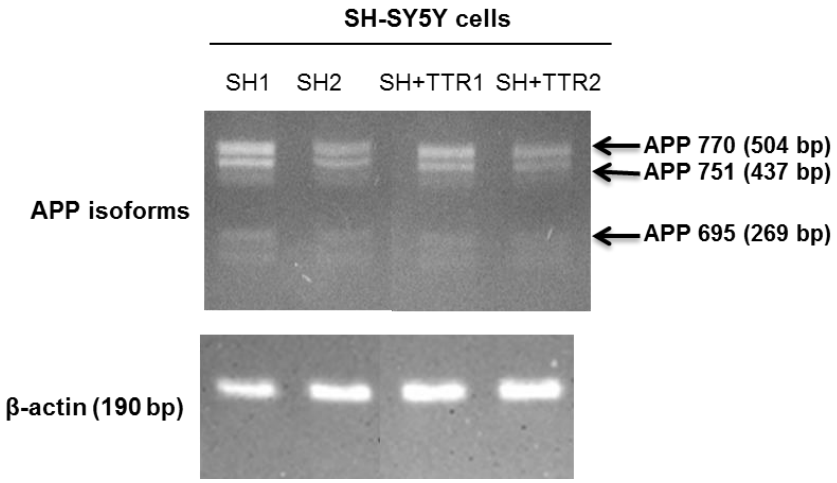

Figure 31 – RT-PCR analysis of TTR and APP genes expression. SH-SY5Y cells were incubated with 1 μM hrecTTR (SH+TTR1 and SH+TTR2), and as control (SH1 and SH2), cells were incubated only with 0% FBS media, during 24 hours. Total RNA was extracted and subjected to RT-PCR, using primers for APP isoforms (APP 770 (504 bp), APP 751 (437 bp) and APP 695 (269 bp)), and β-Actin (190 bp).

Discussion – chapter II

AD is characterized as an inevitably progressing dementia. In susceptible brain regions, such as the hippocampus and cortex, there is an accumulation of Aβ peptides into extracellular neuritic plaques. Previous studies have shown an important function of TTR as an Aβ carrier suggesting that TTR binds and rescues the peptide in the CSF (Schwarzman *et al.* , 1994; Schwarzman *et al.* , 1996). In fact, Aβ clearance mechanisms are becoming an important aspect in the prevention of AD pathology, since it is now believed that in AD sporadic cases, accounting for the larger majority of AD cases, Aβ clearance is impaired, contrarily to older believes which considered Aβ production to be increased.

TTR is predominantly synthesized by the liver where more than 90% of the protein is produced. The remaining is produced by the choroid plexus and the retina, but other sites of synthesis have been described in mammals (Oliveira *et al.* , 2011). For a long time, investigators supposed that the locally secreted TTR protein acted as a carrier of thyroid hormones between blood and brain, seeing that the BBB is not permeable to these molecules. TTR production within the CNS was ascertained by immunochemical techniques demonstrating the participation of organelles involved in the CSF secretory pathway (Aleshire *et al.* , 1983). Herbert and colleagues showed in 1986 that TTR, within the CNS, is solely synthesized by the choroid plexus, suggesting a special role for TTR in the brain or CSF, also speculating if this function differs or not from its established plasma transport functions (Herbert *et al.* , 1986). However, recent reports raised controversy on this issue describing TTR synthesis in other areas of the brain, namely in the hippocampus. Stein and Johnson described that the lack of degeneration in Tg2576 mice, was associated with increased level of TTR in the hippocampus (Stein *et al.* , 2002), and other group demonstrated that neurons transcribe TTR mRNA, which is subsequently translated to produce TTR protein (Li *et al.* , 2011b). Moreover, intraneuronal TTR was seen in the brains of normal humans and mice and in AD patients and APP23 AD transgenic mice. The APP23 brains showed co-localization of extracellular TTR with Aβ in plaques (Buxbaum *et al.* , 2008).

Very recently, some suggestions on TTR neuronal synthesis have emerged and Kerridge et al. showed that TTR is expressed in SH-SY5Y cell line, and that it is up-regulated by the AICD fragment of APP, which derived from the APP695 isoform (Kerridge *et al.* , 2014). The accumulation of functional AICD resulted in TTR up-

regulation and Aβ decreased levels. Wang and colleagues verified that TTR expression is enhanced by HSF1 in SH-SY5Y cells, primary hippocampal neurons and the hippocampus of APP23 mice (Wang et al., 2014). Nevertheless, the mechanism of neuronal TTR induction, both in biological and pathological conditions is far from being unraveled and in this view, this study has focused in the investigation of certain stimulus, such as the different Aβ species (soluble, oligomers and fibers), at inducting TTR gene expression and at altering the profile of APP isoforms gene expression, using a human SH-SY5Y neuroblastoma cell line. In addition, it was evaluated also if SH-SY5Y cells over-expressing 695APPswe were relevant to induction TTR gene versus the influence of exogenous TTR in APP gene expression.

Analysis by RT-PCR revealed no TTR expression after treatment with soluble Aβ, both at low and high concentrations. On the other hand, Aβ1-42 oligomers and fibrils, the neurotoxic elements in AD, induced neuronal TTR expression. This finding suggests that TTR can be produced in neurons under certain conditions. Thus, when neuronal cells are in need, TTR is synthesized and then bind to oligomeric and fibrillar forms of Aβ, promoting their clearance at the BBB (as evaluated in chapter I) to prevent neurotoxicity. As previously described, *in vitro*, TTR is able to inhibit and to disrupt Aβ fibrils (Costa *et al.* , 2008b). Furthermore, as described in several studies, TTR has protease activity and cleaves Aβ producing fragments non-toxic or less toxic than the full-length peptide (Liz *et al.* , 2004; Costa *et al.* , 2008a). Thus, it is possible that TTR acts in several ways aiming at protecting against AD. Our results contradict other studies, which reported that TTR mRNA levels are significantly increased in SH-SY5Y cells over expressing 695APPswe (Li *et al.* , 2010; Kerridge *et al.* , 2014). Furthermore, Li and co-workers showed that increased TTR mRNA and protein in cultured hippocampal neurons from AD model mice and also demonstrated an increase in TTR-stained neurons in human AD brains, suggested that neuronal TTR transcription might be substantially induced by Aβ or its precursors (Li *et al.* , 2011b). Others reported that, sAPPα or sAPPβ leads to increased expression of protective genes, such as TTR, to confer neuroprotection (Stein *et al.* , 2004; Li *et al.* , 2010). Indeed, transfected cells, due to the presence of the human APPswe gene, should generate large amounts of Aβ peptide compared to the non-transfected cells, and our results might indicate that either 1) Aβ did not reach a concentration high enough, or 2) the peptide is not sufficiently aggregated, to induce TTR expression.

The APP gene is alternatively spliced to produce three major isoforms including APP695, APP751 and APP770. APP695 is predominantly expressed in neurons and

lacks the KPI domain, contrarily to APP751 and APP770 which contain this domain (Matsui *et al.* , 2007). Studies show that both protein and mRNA levels of KPI-containing APP isoforms are elevated in AD brains and are associated with increased Aβ deposition (Menendez-Gonzalez *et al.* , 2005). In light of this, we investigated if Aβ could induce such alteration in total APP and APP isoforms. With regard to soluble Aβ no significant variation was observed. By contrast, Aβ oligomeric and fibril forms caused an increase in APP gene expression as deduced by increase observed for all APP isoforms analysed, suggesting a mechanism of positive feedback while disease develops: when Aβ starts to aggregate and accumulate in brain, the APP transcription is signalized and then, more Aβ is produced, contributing to plaque formation in the brain and disease progression.

We also evaluated if, on the contrary, TTR as a protective molecule could decrease APP expression, namely the KPI-containing isoforms. However, we found no differences in SH-SY5Y cells incubated with or without human recTTR, suggesting that TTR protection in AD is mostly associated with Aβ binding and clearance but not with Aβ precursor.

In summary, our results demonstrated that TTR gene expression is induced by aggregated and fibrillar Aβ forms suggesting that neuronal synthesis of this protein might be a natural protective response to the cellular challenge presented by Aβ aggregation. Thus, protection appears to be a function of increased TTR production with binding of Aβ aggregates and fibrils Aβ and consequently reduction of their toxicity by elimination and/or degradation.

Conclusions and perspectives

This study highlights the importance of TTR as protective protein in AD trough the maintenance of Aβ levels in brain by its elimination across the BBB and/or its degradation.

Based on different approaches used, we can assume that TTR promotes Aβ efflux through the BBB, preventing its accumulation in the brain and deposition in extraneuronal plaques and in this way reduces Aβ-associated toxicity. In the future, it would be interesting to address the precise mechanism underlying TTR/Aβ interaction at the BBB, namely by testing the participation of LRP1 and P-gp, by blocking these molecules with specific antibodies or through the use of interference RNA; it will be important to assess TTR binding these molecules, directly or via Aβ, for instance by co-immunoprecipitation approaches. Given that TTR has proteolytic activity proposed to be relevant in the CNS, the studies should not just focus on Aβ transport but also on its degradation, for instance by performing TCA precipitation of labelled Aβ after the transport assays. In future experiments, the importance of TTR stability in AD and in particular in its assistance in Aβ transport across the BBB, should also be explored. For that, the permeability studies in the transwell system can be performed in the presence of TTR stabilizers, such as IDIF.

Our study presented for the first time evidence that neuronal TTR expression can also be induced by Aβ oligomers and fibrils, indicating that neuronal synthesis of TTR can be a natural mechanism to protect neurons against Aβ toxicity. It will be important to unravel the mechanism behind TTR neuronal synthesis, by confirming our findings *in vivo*. It is also important to understand how our data relates to other reports on the regulation of TTR expression in the brain.

In summary, TTR role in the CNS might encompass, among other effects, assistance in Aβ brain efflux at the BBB. Under a pathological situation, such as AD, and in the presence of threshold of Aβ toxic species, TTR neuronal synthesis is induced to confer protection by binding to Aβ aggregates and fibrils and consequently by reducing their toxicity (for instance, by elimination across the BBB and/or degradation).

Finally, because Aβ is also transported across the BCSFB and since TTR is the major Aβ binding protein in the CSF, it is fundamental to research on the effect of TTR in Aβ efflux at the BCSFB. For that we propose to use primary cultures of choroid plexus epithelial cells and also in vivo studies.

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Annexes

Annex I – Evaluation of integrity of hCMEC/D3 cells monolayer

During basolateral-to-apical experiment, the tightness and integrity of the BBB cells monolayer, was monitored by the permeability of FITC-labeled dextran across the monolayers, as previously described in material and methods section, in chapter I.

An example of integrity and permeability assay is demonstrated in table 3. As it can be seen, the transwell 6 was excluded from the study, because the concentration of FITC-dextran in basolateral side exceeded 125 ng/mL, suggesting that cell monolayer was disrupted, leaving FITC-dextran molecules pass freely. The remaining transwells presented less than 125 ng/mL of FITC-dextran in basolateral side after 1 hour of incubation. Thus, these monolayers were well organized and cell-cell interactions were formed, preventing free passage of FITC-dextran across the cells, and therefore, transwells were used in hCMEC/D3 basolateral-to-apical permeability to Aβ.

Table 3 – Evaluation of integrity of hCMEC/D3 cells monolayer in transwells filters.

Transwells	Concentration of FITC-dextran in basolateral side (ng/mL)
1	12.2
2	-13.3
3	-17.8
4	36.8
5	20.3
6	499.1
7	-18.07
8	-18.9