Universidade do Minho
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Tiago André Braga Gião

Vascular alterations in Alzheimer's Disease - role of Aß peptide and Transthyretin?

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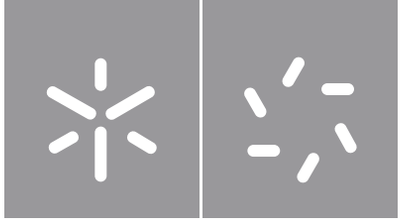

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Vascular alterations in Alzheimer's Disease - role of Aß peptide and Transthyretin?

Master's Thesis
Master's Degree in Applied Biochemistry
Specialization in Biomedicine

Dissertation carried out under the supervision of
Doctor Isabel dos Santos Cardoso
and
Professor Doctor Sandra Cristina Almeida Paiva

October 2019

Despacho RT - 31 /2019 - Anexo 3

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Alterações vasculares na Doença de Alzheimer – papel do péptido Aβ e da Transtirretina?

RESUMO GERAL

Na Doença de Alzheimer (DA), a barreira hematoencefálica (BHE) é disfuncional, contribuindo para a acumulação do péptido β-amilóide (Aβ) no cérebro. Além disso, a membrana basal (MB) do cérebro, que regula as caraterísticas da BHE e a angiogénese é mais espessa na DA, como é evidenciado pelo aumento da camada de colagénio IV, e suspeita de afetar o efluxo cerebral do Aβ.

A transtirretina (TTR) é uma proteína neuroprotetora na DA, no entanto, os seus níveis estão diminuídos devido à sua instabilidade tetramérica, afetando a capacidade da TTR se ligar ao Aβ. Contudo, os níveis de TTR podem ser aumentados e a interação TTR/Aβ melhorada por pequenos ligandos, sendo a base para a criação de um programa de triagem de drogas, focado na procura de pequenos compostos potenciadores da interação TTR/Aβ, como estratégia terapêutica na DA.

Este projeto teve como objetivo avaliar o envolvimento da TTR na vasculatura cerebral, em contextos fisiológicos e patológicos. O trabalho realizado também pretendeu avaliar o efeito de dois estabilizadores tetraméricos da TTR, administrados a ratinhos com DA, nos depósitos amiloide de Aβ.

A atividade angiogénica da TTR foi confirmada usando o ensaio *in vivo* da membrana corioalantóica de embrião de galinha e a análise *in vitro* revelou que a TTR regulou positivamente a expressão de IL-6, IL-8, VEGF e Ang-2. Os níveis de colagénio IV nos microvasos cerebrais aumentaram em ratinhos TTR+/- com 3 meses de idade relativamente a TTR+/+. A comparação entre DA/TTR+/- e TTR+/- revelou maior conteúdo de colagénio IV em ratinhos com DA, sugerindo que a redução da TTR tem um impacto maior na BM no contexto da doença. Células endoteliais do cérebro incubadas com oligómeros ou fibras de Aβ expressaram mais colagénio IV do que as células controlo. Esse efeito foi neutralizado pela TTR, resultando numa menor expressão de colagénio IV.

Os efeitos do iododiflunisal e tolcapone, foram avaliados *in vivo* num estudo longitudinal por tomografia por emissão de positrões (TEP), em ratinhos AD/TTR+/-. Aqui é descrito apenas a análise final dos depósitos amiloide no cérebro, por imunohistoquímica, após o sacrifício dos animais, aos 15 meses de idade. Os resultados não mostraram diferenças, embora só possamos concluir acerca do efeito desses compostos após a análise dos resultados da TEP nas diferentes idades.

No seu conjunto, os nossos resultados sugerem que a TTR é angiogénica e os seus baixos níveis na DA parecem ter impacto nas alterações vasculares que ocorrem na DA, corroborando a atividade neuroprotetora da TTR.

Palavras chave: Alterações vasculares; Angiogénese; Colagénio IV; Doença de Alzheimer; Transtirretina

Vascular alterations in Alzheimer's Disease – role of Aβ peptide and Transthyretin?

GENERAL ABSTRACT

In Alzheimer's Disease (AD), the blood-brain barrier (BBB) is dysfunctional, contributing to the accumulation of amyloid-β peptide (Aβ) in the brain. In addition, the brain basement membrane (BM), which regulates features of the BBB and of angiogenesis, is thicker in AD, as evidenced by an increase in the collagen IV layer, and is thought to impair Aβ brain efflux.

Transthyretin (TTR) is a neuroprotective protein in AD, however, its levels are decreased due to its tetrameric instability, affecting TTR ability to bind to Aβ. Despite this, TTR levels can be raised and the TTR/Aβ interaction enhanced by small ligands, being the basis for settling a drug screening program focused in the discovery of small compounds enhancers of the TTR/Aβ interaction, as a therapeutic strategy in AD.

This project aimed at assessing the involvement of TTR in brain vasculature, in physiological and in pathological contexts. The work performed also intended at evaluating the effect of two TTR tetrameric stabilizers, administrated to AD mice, in Aβ amyloid burden.

The angiogenic activity of TTR was confirmed using the *in vivo* chick embryo chorioallantoic membrane assay and *in vitro* analysis revealed that TTR up-regulated the expression of IL-6, IL-8, VEGF and Ang-2. Levels of collagen IV in brain microvessels were increased in TTR+/- 3-month old mice relatively to TTR+/+. Comparison between AD/TTR+/- and TTR+/-, revealed higher collagen IV content in AD mice, suggesting that TTR reduction has a greater impact in the BM in the context of disease. Brain endothelial cells incubated with Aβ oligomers or fibrils expressed more collagen IV than control cells. This effect was counteracted by TTR, resulting in decreased expression of collagen IV.

The effects of the iododiflunisal and tolcapone, were evaluated *in vivo* in a longitudinal study by positron emission tomography (PET), using AD/TTR+/- mice. Here is reported only the final evaluation of amyloid burden in the brain, after animal sacrifice, at the age of 15 months, by immunohistochemistry. The results showed no differences, although one can only conclude about the effect of these compound after examination of the PET results, at the different ages.

Altogether, our results suggest TTR is angiogenic and its decreased levels in AD seem to impact in the vascular alterations occurring in AD, further corroborating the neuroprotector activity of TTR.

Keywords: Alzheimer's Disease; Angiogenesis; Collagen IV; Vascular alterations; Transthyretin

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AD/TTR/+/+	AD transgenic mice with both copies of TTR gene
AD/TTR/+/-	AD transgenic mice with one copy of TTR gene
AD/TTR/-/-	AD transgenic TTR KO with no copy of TTR gene
AICD	APP intracellular domain
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
ApoA-I	Apolipoprotein A-I
ApoE	Apolipoprotein E
ApoJ	Apolipoprotein J
APP	Amyloid-ß-precursor protein
Aß	Amyloid-ß peptide
BBB	Blood-brain barrier
bEnd.3	Immortalized mouse brain endothelial cell line
bFGF	Basic fibroblast growth factor
BM	Basement membrane
BSA	Bovine serum albumin
CAA	Cerebral amyloid angiopathy
CAM	Chick chorioallantoic membrane
CNS	Central nervous system
CSF	Cerebrospinal fluid
EC	Endothelial cell
EOAD	Early-onset AD
EGF	Epidermal growth factor
EDD	Embryonic development day
FAD	Familial AD
FAP	Familial amyloidotic polyneuropathy
FBS	Fetal bovine serum
$GABA_A$-Rs	$GABA_A$ receptors
GLUT-1	Glucose transporter 1
hCMEC/D3	Immortalized human cerebral microvascular endothelial cell line

HIF-1	Hypoxia-inducible factor-1
hRECs	Human retinal microvascular endothelial cells
HUVECs	Human umbilical vein endothelial cells
IDIF	Iododiflunisal
IL-6	Interleukin-6
IL.8	Interleukin-8
IGF	Insulin-like growth factor
LOAD	Late-onset AD
LB	Lubia-Bertani
LRP1	Low-density lipoprotein receptor-related protein 1
LRP2	Low-density lipoprotein receptor-related protein 2
MMP-2	Metalloprotease-2
MMP-9	Metalloprotease-9
NFT	Neurofibrillary tangles
NPY	Neuropeptide Y
NVU	Neurovascular unit
NSAIDs	Non-steroid anti-inflammatory drugs
NT	Non-transgenic mice
ON	Overnight
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline with Tween
PECAM-1	Platelet endothelial cell adhesion molecule
PlGF	Placental growth factor
Pgp	P-glycoprotein
PSEN1	Presenilin 1
PSEN2	Presenilin 2
RAGE	Receptor for advanced glycation end products
RBP	Retinol binding protein
RT	Room temperature
SEM	Standard error of the mean
SP	Senile plaque
TEM	Transmission electron microscopy

TNF-α	Tumor necrosis factor α
TTR+/+	Non-transgenic mice (with both copies of TTR gene)
TTR+/-	Non-transgenic heterozygous mice (with one copy of TTR gene)
TTR-/-	Non-transgenic knockout mice (witch no copy of TTR gene)
T4	Thyroxine
VEGF	Vascular endothelial growth factor
WT	Wild-type

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1. GENERAL INTRODUCTION

1.1 Angiogenesis

1.1.1 Overview

The vascular system is an important element during the organism development, ensuring the proper nutrient and oxygen distribution for the formation, maintenance and regeneration of multiple organs and systems.

The formation of new blood vessels may occur through one of two pathways, namely vasculogenesis or angiogenesis. During embryonic development, vasculogenesis occurs through the differentiation and proliferation of angioblasts in endothelial cells (EC), then joining to form primitive blood vessels (vascular plexus). Further expansion of the later occurs by the process of angiogenesis (or neovascularization) which refers to the formation of new vessels by splitting or branching the pre-existing ones [1].

Just as vasculogenesis, angiogenesis starts during embryonic development, although it can also occur in the course of adulthood in specific physiological situations such as adaptation to physical exercise, wound healing or pregnancy, in a tightly regulated manner. Nevertheless, in pathological conditions such as cancer, cerebral arteriovenous malformations or ischemic disease, angiogenesis is considerably less regulated [2].

A balance of pro-angiogenic and anti-angiogenic factors, which are mostly growth factors and cytokines, control both induction and suppression of angiogenesis, coordinating all steps during this complex process. These molecules are produced by ECs, stromal cells, leukocytes or simply by fragmentation of proteins from the extracellular matrix. Angiogenesis is then induced if the expression of pro-angiogenic factors is increased or the expression of anti-angiogenic factors is decreased [3].

The major inducer of angiogenesis is hypoxia, which leads ECs to express hypoxia-inducible factor-1 (HIF-1). This factor is capable of up-regulating several angiogenic molecules, namely the pro-angiogenic vascular endothelial growth factor (VEGF) that modulates EC migration and proliferation (Figure 1, 1), the degradation of the basement membrane (BM) and vessel permeability [4,5].

The growth of a new vessel starts with a stimulus that leads to local breakdown of the BM and detachment of mural cells (pericytes or vascular smooth muscle cells), forming a breach in the BM. The proteolytic breakdown of the BM is mediated by proteases under tight regulation, releasing matrix-bound angiogenic growth factors and cytokines that stimulate vessel sprouting [1].

Attracted by pro-angiogenic signals, the nascent vessel starts to sprout through the BM. This involves EC proliferation, migration, tube formation and the establishment of connections with other

reminiscent vessels [1] (Figure 1, 2). Plasma proteins extravasated from leaky vessels serve as a temporary extracellular matrix, allowing the migration of ECs, through the interaction between integrins and extracellular matrix proteins [5]. This phenomenon requires the specification of ECs into a leading tip cell and numerous stalk cells, which exhibit different morphologies and functional properties (Figure 1, 3). The tip cell is a single and highly polarized EC that primarily migrates but exhibits minimal proliferation. This cell possesses filopodia that extend towards the angiogenic stimulus, properly guiding the new vessel branch in a certain direction, preventing unorganized vessel growth. In contrast, the proliferative stalk cells feature fewer filopodia but form tubes and the nascent vascular lumen (Figure 1, 4) [1,6]. The interaction of the filopodia with filopodia of neighbouring tip cells allows the fusion of nascent vessels, leading to the addition of a new vessel circuit, in a process named anastomosis (Figure 1, 5) [6].

In order to become fully functional, with the suppression of proliferation, the new branch needs to go through a maturation process. This requires the recruitment of mural cells and deposition of the extracellular matrix, allowing vessel stabilization (Figure 1,6). During this phase, remodelling and tissue-specific differentiation of vessel occur to allow adaptation of vascular patterning to local nutritional and oxygen requirements. Remodelling involves the growth of new vessels and the regression of others, establishment of directional flow, changes in the diameter of vessel lumens and vascular wall thickening [1,7].

The sprouting process remains active until pro-angiogenic signals decline, and quiescence is restored [1].

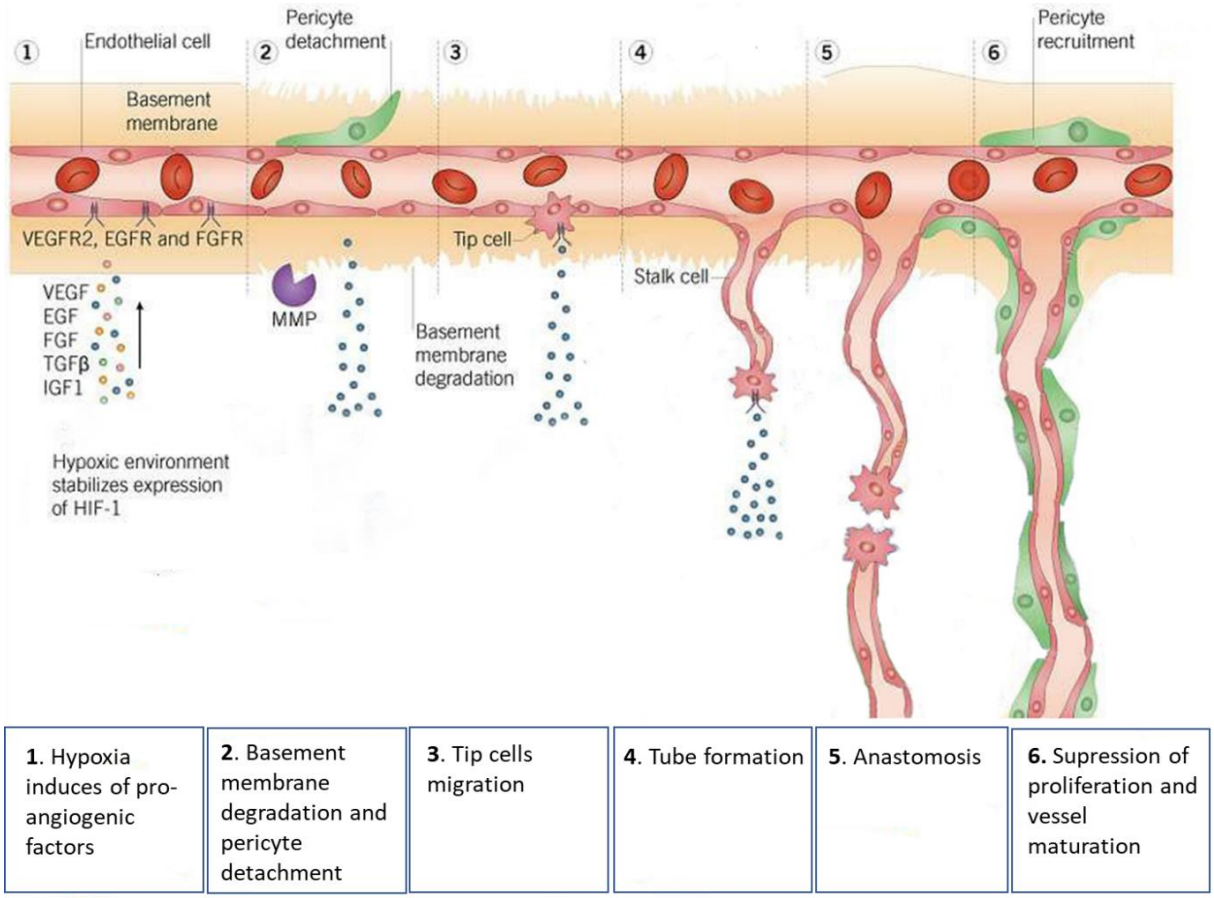

Figure 1. Schematic representation of angiogenesis, highlighting the main steps. Adapted from reference [8].

1.1.1.1 Endothelial cells and the basement membrane

The lumen inside of all the blood vessels on the body is covered by ECs. These cells exhibit multiple phenotypes depending on the local needs of different tissues. Several functions are appointed to these cells such as mediating the transport of molecules through the vessel wall, regulating blood pressure and forming new vessels [9].

The ECs are in contact with a basement membrane, a highly organized extracellular matrix, mainly composed of collagen IV (that accounts for about 50% of all BM components), laminins, nidogen and perlecan. Nevertheless, its molecular composition varies from tissue to tissue and with age, since the BM goes through continuous remodeling. Other molecules associated with the BM such as growth factors and cytokines are commonly found entrapped in this protein network. The BM has a close relationship with ECs providing them with the necessary physical support to maintain vessel organization. Beside, the BM modulates ECs functions by adhesion and through signaling pathways, coordinating its proliferation, migration, tube formation, survival and extracellular matrix components production, evidencing its essential role in angiogenesis [10].

1.1.1.2 Growth factors and cytokines

Angiogenesis is tightly regulated by several molecules, highlighting growth factors and cytokines. These molecules are produced by multiple cells, including the ECs. One of them is VEGF, the master regulator of angiogenesis, that engages in every step of the process and activates the expression of several other angiogenic molecules. Other factors also play important roles in promoting angiogenesis, particularly bFGF (basic fibroblast growth factor), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), tumor necrosis α (TNF-α) and interleukin-8 (IL-8) [11].

1.1.2 Brain vascularization

The brain comprises a highly specialized vasculature to meet the requirements of this highly metabolic active tissue as well as to protect neurons from toxic metabolites and xenobiotics. Beyond the specialized endothelium, brain vessels feature higher coverage of pericytes than vessels from other tissues and possess astrocyte end-feet that cover all vasculature [12]. The angiogenic process in the brain is similar to that reported above, however, in the maturation phase, the vessel acquires the features of the brain barrier – the blood-brain barrier (BBB). In particular, the Wnt signalling pathway has been described as the key regulator of brain angiogenesis and BBB formation. Wtn glycoproteins are secreted by neural progenitor cells during brain angiogenesis. Wnt signalling regulates the induction of brain angiogenesis and the expression of glucose transporter 1 (GLUT-1), the major glucose transporter at the BBB, and claudin-3, a tight junction component [3].

1.1.2.1 The blood-brain barrier

The BBB is formed by a specialized CNS EC layer, essential for its function and integrity. The abluminal surface is surrounded by a layer of pericytes in microvessels or smooth muscle in the other vessels. In the vicinity, support cells such as astrocytes and neurons play essential roles in BBB development and maintenance. The association of all these cells with the BM is called the neurovascular unit (NVU) [13] (Figure 2). The NVU components work together to regulate vessel permeability, nutrient uptake and toxin removal. Any failure on one of the components can contribute to BBB dysfunction [14].

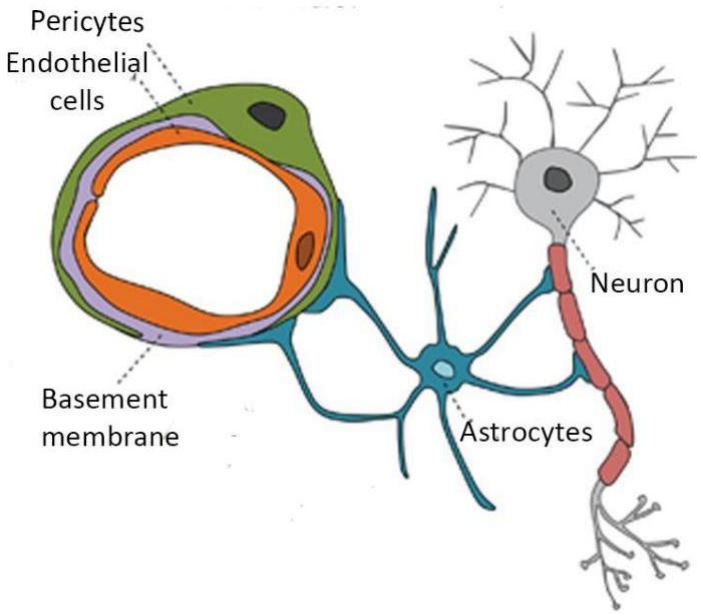

Figure 2. Overview of the NVU, showing the close spatial relationship between ECs, pericytes, astrocytes, neurons and BM. Adapted from reference [15].

The microvessels from the brain have two BMs: an inner endothelial BM between ECs and pericytes, and an outer astroglial BM between pericytes and astrocytic endfeet [16]. Endothelial BM is secreted by pericytes and ECs whereas astroglial BM is secreted by astrocytes. Usually, these two membranes are very close and appear to be only one when observed through an optical microscope, so it is commonly referred to as a single membrane [17]. Regarding the composition of the BM, perlecan, laminin and collagen IV, are the main constituents of this protein layer [18].

The BM is deeply involved in the formation and maintenance of the BBB, also acting as a barrier, retarding the movement of molecules into brain tissue [19]. Cells that constitute the NVU, anchor to the BM, using it as a physical support and allowing its correct positioning. This is mediated by cell adhesion molecules that bind to BM components [16]. Furthermore, the BM is strategically placed between ECs and astrocytes, being capable of initiating several signalling processes in the NVU [20,21].

1.1.3 Angiogenesis in disease

The balance of pro- and anti-angiogenic factors to maintain vascular homeostasis may be disrupted in pathological conditions and acute vascular injury.

However, in response to a stimulus, angiogenesis may be reactivated since ECs retain their ability to proliferate and migrate. During adulthood, generally, vasculature remains quiescent except for situations such as wound healing or pregnancy. In certain cases, this stimulus becomes excessive, leading to pathologies that include cancer, psoriasis, arthritis, blindness, obesity and asthma (Table 1) [22].

Table 1. Diseases characterized or caused by abnormal or excessive angiogenesis.

Organ	Disease in mice or humans
Several organs	Cancer (activation of oncogenes; loss of tumor suppressors); infectious diseases (pathogens express angiogenic genes, induce angiogenic programs or transform ECs); autoimmune disorders (activation of mast cells and other leukocytes)
Blood vessels	Vascular malformation; cavernous hemangioma; atherosclerosis; transplant arteriopathy
Adipose tissue	Obesity (angiogenesis induced by fatty diet)
Skin	Psoriasis, warts, allergic dermatitis, scar keloids, pyogenic granulomas, blistering disease, Kaposi sarcoma in AIDS patients
Eye	Persistent hyperplastic vitreous syndrome; diabetic retinopathy; retinopathy of prematurity; choroidal neovascularization
Lung	Primary pulmonary hypertension; asthma; nasal polyps
Intestines	Inflammatory bowel and periodontal disease, ascites, peritoneal adhesions
Reproductive system	Endometriosis, uterine bleeding, ovarian cysts, ovarian hyperstimulation
Bone, joints	Arthritis, synovitis, osteomyelitis, osteophyte formation

Adapted from reference [22].

Conversely, in certain situations, the angiogenic signal is insufficient causing EC dysfunction, vessel malformation or regression, or even preventing revascularization. Heart and brain ischemia, hypertension, pre-eclampsia, respiratory distress, osteoporosis and neurodegeneration are recognized for insufficient angiogenesis or vessel regression (Table 2) [22]. In the case of Alzheimer's Disease (AD), a neurodegenerative disease that will be here further explained and discussed, the reports are not consensual and other works describe that in AD there is the promotion of angiogenesis that results in concomitant BBB disruption and vessel leakiness [23]

Table 2. Diseases characterized or caused by insufficient angiogenesis or vessel regression

Organ	Disease in mice or humans	Angiogenic mechanism
Nervous system	Alzheimer's Disease	Vasoconstriction, microvascular degeneration and cerebral angiopathy due to EC toxicity by Aß
	Amyotrophic lateral sclerosis; diabetic neuropathy	Impaired perfusion and neuroprotection, causing motoneuron or axon degeneration due to insufficient VEGF production
	Stroke	Correlation of survival with angiogenesis in brain; stroke due arteriopathy
Blood vessels	Atherosclerosis	Characterized by impaired collateral vessel development
	Hypertension	Microvessel rarefaction due to impaired vasodilation or angiogenesis
	Diabetes	Characterized by impaired collateral growth and angiogenesis in ischemic limbs, but enhanced retinal neovascularization secondary to pericyte dropout
	Restenosis	Impaired re-endothelialization after arterial injury at old age
Gastrointestinal	Gastric or oral ulcerations	Delayed healing due to production of angiogenesis inhibitors by pathogens
	Chron disease	Characterized by mucosal ischemia
Skin	Hair loss	Retarded hair growth by angiogenesis inhibitors
	Skin purpura, telangiectasia venous lake formation	Age-dependent reduction of vessel number and maturation due to EC telomere shortening
Reproductive system	Pre-eclampsia	EC dysfunction resulting in organ failure, system thrombosis and hypertension
Lung	Neonatal respiratory distress	Insufficient lung maturation and surfactant production in premature mice
	Pulmonary fibrosis, emphysema	Alveolar EC apoptosis upon VEGF inhibition
Bone	Osteoporosis, impaired bone fracture healing.	Impaired bone formation due to age dependent bone healing decline of VEGF- driven angiogenesis; angiogenesis inhibitors prevent fracture healing

Adapted from reference [22].

1.2 Alzheimer's Disease

1.2.1 General description

By 2015, more than 48 million people worldwide were affected by dementia. Most of the cases are related to AD, accounting for 60-80% of all cases. Age is the main risk factor for developing this pathology [24].

AD is a neurodegenerative disease clinically characterized by the loss of cognition together with a decrease in brain volume [25,26]. This cerebral atrophy is associated with loss of neuronal death and loss

of synapses, particularly in the hippocampus, neocortex and other subcortical regions [27]. In these cerebral regions, there is an accumulation of extracellular senile plaques (SP) and intracellular neurofibrillary tangles (NFT), composed by aggregates of the amyloid-β peptide (Aβ) and hyperphosphorylated Tau protein, respectively. These aggregates are considered the key hallmarks of AD [27–29].

For the purpose of this thesis, only the senile plaque and its main constituent, the Aβ, thought to be the causative agent of AD, will be further approached in the following sections.

1.2.2 Amyloid-β peptide and senile plaques

Aβ is the main component of SP in AD. This peptide has approximately 4 kiloDalton (kDa) and is formed upon the sequential cleavage of the β-amyloid protein precursor (APP) by β- and γ- secretases (Figure 3). The peptide is normally present in its soluble form in biological fluids, such as blood and cerebrospinal fluid (CSF) [30]. Its constitutive formation and the normal presence in the brain seem to indicate a normal physiological role that was not uncovered, at least until now.

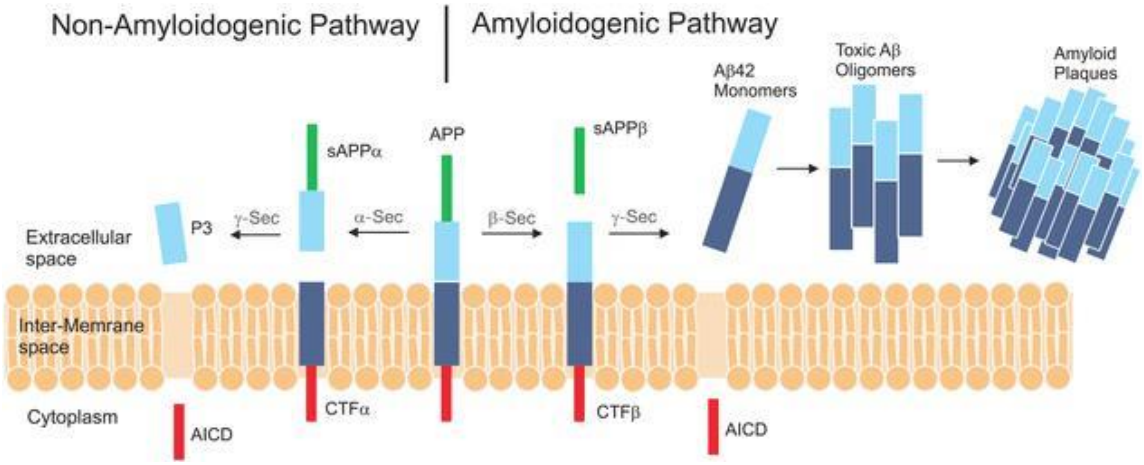

Figure 3. Representation of APP processing by non-amyloidogenic and amyloidogenic pathways. Aβ peptide is formed via the amyloidogenic pathway. Reprinted from reference [31].

In SP, Aβ species of several lengths (37 to 43 residues) are present. However, the monomers of $A\beta_{40}$ and $A\beta_{42}$, remarkable for their toxicity and aggregation capacity, are in a larger quantity [32,33]. Nevertheless, beyond the fact that $A\beta_{42}$ peptide is formed to a lesser extent under physiological conditions, it is more susceptible to aggregation than $A\beta_{40}$ owning to its two additional hydrophobic residues [34].

Due to the great tendency of the Aβ to form aggregates, it easily deposits in the form of insoluble fibrils. These structures are formed by the aggregation of 15-20 Aβ monomers, giving rise to oligomers [35]. Oligomers join to form protofibrils, that aggregate to produce fibrils [36] (Figure 4). Fibrils have a cross-β

structure, with individual β-strands oriented perpendicular to the fibril axis [37]. Thus, intermediates of this process are always present at SP, in a mixture of Aβ monomer, oligomers and fibrils [38].

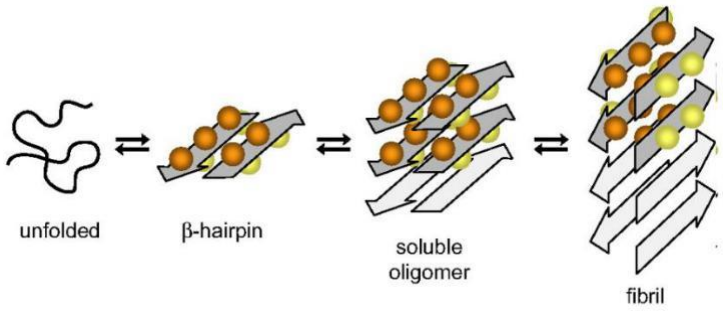

Figure 4. Schematic model of Aβ fibril formation. Reprinted from reference [39].

1.2.3 Amyloid cascade hypothesis and molecular bases of AD

Since Aβ was found in its soluble form in healthy brains but in fibrillar aggregates in AD brains, a hypothesis was formulated establishing that these insoluble aggregates triggered all pathological events characteristic of the disease [29], the amyloid cascade hypothesis [40]. In resume, it postulates that an abnormal extracellular increase of Aβ levels in the brain can lead to Aβ aggregation into fibrils, triggering the formation of SP. These aggregates lead to neuronal dysfunction and death, as well as the formation of NFT resulting in the dementia characteristic of AD patients (Figure 5).

Over the years the hypothesis has undergone some changes since levels of soluble Aβ have a better correlation with cognitive impairment of patients with AD than SP density [41]. Thus, the intermediates of fibrils formation have been suggested as primary neurotoxic species in AD, particularly those that are in the oligomeric form [42].

Under physiological conditions, there is a balance in the brain between the production and clearance of Aβ. However, with ageing and over pathological conditions, equilibrium is not maintained resulting in the accumulation of Aβ and leading to SP formation. Moreover, in the AD brain, it is possible to detect an increase in the $A\beta_{42}/A\beta_{40}$ ratio, suggesting that the increased level of $A\beta_{42}$ is crucial for the pathology [43,44]. Due to its greater hydrophobicity, $A\beta_{42}$ is likely to provide the core for Aβ assembly into oligomers and fibrils [34].

Depending on the age of onset, AD can be classified into two types: early-onset or late-onset AD, EOAD and LOAD, respectively. The EOAD cases are rare, comprising 5-10% of the total AD patients, and are usually familial cases (FAD) [45], in an autosomal dominant inheritance manner and with the onset of symptoms appearing around 30-50 years. In most cases, patients have mutations in one of the encoding

genes for amyloid-β precursor protein (APP), Presenilin 1 (PSEN1) or Presenilin 2 (PSEN2), the two latter are part of the γ-secretase complex [46–48]. Hundreds of mutations associated with these genes have been reported with at least one of them present in 80% of patients with this type of AD [49]. These mutations can lead to scenarios such as the increased formation of $A\beta_{42}$ compared to $A\beta_{40}$, increased expression of APP that may enhance Aβ synthesis or even increased capacity of Aβ to aggregate [50–52].

LOAD accounts for most of the cases of AD (more than 95%) and disease symptoms usually appear after the age of 65, with no autosomal-dominant inheritance nor any other type of transmission, as far it's known [53]. Thus, it is believed that a conjunction of genetic and environmental risk factors defines the propensity to this pathology. The presence of the ε4 allele of the apolipoprotein E (ApoE) gene is recognized as a major risk factor in LOAD [54]. The ApoE gene can appear as three polymorphic alleles - ε2, ε3, and ε4 - with a world population frequency of 8.4%, 77.9%, and 13.7%, respectively. However, in AD, ε4 allele frequency reaches 40 % of the world population [55,56]. ApoE is the main cholesterol transporter in the brain and may also play a role in the degradation of Aβ. Interestingly, ApoE3 has been shown to promote more Aβ degradation than ApoE4, due to the lower affinity of ApoE3 for the Aβ peptide [57,58]. Furthermore, ApoE4 appears to promote Aβ oligomerization [59].

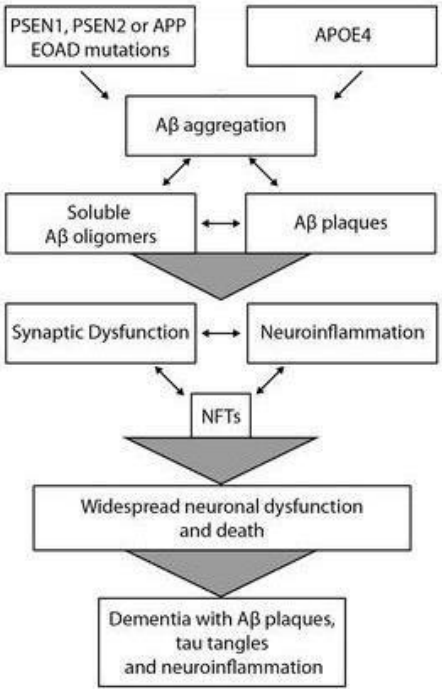

Figure 5. Schematic overview of the amyloid cascade hypothesis. The accumulation of Aβ species trigger a cascade of events involving the formation of the NFT leading to neuronal dysfunction and death. Adapted from reference [60].

1.2.4 Mechanisms of Aβ clearance at the BBB

The dysfunction of the mechanisms of Aβ clearance is decisive for the accumulation in the brain. In a healthy brain, the production of Aβ is counterbalanced by processes such as proteolytic degradation, cell-mediated degradation, perivascular drainage and efflux across the BBB into the peripheral circulation (Figure 6) [13]. The transport of Aβ peptide out of the brain is performed mostly by receptor-mediated transcytosis. The low-density lipoprotein receptor-related protein 1 (LRP-1) is the main responsible for the transport of Aβ through the BBB into the systemic circulation. LRP-1 is expressed on the abluminal side of the vessel and although it acts normally as an endocytic receptor, it has been reported to mediate Aβ transcytosis across the BBB [61,62]. In plasma, the soluble LRP is the main Aβ binding protein and can sequester 70% of $A\beta_{40}$ and about 90% of $A\beta_{42}$ [63,64]. These complexes are transported to the liver that conducts systemic elimination of Aβ [65]. Other receptors such as low-density lipoprotein receptor-related protein 2 (LRP-2) and P-glycoprotein (P-gp) also appear to mediate the efflux of Aβ across the BBB to plasma [66,67].

These receptors bind Aβ directly or to its carrier protein, transporting the peptide across the EC. Alpha-2-Macroglobulin (α2M), ApoE (different isoforms have a different impact in Aβ clearance), apolipoprotein J (ApoJ) and transthyretin (TTR) are examples of proteins that bind and carry Aβ for elimination [68].

However, some carriers at BBB endothelium mediate the influx of Aβ from blood to brain. In particular, the receptor for advanced glycation end product (RAGE) has been demonstrated to be the main Aβ transporter from the systemic circulation into the brain [69].

The balance between efflux and influx of Aβ to the brain is then critical to the maintenance of its homeostasis. The disruption of this balance may be the key factor in the initiation of the pathology promoted by Aβ.

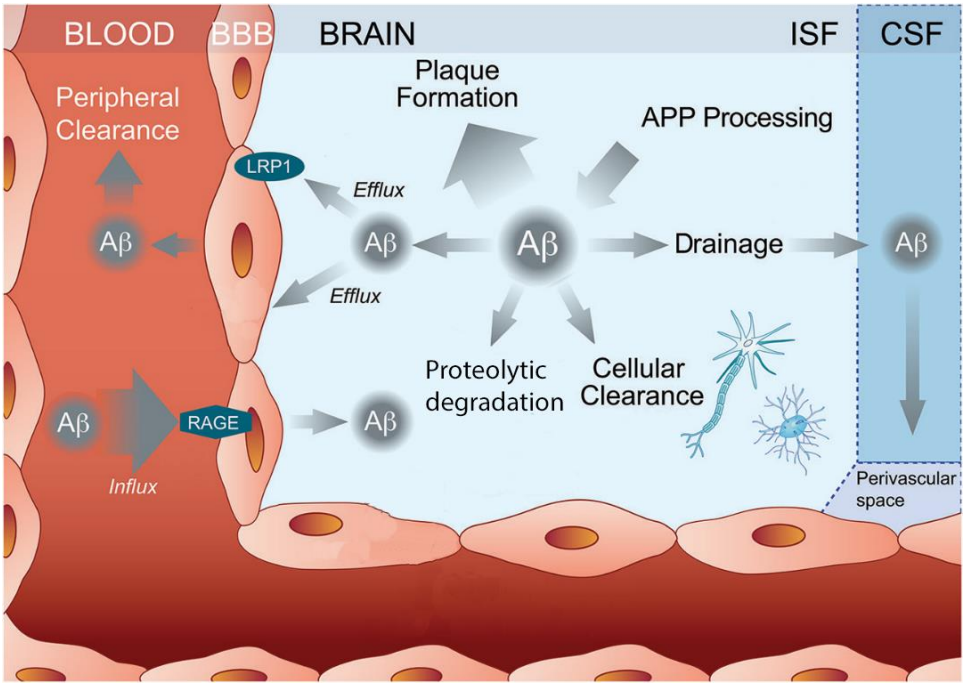

Figure 6. Schematic picture of the clearance and degradative mechanisms of Aβ. Adapted from reference [70].

1.2.5 BBB dysfunction in AD

AD patients undergo several neurovascular changes at different levels. Decreased expression of LRP-1 and P-gp, as well as RAGE up-regulation, are mechanisms reported to be changed in AD patients, leading to Aβ accumulation in the brain [71,72].

In addition to defective clearance mechanisms, an increase in endothelial pinocytosis, a decrease in the number of mitochondria, a decrease in GLUT-1 and a loss of tight/adherents junctions are features detected in AD [73]. These BBB changes may promote the entry of unwanted and potentially neurotoxic, molecules as well as disrupt the normal transport of nutrients and other essential molecules for proper neuronal functioning.

Finally, the reduction of the capillary density is also characteristic of the AD brains [74]. This is due to an aberrant angiogenesis with premature pruning of capillary networks. This defective angiogenesis may be caused by a lack of angiogenic stimuli and unresponsive endothelium [75]. Although other authors describe increased vascular density in AD [23], they all agree that, in AD, angiogenesis has pathological characteristics.

1.2.6 Cerebral amyloid angiopathy and remodelling of the neurovasculature

As concluded by the description of this disorder, AD is the consequence of ageing-related multifactorial biological dysfunction. In recent years it has been reported that ageing-related vascular

alterations are key features of neurodegenerative processes in AD, playing a central role in the onset and progression of the pathology. Despite this, the importance of vascular changes in AD is not fully understood as they are also present in normal ageing.

The involvement of vascular alterations in AD was evidenced in a large autopsy-based study that included more than 6000 brain analysis. It was shown that almost 80% of the analyzed subjects with AD and no signs of vascular dementia had brain vascular pathology. In these brains, it was possible to found stroke-related injuries (lacunes, microinfarcts, large infarcts and hemorrhages), atherosclerosis, arteriolosclerosis and cerebral amyloid angiopathy (CAA) [76]. Another study with 1171 participants was performed to understand the chronological order of late-onset AD progression, through the analysis of brain images, plasma and CSF biomarkers. The results suggest that brain vascular dysregulation is the earliest and strongest factor during the disease progression and is followed by Aβ deposition, glucose metabolism dysregulation, functional impairment and gray matter atrophy, in this order [77]. Other studies have suggested that both ageing and AD are associated with a decrease in resting perfusion, vascular tone, stimulus induced hemodynamic responses and angiogenesis [68].

The major insult to the blood vessels in AD is the aforementioned CAA, characterized by the deposition of Aβ in the brain vessels. This pathology has a prevalence of 10-30% in elderly people and more than 80% in patients with AD [78,79]. CAA likely develops as a result of the ineffective perivascular clearance of Aβ. This event occurs in the tunica media and adventitia of arteries and arterioles as well as in the BM of leptomeninges and cerebral cortex capillaries. Its deposition occurs in fibrils form, causing EC dysfunction [80].

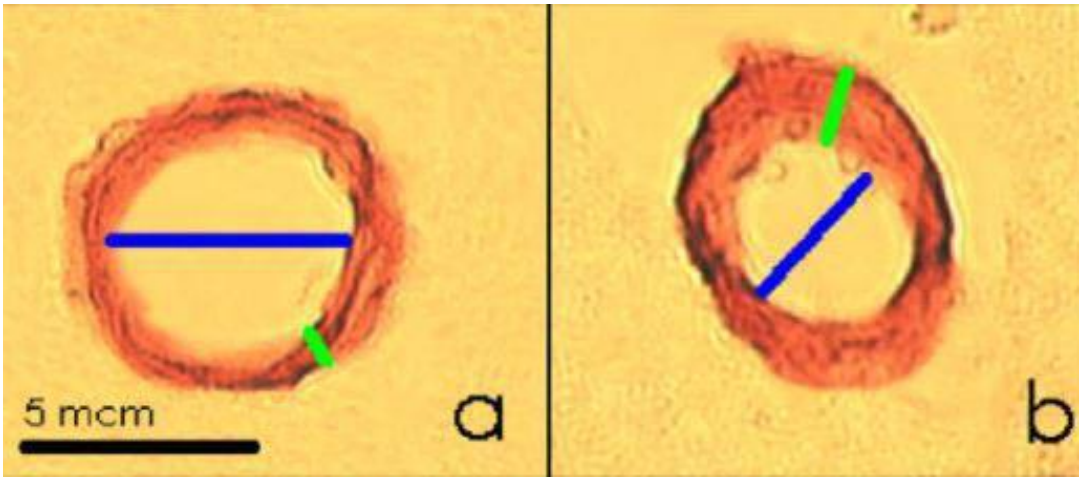

Figure 7. Analysis of brain microvessel inner diameter (blue line) and wall thickness (green line) in (a) young patients; (b) old patients. Old people show thickening of the microvessel wall and larger vessel lumen. Reprinted from reference [81].

The BM becomes thicker in AD [82] and ageing [81] (Figure 7). Since the increase in BM density occurs before Aβ deposition, it is speculated that the thick BM functions as a physical barrier to the Aβ clearance across the BBB [83]. Some studies have related BM thickening with increased collagen IV content, in both AD and normal ageing [81,84]. Besides collagen IV, perlecan and fibronectin were found to be more abundant in vessels of patients with AD [85]. It has been reported that collagen IV, laminin and entactin can promote the disassemble and disruption of Aβ fibrils, in a dose-dependent manner [86,87]. The matrix metalloproteases may also contribute to this phenomenon since they regulate the metabolism of the BM, and changes in the activity of these proteases can contribute to change the abundance of BM components [88]. As in angiogenesis, this BM thickening may be regulated by a complex interaction between ECs, angiogenic factors and extracellular matrix proteins. Nevertheless, Aβ may have a key role in this remodelling.

1.3 Transthyretin

1.3.1 TTR structure and synthesis

TTR is a 55 kDa homotetramer, consisting of four identical subunits of 127 amino acids each [89]. Regarding the secondary structure, each monomer is comprised of 8 antiparallel β-strands, which are arranged in 2 β-sheets (an inner sheet of strands D, A, G, and H, and an outer sheet of strands C, B, E and F) forming a β-barrel and a small α-helix between E and F strands. With the twofold axis of symmetry, it can structurally be considered a dimer of dimers. The dimerization is achieved through the interaction of the strands H and F of each monomer. These dimers combine to form the tetramer through a connection between the AB loop of one monomer and the H strand of the other. At the interface of the dimers emerges a hydrophobic channel that can accommodate ligands such as thyroxine (T4) [90].

TTR is mainly synthesized by the liver (90% of plasma TTR in humans) [91] and the choroid plexus, in the brain [92]. In new-borns, TTR levels in plasma are lower when compared with adults, starting to decline after about 50 years of age. In humans, the biological half-life of TTR is about 2-3 days, and the main sites of TTR degradation are the liver, muscle and skin. Besides this, no evidence has been found of TTR degradation in the nervous system [93].

1.3.2 TTR mutations associated with disease

TTR tetrameric structure and its stability are extremely important for TTR functions. TTR instability, caused by mutations or by other factors, leads to dissociation of the tetramer into altered monomers [94] which are prone to aggregate and deposit as amyloid fibrils, leading to toxicity and death.

Mutations in TTR are associated with hereditary forms of TTR amyloidosis and the most frequent TTR variant is TTR V30M that causes ATTRV30M amyloidosis, also known as familial amyloid polyneuropathy (FAP)) [95]. FAP is an autosomal dominant neurodegenerative disorder with its largest focus in Northern Portugal. FAP is characterized by the extracellular deposition of amyloid fibrils from mutated TTR in peripheral nerves [96]. More than 100 mutations in TTR with amyloidogenic potential have already been described [97]. The V30M TTR variant is the most common amyloidogenic form in the pathology [98], but it is also important to highlight the highly clinically aggressive L55P TTR mutant [99]. Another TTR mutation very frequent, Val122Ile leads to cardiac amyloidosis [100]. Conversely, the T119M mutant is recognized as having a protective role against the disease [101]. Nevertheless, a non-hereditary form of TTR amyloidosis also exists and is mainly associated with cardiomyopathy of aged people, over 80 years old, and the amyloid deposits are composed of WT protein [102].

It should be noted that the formation of TTR aggregates is rarely observed in the brain, which may be due to the stabilization provided by T4. TTR transports 80% of T4 in the brain as opposed to plasma TTR which only carries 15%, making the TTR in the plasma more sensitive to destabilization [103,104].

TTR instability also leads to accelerated clearance, resulting in lower levels of the protein [105], which in turn prevents TTR from fully exerting its functions, namely its neuroprotection roles, that will be further detailed ahead in this thesis.

1.3.3 Roles of TTR

1.3.3.1 TTR as a transporter protein

This protein is essentially recognized by its roles as a carrier in plasma and CSF, but in recent years it has been associated with other functions.

Plasma retinol (vitamin A) is bound to retinol-binding protein (RBP), which in turn forms a complex with TTR, avoiding the loss of the low molecular mass RBP (21 kDa) by glomerular filtration in the kidneys [106,107]. TTR has four binding sites for the RBP on the TTR surface. However, only one RBP is connected due to the steric hindrance and limiting concentration of this ligand [108].

TTR is also involved in the distribution of T4, carrying the thyroid hormone in blood to the tissues [103]. Of note, this interaction with T4 is independent of that with RBP [106]. Even though TTR contains two binding sites for T4 in the hydrophobic channel of the dimer-dimer interface, it normally only carries one ligand due to the existing negative cooperativity [90,109].

Figure 8 displays the structure of TTR alone (A), or in complex with RBP containing retinol (B) or with T4 (C).

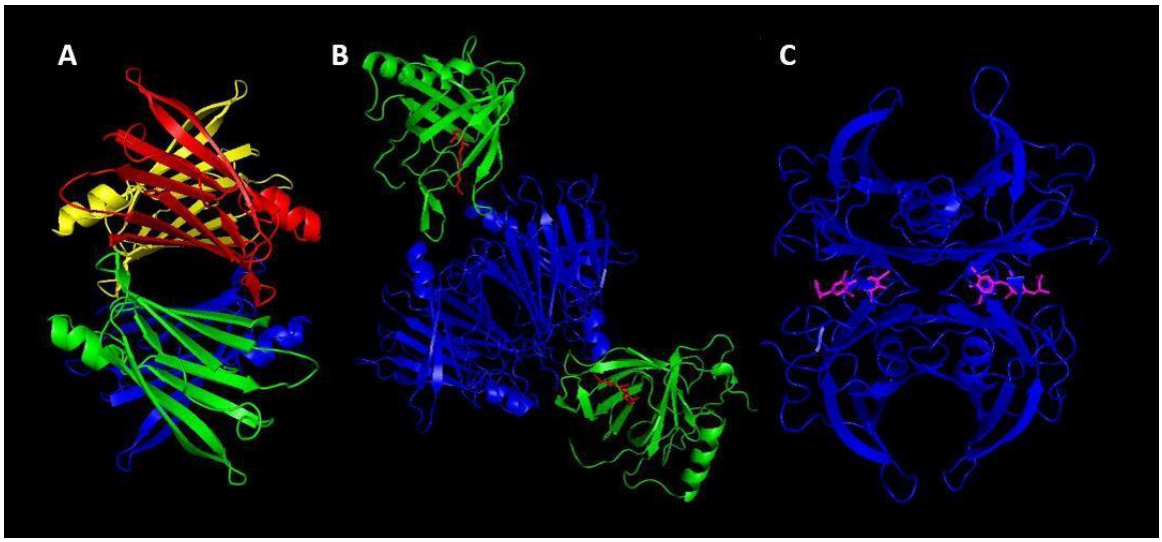

Figure 8. Structure of TTR in complex with its main ligands. (A) Tetramer (PDB ID: 1DVQ); (B) TTR (blue) in complex with RBP (green) containing retinol (red) (PDB ID: 1RBL); (C) TTR (blue) in complex with T4 (pink) (PDB ID:1IE4). Images captured from PyMOL software.

1.3.4 Newly described TTR functions

1.3.4.1 Proteolytic activity

In recent years, a novel cryptic metalloprotease activity of TTR was described. Liz and colleagues demonstrated that TTR was able to cleave the C-terminal domain of Apolipoprotein A-1 (ApoA-I), interfering in lipid metabolism since ApoA-I loses its ability to promote cholesterol efflux [110,111]. Besides this, TTR-cleaved ApoA-I is more prone to form aggregated particles and fibrils than full-length Apo-I. This suggest that TTR may have a role in the development of atherosclerosis by decreasing cholesterol efflux and increasing the ApoA-I amyloidogenic potential [111].

The same authors demonstrated *in vitro* that TTR is also capable of cleave the neuropeptide Y (NPY) and possibly contributing to the increased NPY levels observed in TTR knockout (KO) mice [112,113].

Moreover, TTR proved to be able to cleave Aβ peptide, *in vitro*, generating fragments less amyloidogenic and less toxic than the full-length peptide [114,115].

1.3.4.2 Neuroprotective Functions

Several reports attribute neuroprotective functions to TTR, in different contexts. TTR was found to be involved in the modulation of depressive behaviour by increasing levels of NPY, known as an antidepressant neurotransmitter, in TTR KO animals [113]. The effect of TTR in NPY has already been referred above, concerning the proteolytic activity of TTR [112].

Besides, TTR KO mice present memory impairment compared with WT animals [116], demonstrating that the absence of TTR accelerates cognitive deficits usually associated with ageing.

TTR was also associated with nerve regeneration [117]. Fleming and co-workers revealed that TTR acts as an enhancer of nerve regeneration, following the observation that TTR KO mice have decreased ability to regenerate from a sciatic crushed nerve. Moreover, *in vitro* experiments showed that neurite outgrowth and extension were decreased in the absence of TTR. Revealing the mechanisms behind TTR action in nerve regeneration, the authors demonstrated that the absence of TTR leads to impaired retrograde transport. Besides this, the effect of TTR in neurite outgrowth and nerve regeneration was found to be mediated by megalin-dependent internalization [118]. Curiously, cells grown with proteolytically inactive TTR showed decreased neurite length suggesting that the proteolytic activity of TTR is important for the capacity of TTR to promote neurite outgrowth [112].

Assessing the role of TTR in ischemia, Santos and colleagues proposed that in a compromised heat-shock response, CSF TTR contributes to control neuronal cell death, edema and inflammation [119] via megalin [120].

The involvement of TTR against Aβ aggregation and toxicity in the context of AD is now well established and will be explored in this thesis, further ahead.

1.3.4.3 TTR as a gene modulator

Some years ago, TTR was characterized to be involved in the regulation of insulin-like growth factor I (IGF-I) receptor in the hippocampus by increasing IGF-I receptor levels [121]. Subsequently, it was found that TTR has a synergistic action with IGF-I over the IGF-I receptor, activating the Akt, implying a role of TTR in cell survival [122].

Alemi and colleagues showed that LRP-1 is increased in TTR+/+ mice compared to TTR-/- littermates in the brain and liver. *In vitro*, LRP-1 expression was higher in the immortalized human

cerebral microvascular endothelial cell line, hCMEC/D3 cells, and in the human liver cancer cell line, HepG2 cells, in the presence of TTR, suggesting modulation of this receptor by TTR [123]. This is important since LRP-1 regulates the Aβ clearance from the brain to the periphery in the BBB [61], as already discussed. Besides this, LRP-1 is one of the main Aβ receptors in the liver [65].

Very recently, Zhou and colleagues studied the interplay between TTR and δ subunit of $GABA_A$ receptors ($GABA_A$-Rs) [124]. $GABA_A$-RS are activated by GABA (γ-aminobutyric acid) and play an important role in brain development and synchronization of neural network activity. These receptors are located on both synaptic and extrasynaptic membranes to mediate phasic and tonic inhibition (short- and long-lasting inhibition, respectively) of neuronal activity. δ-$GABA_A$-Rs are one of the major subtypes of extrasynaptic $GABA_A$-Rs, mediating tonic inhibition [125]. In this work TTR KO mice revealed a significant decrease of the surface expression of the δ-$GABA_A$-Rs in cerebellar granule neurons suggesting that TTR interacts with δ-$GABA_A$-Rs regulating their expression and function, especially by modulating the tonic and phasic inhibition, since the increase of the extrasynaptic δ-$GABA_A$-Rs in the cortical neurons results in a decrease of synaptic GABA transmission. This work also highlights that this newly discovered function is dependent on the presence of the TTR tetramer and cannot be achieved by the monomeric species.

1.3.5 TTR and angiogenesis

Previous work has implicated TTR in the angiogenesis process, but the pathways involved are far from being unravelled. A study has compared the expression of Ang-2 and VEGF receptor 1 and 2, that are recognized as pro-angiogenic genes, using WT TTR and the most common TTR mutation, V30M, in human umbilical vein endothelial cells (HUVECs). They show that the V30M mutation downregulated the expression of the genes under study, as compared to WT TTR. Beyond this, WT TTR promoted more cell migration and survival relatively to the TTR variant [126]. However, this study did not analyze the effect of WT TTR *per se*. Instead, the work studied a possible toxic effect of the V30M variant, concluding that it impacts in angiogenesis, by inducing apoptosis. In a different study, Shao and colleagues, trying to understand the effects of TTR in diabetic retinopathy (DR), compared several ECs functions related to angiogenesis in human retinal microvascular endothelial cells (hRECs) cultured with TTR in natural and simulated DR environments (hyperglycemia and hypoxia). It was observed that TTR led to the repression of cell proliferation, migration and tube formation in a DR environment [127]. Conversely, in a low glucose environment, the angiogenesis-related features above mentioned were improved by TTR. Taking into account that TTR may be increased in Diabetes type II, this protein might have inhibitory functions under hyperglycaemic conditions from the early stage of DR [128].

TTR has also been consistently referred in tumour research as a differentially expressed gene or protein, however, the processes in which TTR is involved were not precisely addressed. Recently, it was reported that TTR concentration was highly increased in human serum of lung cancer patients. TTR concentration was also enhanced in the serum, bronchoalveolar lavage fluid, alveolar epithelial cells, and alveolar myeloid cells of a lung tumour mouse model. Moreover, TTR was shown to regulate several ECs functions as migration, permeability and tube formation. TTR also played a role in immune cell differentiation as well as in stimulating cancer cell proliferation and growth.[129].

However, TTR potential in angiogenesis has never been addressed *in vivo* and possible participation of TTR in brain angiogenesis and vascular alterations has never been addressed, neither *in vivo* nor *in vitro*.

1.3.6 TTR as a neuroprotector in AD

TTR has been reported as a neuroprotector in AD with several evidences supporting this claim. Comparative analysis of TTR evidenced a significant decrease in the concentration of this protein, not only in the CSF but also in plasma [130–132] in AD patients, compared to age-matched healthy subjects. Notwithstanding, this decrease is also associated with ageing [133]. Additionally, TTR levels in the CSF and plasma are negatively correlated with the abundance of SP and with disease stage, respectively [131,134].

Moreover, it was reported that AD TTR-hemizygous mice have increased Aβ production and deposition, compared to AD hemizygous TTR littermates, whereas overexpressing human WT TTR in an AD mouse model decreases neuropathology and Aβ deposition [135].

Prior to these studies, Stein and Johnson evaluated gene expression profiles in the hippocampus and cerebellum of 6-month-old AD mice, which overexpressed a mutant form of APP yielding high Aβ levels in the brain. Results have indicated that, compared with age-matched controls, levels of TTR and other important proteins for certain survival pathways were upraised and could be the explanation for the slow progression and lack of some important hallmarks of AD pathology as plaque deposition, that characterizes this model [136]. The authors determined that the expression of these protective genes were up-regulated by sAPPα [137], one fragment resulting from the proteolytic cleavage of APP. Earlier, it was demonstrated that transgenic mice overexpressing APP Swedish and presenilin mutations (APPSwe/PS1ΔE9) exhibited lower Aβ levels in the cortex and hippocampus when exposed to an "enriched environment" relatively to animals maintained in standard house conditions [138].Interestingly, DNA microarray studies revealed an increase in the TTR expression levels in mice maintained in "enriched" conditions.

1.3.6.1 The earlier observations

Some decades ago, Wisniewski and co-workers verified that $A\beta_{40}$ fibril formation was inhibited by the presence of human CSF [139]. At the time, the event was associated with the sequestration of Aβ by extracellular proteins circulating in CSF, such as ApoE and ApoJ [140–142]. Among several proteins determined as Aβ carriers, Schwarzman and colleagues concluded that TTR was the main protein able to sequester the peptide in the CSF. This was the first evidence implicating TTR in AD, specifically in Aβ transport and its clearance [143]. The authors proposed the hypothesis of sequestration as a possible explanation for the peptide aggregation and consequent progression of AD. This hypothesis suggested that certain extracellular proteins sequester normally produced Aβ, thereby preventing amyloid formation and its toxicity. Amyloid formation would occur when sequestration failed [143–145], which could be related either with an Aβ overproduction, a reduction in the levels of sequestering proteins, inability of those proteins to interact with the peptide, deficient clearance mechanisms or a combination of all the events above described.

Although contradictory literature reporting on the localization of TTR within senile plaques has been reported, some authors detected the presence of TTR within plaques [137,146].

In another early report, cultured brain vascular smooth muscle cells from dogs and AD patients, which were characterized by the presence of intracytoplasmatic granules containing Aβ, were incubated with TTR. Results demonstrated that TTR was able to prevent Aβ accumulation as verified by the reduction of Thioflavin S staining [147]. In a different study, Tsuzuki and colleagues have found $A\beta_{40}$ and $A\beta_{42}$ mostly in proximal and distal renal epithelial cells from kidneys obtained from patients that did not suffer from neurologic pathologies. TTR was co-localized in proximal tubules with $A\beta_{42}$ [148].

Link and colleagues produced a *Caenorhabditis elegans* highly expressing $A\beta_{42}$ in muscle cells that triggered amyloid deposition positive for Thioflavin S staining. To determine whether TTR expression would inhibit the peptide aggregation, double transgenic strains for Aβ and TTR were generated and results suggested a reduction in the number of positive Thioflavin S deposits [149].

1.3.6.2 TTR/Aβ interaction and importance of TTR stability in AD

Schwarzman and collaborators identified TTR as the major Aβ-binding protein in the brain and hypothesized that this protein binds to and sequesters Aβ peptide, therefore avoiding the formation of aggregates. Costa and colleagues demonstrated that TTR is able to interact with monomeric soluble Aβ, as well as with oligomers and fibrils, with similar binding affinities. Besides neutralizing Aβ toxicity, TTR inhibited oligomerization and promoted Aβ fibril disruption [150].

The implication of TTR mutations in Aβ interaction was also investigated. Schwarzman and co-workers synthesized approximately 40 recombinants amyloidogenic/not amyloidogenic mutated TTRs and, by applying several *in vitro* techniques, they were able to demonstrate that TTR variants bound differently to Aβ. TTR variants E42G and L55P, strong amyloidogenic variants, were the only completely failing the binding to the peptide[143]. Costa and co-workers also used amyloidogenic and non-amyloidogenic TTR variants and obtained the following profile, regarding the strength of the interaction with soluble Aβ peptide: T1119M>WT>V30M≥Y78F>L55P, indicating that the higher the amyloidogenic potential of TTR, the weaker the interaction with the peptide is. Since the amyloidogenic potential of TTR correlates negatively with its tetrameric stability, those results also indicate that the lower the stability of TTR, the weaker the TTR/Aβ interaction is [114]. Further, the L55P TTR variant also could not prevent Aβ toxicity in culture, confirming that one of the aims of the TTR/Aβ interaction is to prevent the noxious effects of the peptide[151]. These observations also imply that the TTR species needed for this interaction is the tetramer. Somehow supporting this idea is the report that genetic stabilization of TTR through the presence of the T119M allele which renders a more stable tetramer, has been associated with decreased risk of cerebrovascular disease and with increased life expectancy in the general population [152], further demonstrating the importance of the TTR tetramer in the protein biological activity.

This tetramer stability is important since TTR variants with more tendency to dissociate into altered monomers show a fast clearance, resulting in lower levels [105]. Although no TTR mutations have been found in AD patients, destabilization of TTR may result from other events such as medium acidification or interaction with components such as metal ions and carbohydrates [153–156]. Thus, it is hypothesized that, in AD, TTR is destabilized and consequently is more quickly cleared. Ribeiro and colleagues, described a 15% decreased in the ability of TTR from AD patients to carry T4, as compared to healthy controls, suggesting TTR instability [131]. More recently, the status of TTR was measured in plasmas from AD patients, and results showed a significant decreased in the ratio folded/monomeric TTR [156]. This loss of structure, decreases Aβ affinity and therefore prevents TTR from exerting its protective effect in AD.

Fortunately, small compounds such as iododiflunisal (IDIF) and resveratrol have been reported to bind and stabilize TTR, occupying the same binding sites as T4, the two funnel-shaped pockets at the dimer-dimer interface of the protein. Both IDIF and resveratrol show negative cooperativity however resveratrol has a preference for one of the binding sites for T4 [157,158]. This interaction and consequent TTR stabilization increase the strength of the binding between Aβ and TTR [151]. Figure 9 displays the structure of TTR in complex with resveratrol and T4 (A), or in complex with IDIF (B).

In vivo tests have shown that the administration of IDIF to an AD mouse model resulted in decreased Aβ levels and deposition in the brain, and in amelioration of the cognitive deficits, characteristic of this AD model [159]. Very recently, it has been shown that the formation of TTR-IDIF complexes enhances BBB permeability of both IDIF and TTR [160], adding more interest in the use of the TTR stabilization as a therapeutic strategy in AD. *In vitro*, resveratrol was able to decrease Aβ levels [161] and its administration to an AD mouse model resulted in increased TTR expression in mice brain [162] and in plasma [163]. Also, instable TTR pre-incubated with IDIF or resveratrol, induced increased Aβ internalization by primary cultures of hepatocytes and by brain cells [156].

However, in the context of TTR neuroprotection in AD, there is no consensus on the TTR species that provides the best effect and thus, whether TTR instability demonstrated in AD [151,156] is a negative consequence or is a protective mechanism to deal with the excess of Aβ, is not known, yet. In fact, and in opposition to what was described so far, some authors reported that a monomeric TTR mutant binds strongly to Aβ and is stronger at avoiding its aggregation and toxicity [164–166]. As a consequence, TTR interaction with Aβ aggregates leads to the formation of amorphous deposits, that are probably more inert than fibrillar deposits [167]. Despite this, *in vivo* tetrameric TTR concentration is one thousand-fold higher than that of the monomer [165]. Thus, it's likely that the tetrameric form is the major intervenor in avoiding Aβ aggregation and toxicity.

Data on the structural nature of the TTR/Aβ interaction was initially obtained from computer-assisted modelling [143]. The model predicts the existence of an Aβ binding domain on the surface of each TTR monomer. Other studies by the same group reported that residues 30-60, especially the 38-42 region of TTR are the key structure of the binding domain to Aβ [144,168]. Further studies identified TTR residues in the strand A and G, in or near the hydrophobic T4-binding site, as involved in TTR/Aβ interaction. The involvement of EF helix and loop, which is highly-solvent exposed and prone to conformational changes, was also detected [164,169]. Conversely, another study has shown that binding of the Aβ peptide is likely to occur on the surface of the protein, although the EF helix and the loop may play an important role in this interaction. More, the stabilizer IDIF was capable of binding to TTR at the central pocket without affecting Aβ peptide binding [170].

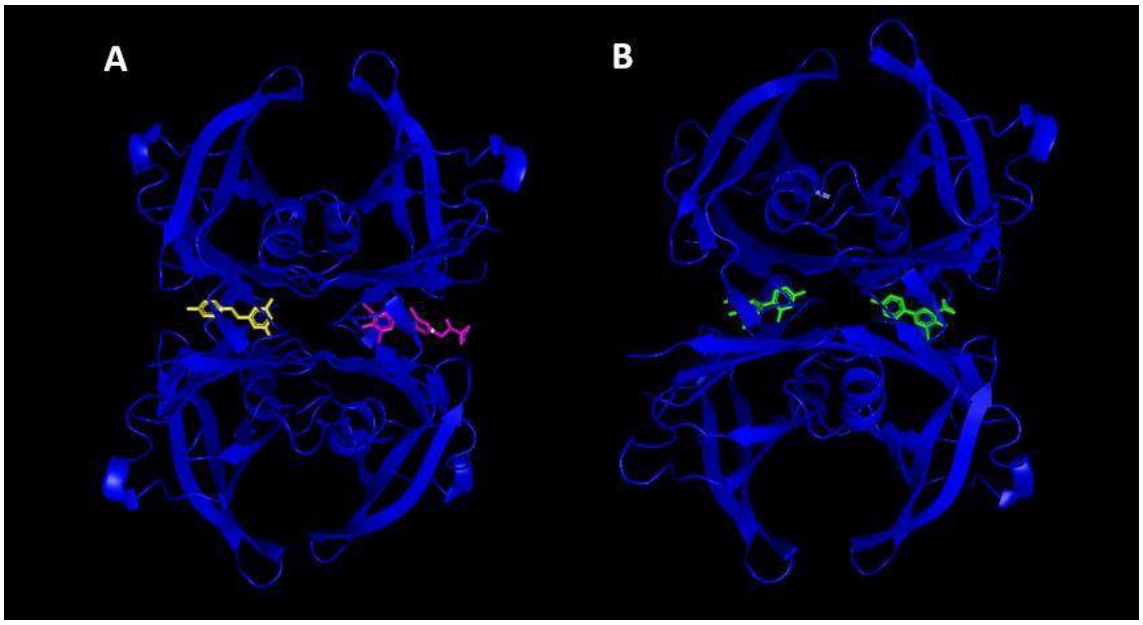

Figure 9. Structure of TTR in complex with stabilizers. (A) Tetramer (blue) in complex resveratrol (yellow) and T4 (pink) (PDB ID: 5CR1); (B) TTR in complex with IDIF (PDB ID:1Y1D). Images captured from PyMOL software.

1.3.6.3 Mechanisms Involved in TTR neuroprotection in AD

In vitro and *in vivo* studies suggest a direct interaction between TTR and Aβ peptide, as already discussed, resulting in the inhibition of Aβ aggregation, fibril disruption or both, and thus, in this view, TTR is an Aβ carrier.

However, other mechanisms involving TTR in AD have been disclosed. As already mentioned, the proteolytically active form of TTR is able to cleave Aβ peptides as well as their aggregates, *in vitro*. The resulting peptides show lower or no amyloidogenic potential, as compared to the full-length peptide $A\beta_{42}$ [114,115]. Nevertheless, it is necessary to determine whether this proteolytic activity is relevant for neuroprotection *in vivo*.

More recently and following the observation that AD mice with only one copy of the TTR gene have higher Aβ brain levels than AD animals with the two TTR copies, Alemi and colleagues studied the involvement of TTR in Aβ brain efflux at the BBB. The authors showed that TTR promotes Aβ internalization and efflux in hCMEC/D3 cells, when added to the brain compartment of the transwell, together with Aβ. However, TTR added to the blood side, did not impact in the efflux of the peptide, suggesting that TTR interacts directly with Aβ at the BBB. It was also suggested that the TTR/Aβ complex uses LRP-1 to cross the barrier, since TTR modulates its levels both in the brain and liver [123]. TTR stability was shown to be crucial in this TTR function [156].

Although choroid plexus is commonly credited as the only source of TTR in the CNS, it has been reported, in recent years, that hippocampal and cortical neurons are able to produce the protein [171,172]. A neuroblastoma cell line overexpressing the APP intracellular domain (AICD), evidenced that TTR is epigenetically regulated by this fragment. As a result, there is an increase in TTR expression accompanied by a decrease in Aβ levels [172]. Additionally, it is reported that TTR binds to the CTFβ fragment, a product of AICD fragment, preventing γ-secretase from cleaving and releasing Aβ peptide. This binding reduces levels of Aβ secretion, thus suggesting that TTR regulates the metabolism of APP [173]. Another work, described that the expression of TTR in the hippocampus, in primary murine hippocampal neurons and SH-SY5Y neuroblastoma cell line is significantly enhanced by the heat shock factor 1 (HSF1) [174]

Taking all this evidence into consideration, this project aimed at further unravelling the neuroprotective of TTR by investigating its involvement in angiogenesis and in vascular alterations, under physiologic and pathologic conditions.

2. RESEARCH PROJECT

Objectives

This project aimed at investigating the potential role of TTR in angiogenesis *in vivo* and at identifying other angiogenic molecules affected by TTR. The project also aimed at understanding TTR participation in vascular alterations, namely at the level of the BM, both in physiologic and in pathologic conditions. Finally, a longitudinal *in vivo* study, aiming at evaluating the effect of TTR stabilizers in its neuroprotective function in AD, was also covered in this project.

The experiments performed had the following purposes:

1. To investigate the angiogenic potential of TTR, under physiologic conditions, by:

1.1. Using an *in vivo* model, the Chick Chorioallantoic Membrane (CAM) assay, to assess the angiogenic potential of TTR

1.2. Using the *in vitro* tube formation assay, with hCMEC/D3 cells (human brain endothelial cells) to identify the molecular pathways involved.

1.3. Ascertaining the effect of TTR genetic reduction in the BM of brain microvessels, by evaluating the thickness of the collagen IV layer in mice 3-month old, carrying either the 2 copies of the mouse TTR gene (TTR+/+) or only 1 copy (TTR+/-).

2. To investigate the participation of TTR in brain vascular alterations, using an AD mouse model, by:

2.1. Evaluating the thickness of the collagen type IV layer in AD transgenic mice 3-month old, with different TTR genetic backgrounds, AD/TTR+/+ and AD/TTR+/-,

2.2. Comparing the thickness of the collagen IV layer in TTR+/- and in AD/TTR+/-, thus evaluating if any alteration detected happens early, before the onset of AD-like disease in this AD model.

3. To investigate the relevance of TTR stabilization in collagen type IV in AD, by measuring collagen IV levels in brain vessels in AD/TTR+/- mice treated with resveratrol, a TTR stabilizer, and in control (non-treated) animals.

4. To determine if Aβ can trigger collagen IV overexpression and consequent thickening of the BM, by:

4.1. Studying the effect of different Aβ species (soluble, oligomeric and fibrillar) on collagen IV levels, in bEnd.3 (Immortalized mouse brain endothelial cell line) cells.

4.2. Investigating if TTR is able to abrogate the effect generated by Aβ peptide.

5. To evaluate the effect of IDIF and tolcapone administrated to AD/TTR+/- mice, by:

5.1 Assessing Aβ amyloid burden in the brain, after the *in vivo* longitudinal study by Positron Emission Tomography (PET).

2.1. Chapter I

Transthyretin as an angiogenic protein: *in vivo* studies and insights from an Alzheimer's Disease mouse model

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2.1.1 Summary

Angiogenesis is the formation of new blood vessels from the pre-existing vasculature. This process is regulated by pro- and anti-angiogenic factors and implies degradation of the BM, EC migration and proliferation and, vessel maturation. This last step compromises the remodeling and tissue-specific differentiation of vessels to allow adaptation to local nutritional and oxygen requirements. This mechanism is crucial during embryonic development and adulthood, as it is involved in processes such as wound healing. On the other hand, aberrant angiogenesis is part of the pathogenicity of diseases as cancer, blindness or AD.

BBB is essential to maintain the homeostasis of the central nervous system. However, in several disorders such as AD, this barrier is dysfunctional which seems to contribute to the accumulation of Aβ species in the brain, leading to the development of the pathology. The brain BM, besides the structural role, seems to regulate angiogenesis and features of BBB. In AD and, to a lesser extent, in older people, the BM is thicker, as also evidenced by an increase in the collagen IV layer. These alterations are thought to impair Aβ peptide brain efflux.

Several studies highlighted TTR as an important neuroprotective protein in AD, through the sequestering and cleavage of Aβ peptide, and by assisting its elimination across the BBB, promoting its clearance. Moreover, TTR also modulates the BBB by influencing the levels of the major Aβ receptor, the LRP1, leading to its increase. Furthermore, 7-month old AD transgenic mice carrying only one copy of the TTR gene (AD/TTR+/-) show increased thickness of the BM evaluated by the collagen IV levels, as compared to littermates with both copies of TTR (AD/TTR+/+), suggesting a role for TTR in angiogenesis and in vascular alterations. However, several evidences showed that TTR levels are decreased in AD due to diminished tetrameric stability, affecting its ability to bind to Aβ peptide. Despite this, some compounds have been shown to stabilize TTR, thus recovering the ability to interact with Aβ peptide and exert its neuroprotective effect.

Taking these evidence into account, this project aimed at assessing the involvement of TTR in brain vasculature, both in the physiological and pathological context. The angiogenic activity of TTR was studied using the *in vivo* chick embryo chorioallantoic membrane (CAM) assay. Quantitative analysis revealed a higher angiogenic response of TTR in relation to the negative control and comparable to the positive control. In vitro, using hCMEC/D3 brain human endothelial cells, grown under conditions of capillary-like-formation, TTR up-regulated the expression of IL-6, IL-8, VEGF and Ang-2, among 10 angiogenic molecules studied.

To understand the impact of TTR on BM, the levels of collagen IV were measured by immunofluorescence in brain microvessels of non-transgenic (non-AD) animals, 3-month old and, as expected, TTR+/- mice presented thicker collagen IV layer. Interestingly, comparison between AD and non-AD in the same TTR genetic background revealed that AD/TTR+/- mice presented collagen IV levels higher than TTR+/- animals, suggesting that TTR insufficiency has a greater impact in the context of disease, and might implicate Aβ in this event.

In fact, bEND.3 brain mouse endothelial cells showed increased collagen IV when incubated with Aβ oligomers or fibrils, as compared to control or cells incubated with soluble Aβ. Furthermore, the effect of Aβ oligomers on collagen IV production was counteracted by TTR, resulting in decreased expression of this basement membrane component.

Resveratrol, a TTR tetrameric stabilizer, administrated to AD/TTR+/- 7-10-month old mice did not impact in collagen IV levels, when compared with control non-treated animals, suggesting that, while TTR may have a direct involvement in collagen IV content, its stabilization is not important for this purpose.

Altogether, our results implicate TTR in angiogenesis and in vascular alterations occurring in AD. Future studies are necessary to unravel the specific molecular pathways involved and to determine if TTR participation is protective or it contributes to disease development and/or progression.

2.1.2 Material and Methods

2.1.2.1 Production and purification of human recombinant TTR

Human recombinant WT TTR was produced in a bacterial expression system using *Escherichia coli* BL21 [175] and purified as previously described [176].

In detail, E. coli BL21 were transformed with pET plasmid carrying WT TTR cDNA and were allowed to grow overnight (ON) in 15-ml starter cultures of Lubia-Bertani (LB) medium containing 50 $\mu g.mL^{-1}$ of ampicillin at 37°C and 180 rpm. One-liter cultures with LB containing ampicillin were inoculated with starter culture and incubated at 37°C and 180 rpm until reach an optical density of 0.4-0.5. Then, TTR expression was induced with 500 μM of Isopropyl β-D-1- thiogalactopyranoside (IPTG), and the culture was grown for 6 h at 37°C and 180 rpm. Then, the culture media was centrifugated at 4000 rpm for 10 min at 4°C and the pellet was resuspended in lysis buffer (1M Tris pH 7.5, 0.5 M Ethylenediamine tetraacetic acid (EDTA), Triton X-100, Phenylmethylsulfonyl fluoride (PMSF) and 10 $\mu g.mL^{-1}$ DNAse). Cells were disrupted by successively freezing-thawing the solution and by sonication, and then the solution centrifugated at 15000 rpm for 25 min at 4°C. The pellet containing cell debris was discarded and the supernatant was dialyzed ON in glycine-acetate at 4°C.

Regarding TTR purification, the dialyzed solution was passed through an ion exchange column of diethylaminoethyl (DEAE)-cellulose (Sigma). The column was washed with glycine-acetate buffer, pH=7.0, and the fraction containing TTR was eluted by increasing the ionic strength, using a solution of glycine-acetate/3% sodium chloride. The TTR fraction was dialyzed against water followed by a lyophilization.

TTR was finally isolated by preparative electrophoresis in a native Prosieve agarose (Lonza) gel. The band of the gel containing TTR was cut and eluted in 38 mM glycine and 5 mM Tris. TTR purity was analyzed by Blue Safe (NZYTech) staining and protein identification by Western Blot, described below. Protein concentration was determined by Bradford Method, using bovine serum albumin (BSA) as standard.

During this work, the lyophilized preparation obtained after ion-exchange chromatography was already available in the lab. Thus, only the following steps were performed.

2.1.2.2 Western blot for TTR

TTR obtained from the elution was boiled 5 min, loaded in a 15% polyacrylamide gel and then transferred to nitrocellulose membrane (Whatman Ge healthcare – Protan BA 83), using a wet system. The membrane was blocked with 5% milk in Phosphate-buffered saline (PBS) with Tween-20 (PBS-T) and then incubated for 1 h at room temperature (RT) with the primary antibody polyclonal rabbit antibody anti-human TTR (Dako, 1:2000) in 3% milk/PBS-T. After that, the membrane was washed and incubated with the secondary antibody anti-rabbit horseradish peroxidase (1:5000) in 3% milk/PBS-T for 1h. The blots were developed using ClarityTM Western ECL substrate (Bio-Rad), and TTR was detected and visualized using a chemiluminescence detection system (ChemiDoc, Bio-Rad).

2.1.2.3 Preparation of $A\beta_{42}$ species

Synthetic $A\beta_{42}$ (Bachem) was dissolved in hexafluoro-2-propanol (HFIP) (Sigma-Aldrich) and kept at RT over weekend. After, the HFIP was removed under a stream of nitrogen and the powder was dissolved in DMSO at 2 mM. From this stock, three different species of $A\beta_{42}$ were produced: soluble, oligomers and fibrils. $A\beta_{42}$ was diluted to 100 μM in Ham's F12 medium and then incubated at 4°C during two days for oligomer formation or at 37°C for seven days for fibril formation. Soluble $A\beta_{42}$ was obtained by diluting the peptide to 100 μM in Ham's F12 medium immediately before adding to cells. To confirm the presence of the different $A\beta_{42}$ species, samples were visualized by transmission electron microscopy (TEM).

2.1.2.4 Transmission Electron Microscopy

For visualization by TEM, a sample drop was absorbed to carbon-coated collodion film supported on 300-mesh copper grids, and negatively stained with 1% uranyl acetate. The grids were analyzed with a JEOL JEM-1400 transmission electron microscope equipped with an Orious Sc1000 digital camera.

2.1.2.5 Chick Chorioallantoic Membrane Assay

The CAM assay was performed by the "*In vivo* CAM assays Unit", at i3S.

Commercially available fertilized chick (*Gallus gallus*) eggs were horizontally incubated at 37°C, in a humidified atmosphere. On embryonic development day (EDD)3, a square window was opened in the shell after removal of 1.5–2 mL of albumen, to allow detachment of the developing CAM. The window was sealed with a transparent adhesive tape and eggs re-incubated. On EDD10, TTR (2 μM, 1μM, 500

nM), or TTR (1μM) pre-incubated with IDIF(10 μM) for 1 h at 37°C, PBS (vehicle, negative control) or bFGF (positive control) were placed on top of the CAM, into 3 mm silicone rings, under sterile conditions. Eggs were re-sealed and returned to the incubator for an additional 72 h. On EDD13, rings were removed, the CAM was excised from embryos and photographed *ex-ovo* under a stereoscope, using a 20× magnification (Olympus, SZX16 coupled with a DP71 camera). The number of new vessels (<20 μm diameter) growing radially towards the inoculation area was counted in a blind fashion manner. The experiment was repeated 3 times.

2.1.2.6 TTR immunohistochemistry

The presence of TTR in the CAM was evaluated by immunohistochemistry using 3 μm paraffin sections. After deparaffination and hydration in a modified alcohol series, endogenous peroxidase activity was inhibited with 3% hydrogen peroxide (H_2O_2) in PBS for 30 min. Following PBS washes, sections were blocked in blocking solution (4% fetal bovine serum (FBS) and 1% BSA) for 1 h at RT and then incubated with primary antibody polyclonal rabbit antibody anti-human TTR (Dako, 1:2000) in blocking solution ON at 4°C. After washing with PBS, sections were incubated with biotin-labeled anti-rabbit secondary antibody (1:200) in blocking solution for 45 min at RT. Sections were washed with PBS and incubated in Vectastain Elite ABC Reagent (Vector Laboratories), and then washed in PBS followed by development with diaminobenzidine (Sigma-Aldrich), followed by hematoxylin counterstaining. After dehydration, slides were coverslipped with Entellan (Merck & Co.). Stained sections were then viewed on an Olympus DP71 microscope.

2.1.2.7 hCMEC/D3 cell culture and tube formation assay

2.1.2.7.1 Cell culture

The immortalized human cerebral microvascular endothelial cell line, hCMEC/D3 (Tebu-Bio) is a well-characterized *in vitro* model of BBB. The hCMEC/D3 cells were used between passage 25 and 35. The cell line was cultured following the available data sheet. All culture flasks were coated with rat tail collagen type I solution (Sigma) at a concentration of 150 μL/mL and were incubated for 2 h at 37°C. Cells were cultured in EBM-2 medium (Lonza) containing 5% FBS (Gibco), 1% of penicillin-streptomycin (Lonza), 1.4 μM of hydrocortisone (Sigma-Aldrich), 5 μg/mL of ascorbic acid (Sigma-Aldrich), 1% of chemically defined lipid concentrate (Gibco), 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco) and 1 ng/mL of bFGF (Sigma-Aldrich).

2.1.2.7.2 Tube formation assay

Matrigel (Corning) was thawed at 4 °C and 50 μl were quickly added to each well of a 96-well plate and allowed to solidify for 30 min at 37°C. hCMEC/D3 cells, grown till 80-90% confluence, trypsinized and resuspended in conditioned media (1% FBS and bFGF absent) were seeded at a density of 1.5 × 10^4 cells per well in the presence or absence of bFGF (1 ng/ml) or with TTR at different concentrations (2 μM, 1 μM, 0.5 μM and 0.25 μM). Each condition was performed in triplicate and cells were incubated at 37°C with 5% CO_2. One image from each well was photographed at 9 h using the In Cell Analyzer 2000 (GE Healthcare) (magnification, ×10). The supernatants were collected, centrifuged at 14.000 rpm for 10 min and stored at -20°C.

2.1.2.8 Quantification of angiogenesis-related proteins

The angiogenesis-related proteins interleukin-6 (IL-6), IL-8, Ang-1, Ang-2, epidermal growth factor (EGF), bFGF, platelet endothelial cell adhesion molecule (PECAM-1), placental growth factor (PlGF), VEGF and tumor necrosis factor α (TNF-α) were quantified in the supernatants collected from hCMEC/D3 cells used for the tube formation assay, using LEGENDplex Human Angiogenesis Panel (BioLegend) bead-based immunoassay. The assay was performed according to the manufacturer's recommendations. Briefly, 10 different fluorescence-encoded beads were mixed with supernatants (diluted twofold) and were incubated with shaking for 2 h at RT. A cocktail of 10 different biotinylated detection antibodies was added following an incubation with shaking for 1 h at RT. Streptavidin-phycoerythrin was added, the samples were incubated for 30 min at RT.

Analysis was performed using a BD Accuri C6 (BD Biosciences) and LEGENDplex v8.0 analysis software (BioLegend). A standard curve was generated for all analytes from 2.4 pg/mL to 10,000 pg/mL.

2.1.2.9 Animals

Two mouse models were used in this work, an AD transgenic and non-transgenic (NT) mouse models, both established in different TTR genetic backgrounds.

The AD mouse model AβPPswe/PS1A246E/TTR was generated by crossing the AD mouse model AβPPswe/PS1A246E [177] (B6/C3H background) purchased from The Jackson laboratory with TTR-null mice (TTR-/-) (SV129 background) [178] as previously described [179]. F1 animals AβPPswe/TTR+/− and PS1A246E/TTR+/− were crossed to obtain AβPPswe/PS1A246E/ TTR+/+,

AβPPswe/PS1A246E/TTR+/−, AβPPswe/ PS1A246E/TTR−/− and non-transgenic controls TTR+/+, TTR+/−, and TTR−/−. The colony was maintained on a B6/C3H/SV129 genetic background. Hereafter, the AβPPswe/PS1A246E/TTR colony will be referred to as AD/TTR, and the different genotypes AβPPswe/PS1A246E/TTR+/+, AβPPswe/PS1A246E/TTR+/−, and AβPPswe/PS1A 246E/TTR−/− referred to as AD/TTR+/+, AD/ TTR+/−, and AD/TTR−/−, respectively.

Animals were housed in a controlled environment (12-h light/dark cycles, temperature between 22-24°C, humidity between 45–65% and 15-20 air changes/h), with freely available food and water. All the above experiments were approved by the Institute for Research and Innovation in Health Sciences (i3s) Animal Ethics Committee and in agreement with the animal ethics regulation from Directive 2010/63/EU.

In order to study the role of TTR in collagen IV deposition in AD, cohorts of littermates 3 months old female mice AD/TTR+/+, AD/TTR+/-, TTR+/+ and TTR+/- were used.

AD/TTR+/– female mice treated with resveratrol, as previously reported [163], were used to investigate the relevance of TTR stabilization in collagen type IV levels, in AD. AD/TTR+/- were divided into 2 groups: the control group, which received a normal diet, and the treated group, which received for 2 months a diet incorporating resveratrol - a TTR stabilizer. Mice were 5-8 months of age at the beginning of the experiment.

Tissues from the mice described were already available in the lab from previous studies, and thus, these animals were not handled during the current project.

2.1.2.10 Collagen IV Immunohistochemistry

Free-floating 30 μm-thick coronal brain sections of mice were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT, blocked with 5% BSA in PBS for 1h at RT and incubated with anti-collagen IV primary antibody (1:100) (Abcam) in 1% BSA in PBS ON at 4°C. Next, sections were washed with PBS and incubated with Alexa Fluor-568 donkey anti-rabbit IgG antibody for 1h at RT. All steps were performed with agitation. To remove tissue autofluorescence, sudan black B solution (0.3% sudan black B in 70% ethanol) was applied for 5 min at RT, followed by multiple washing steps with PBS at RT with agitation. The brain sections were dried for 20 min at RT mounted on 0.1% gelatin-coated slides with FluoroshieldTM with DAPI (Sigma-Aldrich). Sections were visualized and photographed using a Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axivision 4.9.1 software. A total of fifteen randomly selected vessels the cortex of each mouse was photographed at 100x magnification, and the ratio intensity/area was measured using the ImageJ software.

2.1.2.11 bEnd.3 cell culture and treatment with Aβ species and TTR

2.1.2.11.1 Cell Culture

The immortalized mouse brain endothelial cell line, bEnd.3, another *in vitro* model of BBB, was kindly provided by Dr. Teresa Summavielle (I3S, Porto), and cells were used between passages 25 and 30. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Gibco) and 1% Penicillin-Streptomycin (Lonza), in an incubator at 37°C in a humidified atmosphere and 5% of carbon dioxide (CO_2). Cell culture medium was changed every 2–3 days.

2.1.2.11.2 Treatment with Aβ species and TTR and effect on collagen IV levels

Cells were grown until reach 80% confluence, trypsinized and resuspended in conditioned media (2% FBS). 1×10^4 cells were seeded and left to grow on glass coverslips (Thermo Fisher Scientific) previously autoclaved. When 80% confluence was reached bEnd.3 cells were treated with different species of Aβ (10 μM: monomers, oligomers and fibrils) in culture media (2% FBS) for 24h. Alternatively, bEnd.3 cells were treated, for 24 h with WT TTR alone (2 μM) or previously co-incubated with Aβ in F12 media for 48 h at °C. Additionally, cells were incubated with TTR and Aβ oligomers, simultaneously, for another 24 h, preceded or not by an ON treatment with TTR. Each condition was performed in duplicate and cells were incubated at 37°C. Cells were analyzed for collagen IV expression by immunocytochemistry.

2.1.2.12 Collagen IV Immunocytochemistry

Cells were washed with PBS and fixed with methanol for 15 min at RT. Following fixation, cells were permeabilized with 0.25% Triton X-100 in PBS for 10 min at RT. Next, blocking was performed with 5% BSA in PBS for 1h, followed by an ON incubation with polyclonal rabbit antibody anti-mouse collagen IV (Abcam), at 4 °C. After washing with PBS, cells were incubated with the Alexa Fluor-568 goat anti-rabbit IgG antibody (Invitrogen 1:2000) for 1h at RT. Coverslips were mounted using Fluoroshield with DAPI (Sigma-Aldrich). Visualization and image capture (at 20x magnification) was done with the Zeiss Axio Imager Z1 microscope equipped with an Axiocam MR3.0 camera and Axivision 4.9.1 software. A total of twenty randomly fields was photographed in each condition and the intensity of signal per cell was measured using the ImageJ software.

2.1.2.13 Statistical Analysis

All quantitative data were expressed as mean ± standard error of the mean (SEM). Initially, data was assessed whether it followed a Gaussian distribution. In the cases of non-Gaussian distribution comparisons between two groups were made by Student t-test. When found to follow a Gaussian distribution, differences among conditions or groups were analyzed by one-way ANOVA with the appropriate post hoc pairwise tests for multiple comparisons tests. Differences in vessel number on CAM assay and on collagen IV levels in bEnd.3 cells treated with Aβ oligomers and TTR were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. In opposite, multiple comparisons in bEnd.3 cells treated with Aβ species were performed using Dunnett's post hoc test to assessment of differences between control and conditions tested. Expression of angiogenic related proteins was statistically evaluated again by one-way ANOVA but after with Sidak's post hoc test to compare differences between negative or positive control versus all other groups. P-values lower than 0.05 were considered statistically significant. Statistical analyses were carried out using Graphpad Prism 8 software for Windows.

2.1.3 Results

2.1.3.1 Production and Purification of WT TTR

During this thesis, some assays that will be presented were performed employing human TTR protein. However, the large amounts of protein required, and the associated cost is a major barrier to acquiring commercially available protein. For many years, a bacterial expression system (*E.Coli BL21*) transformed with a pET plasmid has been used by our lab to produce different human recombinant TTR variants. Regarding purification steps, in a first stage, ion-exchange chromatography is applied followed by lyophilization of the protein-containing fraction.

In the current project, the lyophilized preparation of WT TTR obtained from ion-exchange chromatography was already available in the lab. At first, the protein was subjected to a new purification step by preparative electrophoresis, in a native Prosieve agarose gel. The band containing the protein of interest was then excised from the agarose gel and eluted.

After protein purification, two different batches of TTR were analyzed (lanes 2 and 3, Figure 10A and Figure 10B), after resolved by denaturing SDS-PAGE. A different batch of human recombinant TTR, previously tested and validated in the lab, was also loaded in the gel serving as intern control to qualitatively compare protein size and purity (lane 4, Figure 10A and Figure 10B). The gel was either stained with blue safe dye, allowing to check the purity of fractions of the recombinant protein produced and whether the purified protein is of the expected size, or transferred into a nitrocellulose membrane to perform a western blot to ensure that it was, indeed, the protein of interest present there, and not another of similar molecular weight.

Blue safe staining and western blot results are evidenced in Figure 10A and Figure 10B, respectively. Concerning the Blue safe stained gel, it is shown that the two TTR purified batches (lane 2 and 3, Figure 10A) presented similar pattern to internal control: a stronger band appeared with a MW around 15 kDa, as deduced by its proximity to 17 kDa standard band (lane 1, Figure 10A), should be the TTR monomer; a much fainter band appeared with an approximate MW of 30 kDa, possibly corresponding to the TTR dimer. Apart from these 2 bands, no others were detected, indicating a highly pure and suitable for use product. Western blot analysis (Figure 10B) corroborated these results, by showing that the two previously detected bands corresponded to TTR. Once again, no other bands were found, excluding aggregation or degradation of the protein.

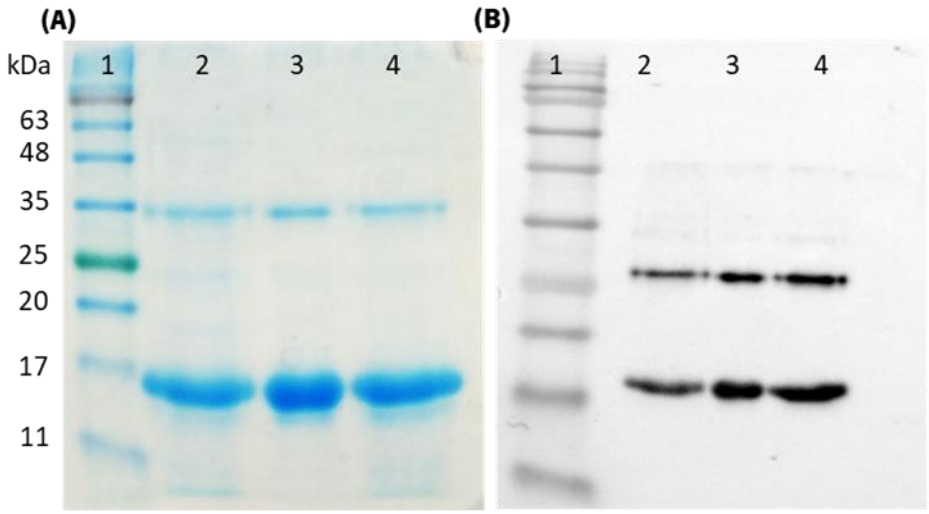

Figure 10. Verifying TTR purity and identity. SDS-PAGE gel was loaded with TTR isolated after production in a bacterial system. Half of the gel was stained with Blue safe **(A)** and the other counterpart was submitted to a western blot **(B)**. Lane 1 corresponds to the protein standards used, and the molecular weight of several of them are presented on the side (in kDa). Lane 2 and 3 represent the purified TTR from two different batches whereas lane 4 represents the internal control TTR.

2.1.3.2 *In vivo* assessment of TTR angiogenic activity

Some studies have associated TTR with angiogenic-related events. Angiogenesis *in vivo* studies are more appealing, however, the commonly used murine model is costly and features a slow vessel growth. An *in vivo* alternative to rodents is the CAM. The easy handling, low cost and high reproducibility are some of the advantages of this model. To further investigate a possible role of TTR in angiogenesis a CAM assay was performed by the "*In vivo* CAM assays Unit", at i3S. Different concentrations of TTR (2 µM, 1 µM and 0.5 µM) were incubated on the CAM, for 72h; bFGF (500 ng) and PBS were used as positive and negative controls, respectively. Figure 11B shows the quantification of the new vessels formed, clearly showing that TTR induced a significative higher angiogenic response than the negative control, as deduced by the higher number of detected new vessels (vessels with a diameter under 20 um). TTR angiogenic response was comparable to that of the positive control for any of the tested concentrations. No significant differences were found between the angiogenic responses of the 3 different concentrations of TTR tested.

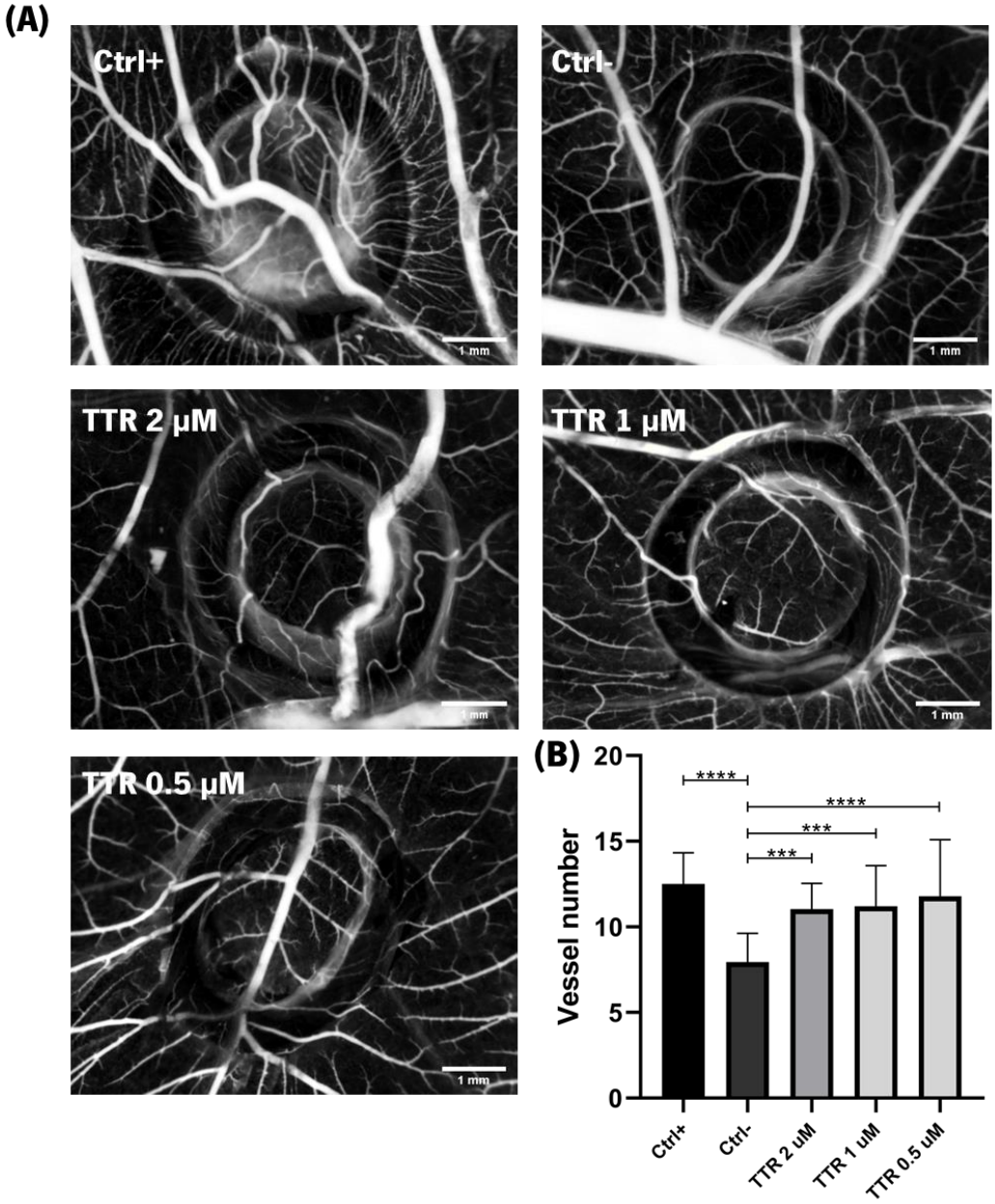

Figure 11. Assessing TTR angiogenic activity by *in vivo CAM* assay. (A) Representative images used in the quantitative analysis of the angiogenic response portraying the inoculation site, delimited by the ring mark, of bFGF (positive control (Ctrl+)), PBS (negative control (Ctrl-)), TTR 2 µM, TTR 1 µM and TTR 0.5 µM. **(B)** Quantification of the number of new vessels (<20 µm diameter) growing towards the inoculation site confirmed that TTR, for any of the tested concentrations, increased significantly the angiogenic response, compared to control negative. Furthermore, this effect is comparable to the positive control. Data are expressed as mean ± SEM. *** $p<0.001$; **** $p<0.0001$. Scale bar = 1 cm.

To confirm the presence of TTR in the membrane, an immunohistochemistry was performed. Representative images of the CAM inoculated with TTR (Figure 12A) and with buffer (Figure 12B) are

depicted here and show, in brown, the TTR located on the CAM outer surface. Furthermore, no evidence of internalization by vessels was detected.

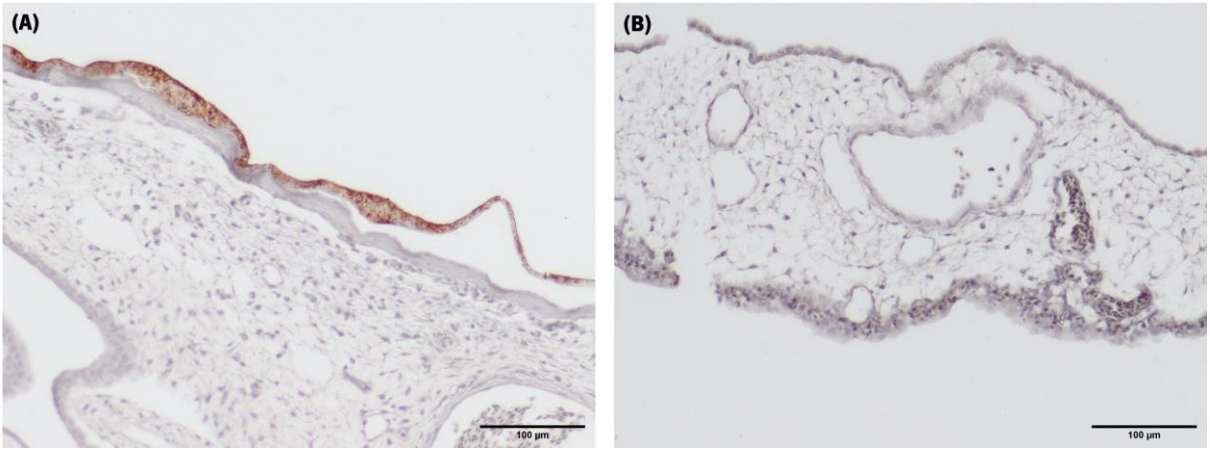

Figure 12. Confirming TTR presence on CAM. Image illustrates immunohistochemical staining of CAM treated with TTR **(A)** and buffer **(B)**. TTR is represented in brown and CAM is counterstained with hematoxylin. TTR is located on the outer surface of the membrane and there is no evidence of its internalization. Scale bar = 100 µm.

2.1.3.3 *In vitro* assessment of TTR angiogenic activity and pathways involved

Despite positive results on CAM assay, and although TTR is a highly conserved protein during the evolution of vertebrates, and studies into the evolution of TTR revealed the existence of a TTR homolog (TTR-like) in all kingdoms [180], specie-related differences need to be considered. Also, and since one of our main interests is angiogenesis in the brain and related alterations during disease, it is necessary to take into account the particular features of brain ECs.

The tube formation assay is a powerful *in vitro* test encompassing EC adhesion, migration, protease activity and tube formation (capillary-like structures), and can be performed with different ECs, including with hCMEC/D3 cell line, also very well known for constituting an *in vitro* BBB model. Figure 13A, evidences that hCMEC/D3 cells, when cultured in Matrigel matrix, hold the ability to form capillary-like structures. In contrast, cells grown under standard conditions, in rat tail collagen I coated plates, form typical monolayers.

Thus, and to further explore the angiogenic activity of TTR, hCMEC/D3 cells grown under tube formation-conditions (grown on Matrigel) were incubated with bFGF (1 ng/ml; positive control), with buffer (negative control), or in the presence of different concentrations of TTR (2 µM, 1 µM, 0.5 µM and 0.25 µM), for 9 h.

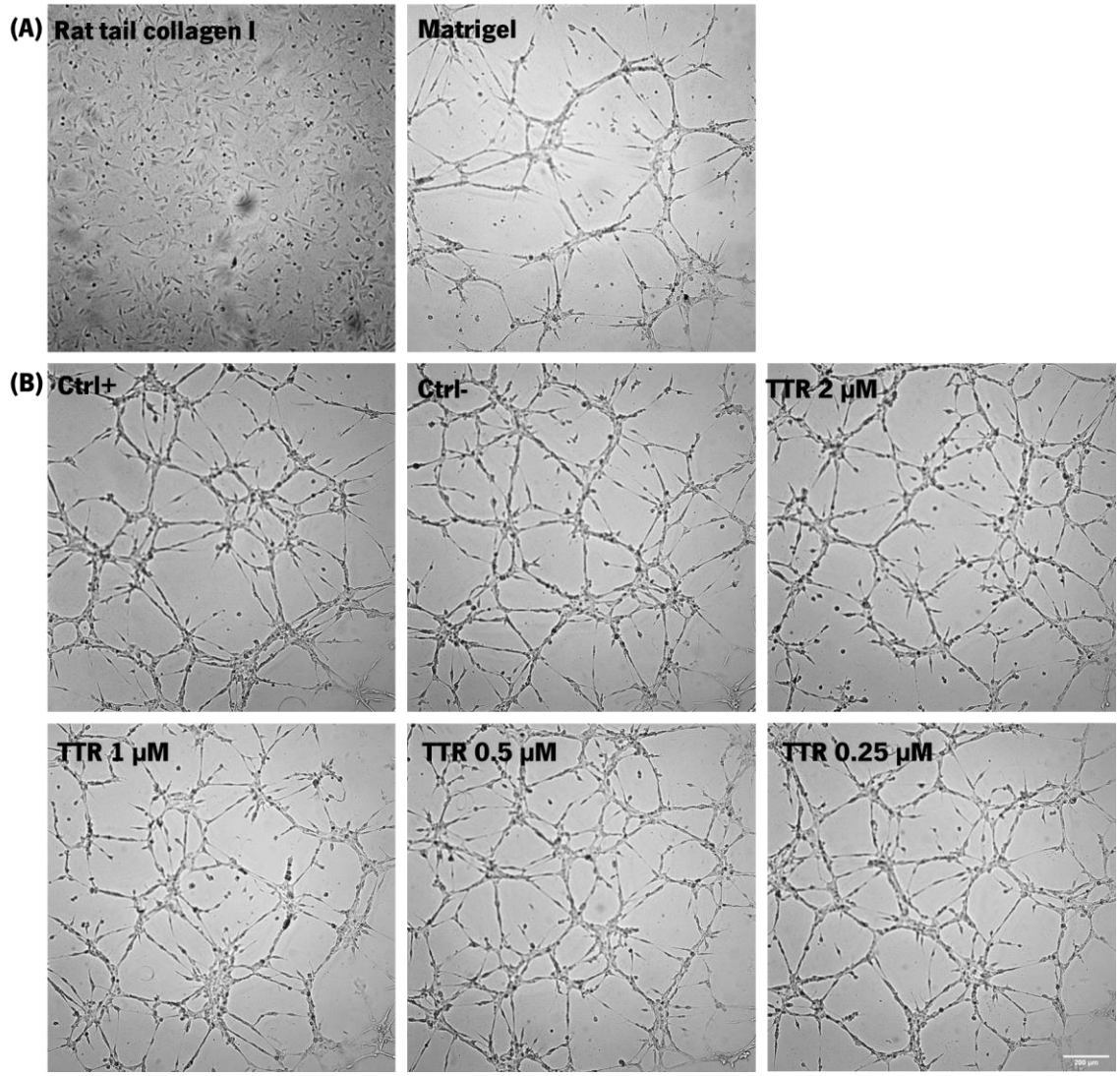

Figure 13. Assessing the angiogenic activity of TTR *in vitro* using the tube formation assay. (A) Representative images of hCMEC/D3 cells grown under standard conditions (left panel), i.e cells cultured in flasks coated with rat tail collagen I, growing in a monolayer, or under angiogenic conditions (right panel), i.e. cells plated on Matrigel forming capillary-like structures. **(B)** hCMEC/D3 cells plated on Matrigel in the absence (negative control) or presence (positive control) of bFGF (1 ng/ml) or with TTR at different concentrations (2 µM, 1 µM, 0.5 µM and 0.25 µM). Scale bar = 200 µm.

Figure 4B shows the results obtained revealing tube formation in all conditions. Then, supernatants collected at 9 h were used to identify key targets involved in angiogenesis which could be modulated/affected by TTR.

To do so, we used the Multi-Analyte Flow assay kit, Legendplex, from BioLegend, consisting of multiplex assays using fluorescence-encoded beads, which allows the simultaneous quantification of 10 key targets involved in angiogenesis, from human origin (Table 3).

Table 3. Panel target of the multi-analyte flow assay kit, Legendplex, from BioLegend.

Target
Il-6
Ang-1
Ang-2
EGF
bFGF
IL-8
PECAM-1
PlGF
VEGF
TNF-α

Next, the results concerning IL-6, IL-8, Ang-2 and VEGF are presented. The results for the remaining 6 molecules are not shown here, either because were their concentration was below the lowest concentration of the standard curve or because they were simply not detected.

As shown in Figure 14, all 4 well-detected molecules had their expression significantly increased relative to the negative control when stimulated with 2 μM TTR. Interestingly, levels of IL-6 and IL-8 in cells incubated with 2 μM TTR was higher than in cells treated with bFGF (positive control), suggesting TTR as an important modulator of these 2 molecules. Regarding Ang-2 and VEGF expression, no differences were found between cells treated with TTR 2 μM and positive control, indicating a similar angiogenic action.

Overall, at a concentration of 2 μM, TTR can modulate the expression of several angiogenesis-related proteins and the decrease in protein concentration correlates with the decrease in the expression of detected molecules.

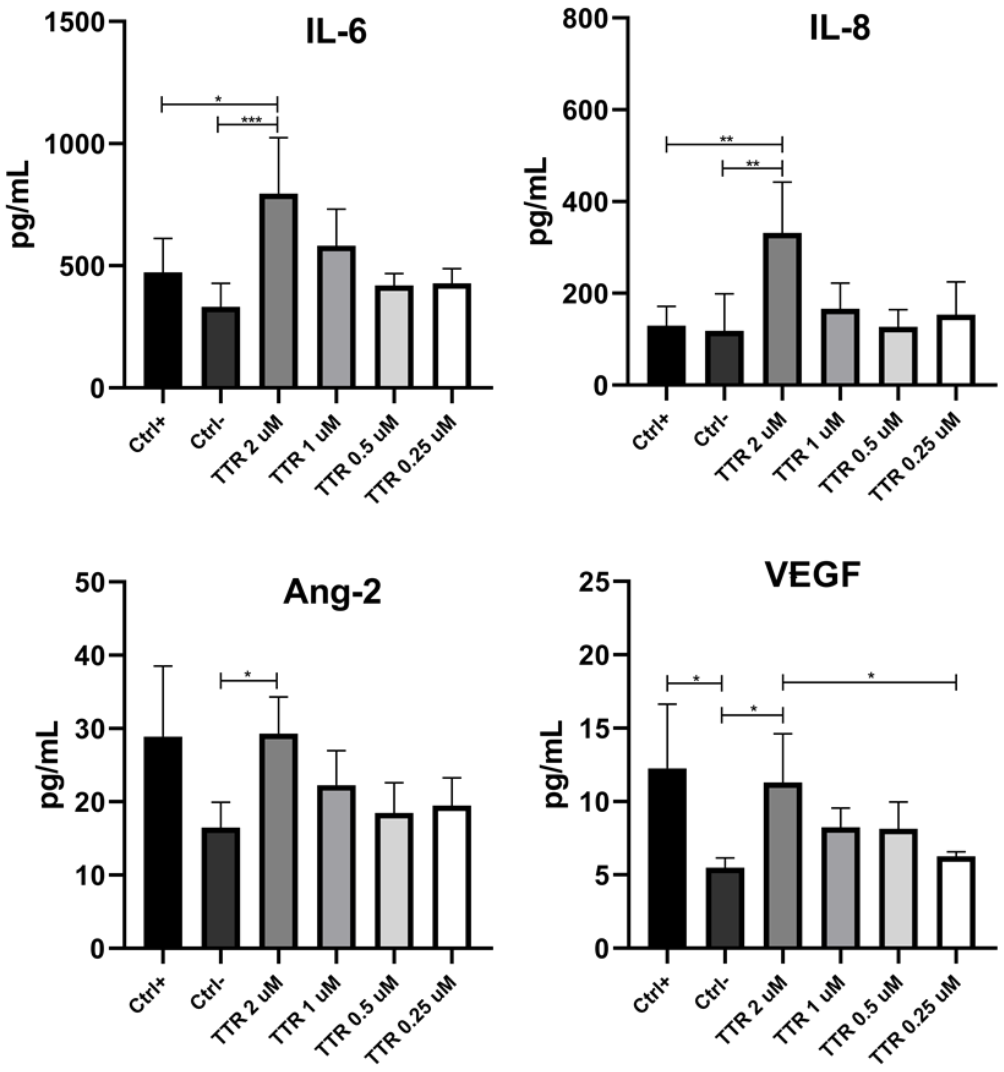

Figure 14. Quantification of angiogenesis-related proteins. Supernatants from cells under conditions of tube formation were collected 9h after the treatment with or without bFGF (1 ng/ml) (Ctrl+ and Ctrl-, respectively) or with TTR at different concentrations (2 μM, 1 μM, 0.5 μM and 0.25 μM). Angiogenesis-related proteins were quantified using bead-based LEGENDplex assay by flow cytometry. TTR 2 μM revealed ability to significantly increase the levels of IL-6, IL-8, Ang-2 and VEGF. Comparisons are relative to the negative control. Data are expressed as mean ± SEM. *p<0.05; **p<0.01; *** p<0.001.

2.1.3.4 Effect of TTR genetic reduction on collagen IV levels in brain microvessels

The BM has an essential role in the development of new vessels as well as in the formation and maintenance of the BBB. Several changes in the molecular composition of the BM are well reported in normal ageing and neuropathology. Current evidence suggests an increase in collagen IV, the major protein matrix protein, during ageing and disease, with concomitant BM thickening. TTR has been suggested to have a neuroprotective role and may also positively modulate the BBB. Altogether, these

observations lead us to question a possible correlation between the neuroprotector role of TTR and the layer of collagen IV in brain microvessels.

Evaluation of collagen IV levels in brain cortical microvessels was performed by immunofluorescence, in brain slices of 3-month old female mice with different genetic TTR backgrounds: TTR+/+, TTR+/-. As Figure 15B depicts, TTR+/- mice showed significantly higher levels of collagen IV in brain microvessels, as compared to TTR+/+ animals, indicating that TTR can be important to control the expression of collagen IV, and consequently in the modulation of the BM.

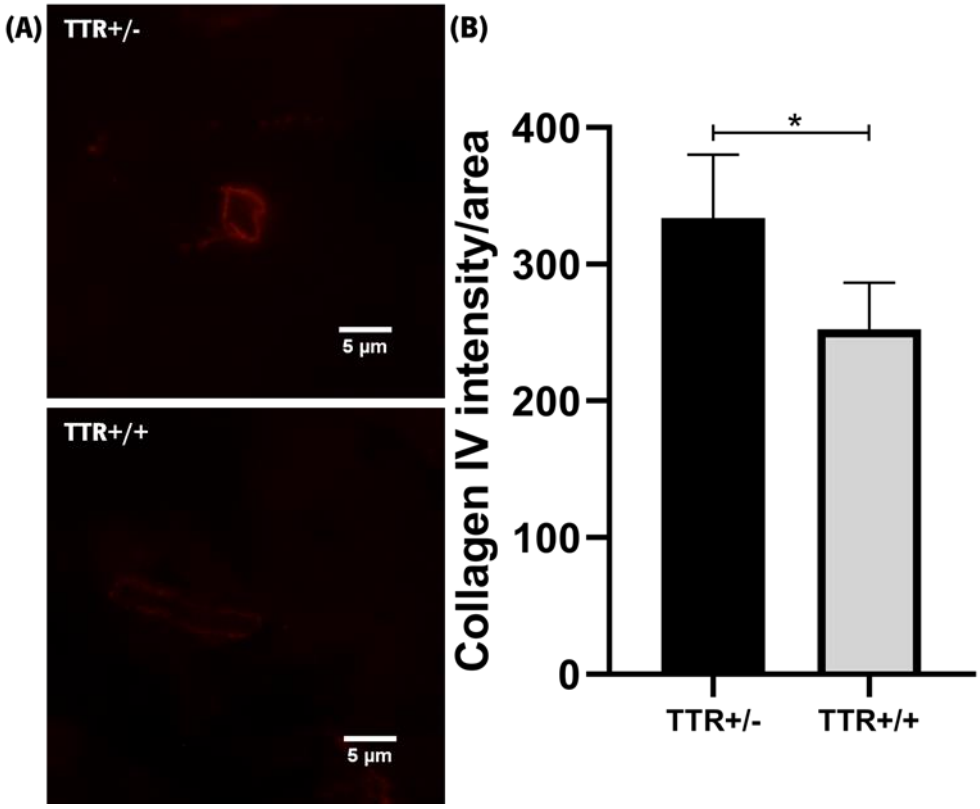

Figure 15. Effect of TTR genetic reduction on collagen IV levels in brain microvessels. (A) Representative images of collagen IV (red) immunostaining depicting cortical microvessels of TTR+/+ and TTR+/- 3-month old mice. **(B)** Quantification of collagen IV intensity/area ratio in cortical brain microvessels revealed an increase in collagen IV expression in TTR+/- mice (n=4) relative to TTR+/+ littermates (n=4). Data are expressed as mean ± SEM. *p<0.05. Scale bar = 5 µm.

2.1.3.5 Effect of TTR genetic reduction on collagen IV levels in AD brain microvessels

During AD, detectable changes in the brain include deposition of Aβ, BM thickening, and changes in the BM protein composition. Taking into account that AD patients show higher levels of collagen IV in

brain microvessels comparing with age-matched controls and that TTR is decreased in AD, it would be interesting to study the effect of TTR in the context of the disease.

Previous studies performed by our group, using AD transgenic mice aged 7 months (in this model, Aβ amyloid deposition starts around the age of 6 months[163]), demonstrated that AD/TTR-/- and AD/TTR+/- animals had a thicker layer of collagen IV when compared to AD/TTR+/+. In order to evaluate the impact of TTR insufficiency on vascular changes in AD and to test if the differences observed in 7-month old animals start early, collagen IV levels in brain microvessels of AD/TTR+/+ and AD/TTR+/- 3-month old animals were compared, by immunofluorescence. Corroborating the results observed for the TTR+/+ and TTR+/- animals, the analysis revealed an increase in expression of collagen IV in AD/TTR+/- mice relative to AD/TTR+/+ littermates, as displayed in Figure 16B.

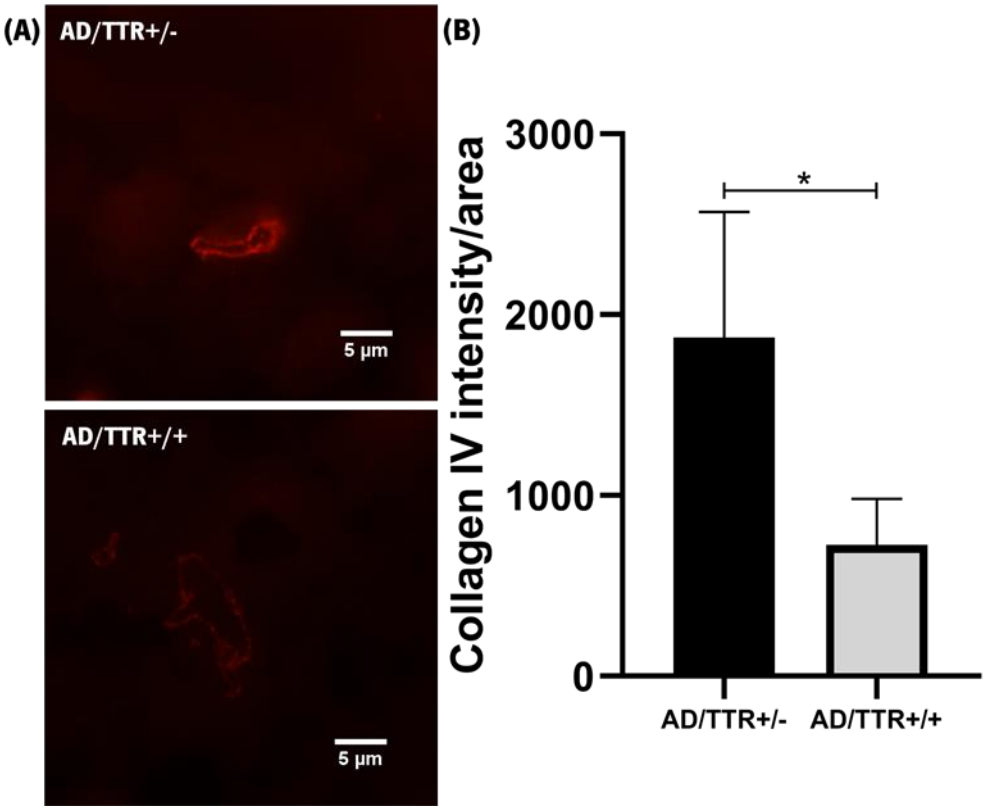

Figure 16. Effect of TTR genetic reduction on collagen IV levels in AD brain microvessels. (A) Representative images of collagen IV (red) immunostaining depicting cortical microvessels of AD/TTR+/+ and AD/TTR+/- 3-month old mice. **(B)** Quantification of collagen IV intensity/area ratio in cortical brain microvessels revealed an increment in collagen IV expression in AD/TTR+/- mice (n=4) compared to AD/TTR+/+ (n=3) littermates. Data are expressed as mean ± SEM. *p<0.05. Scale bar = 5 µm.

2.1.3.6 Comparing collagen IV levels in NT and AD mice with TTR genetic reduction

To understand if the impact in disease, in our AD model, was only the reflex of the effect of TTR reduction, 3-month old TTR+/- and AD/TTR+/- animals were compared.

Interestingly, the results revealed increased collagen IV immunostaining in brain vessels of AD mice as compared to non-AD, in the same TTR genetic background, at the same age (Figure 17B), indicating that the reduction of TTR may impact strongly in its neuroprotective role in AD. Furthermore, this alteration occurs early in the brains of these animals, confirming that vascular changes can precede several of the AD events. However, it remains to be confirmed whether these vascular changes are a cause or a consequence of AD.

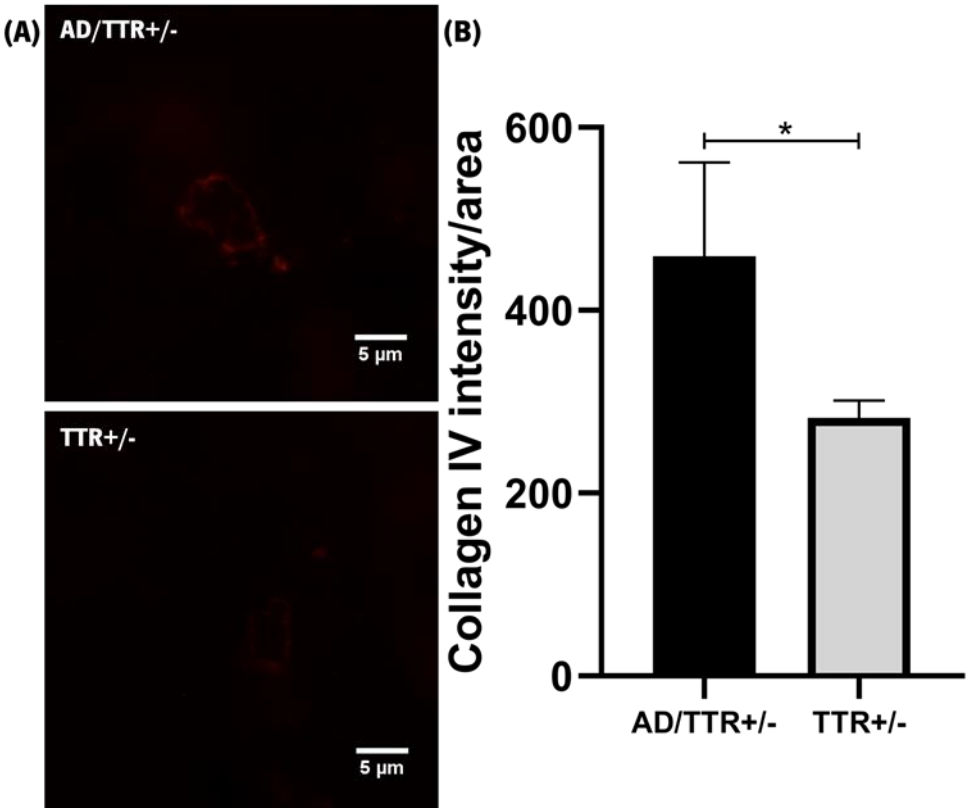

Figure 17. Comparing collagen IV levels in NT and AD mice with TTR genetic reduction. (A) Representative images of collagen IV (red) immunostaining depicting cortical microvessels of AD/TTR+/- and TTR+/- 3-month old mice. **(B)** Quantification of collagen IV intensity/area ratio in cortical brain microvessels revealed an increase in collagen IV expression in AD/TTR+/- mice (n=4) relatively to TTR+/- (n=3) littermates. Data are expressed as mean ± SEM. *$p<0.05$. Scale bar = 5 µm.

2.1.3.7 Impact of TTR stabilization in its angiogenic activity and in collagen IV levels in brain microvessels

TTR instability, which leads to the formation of amyloid fibrils occurring in FAP, leads to accelerated clearance, resulting in TTR lower levels. In AD, the stability of the TTR tetrameric structure is also diminished, preventing its neuroprotective effect from being properly exerted. Certain compounds, by binding to the TTR central pocket, have been used to stabilize TTR's tetrameric structure, thus restoring its clearance and increasing its levels. Administration of IDIF, a potent TTR tetrameric stabilizer, or resveratrol, also a TTR tetrameric stabilizer, both binding in the TTR central pocket, were shown to improve TTR neuroprotection in an AD transgenic mouse model [159,163].

Thus, to explore the effect of TTR stabilization in its angiogenic activity and its importance in the modulation of the BM, the effects of IDF and resveratrol were assessed using the CAM assay and evaluation of the levels of collagen IV, respectively.

Concerning the CAM assay, TTR pre-incubated with IDIF, did not produce any significant difference (not shown), as compared to TTR alone, suggesting that TTR stability is not critical for its angiogenic activity, or that the stability of the WT TTR used was sufficient to achieve its maximum activity.

To test if TTR stabilization can impact on collagen IV, its levels were evaluated in AD/TTR+/- 7-10-month mice that were previously treated with resveratrol, for 2 months, and results showed no significant differences between the groups (Figure 18), in spite of the previously reported beneficial effect on Aβ brain deposition [163], suggesting that TTR stabilization has no influence on the modulation of this component of the BM.

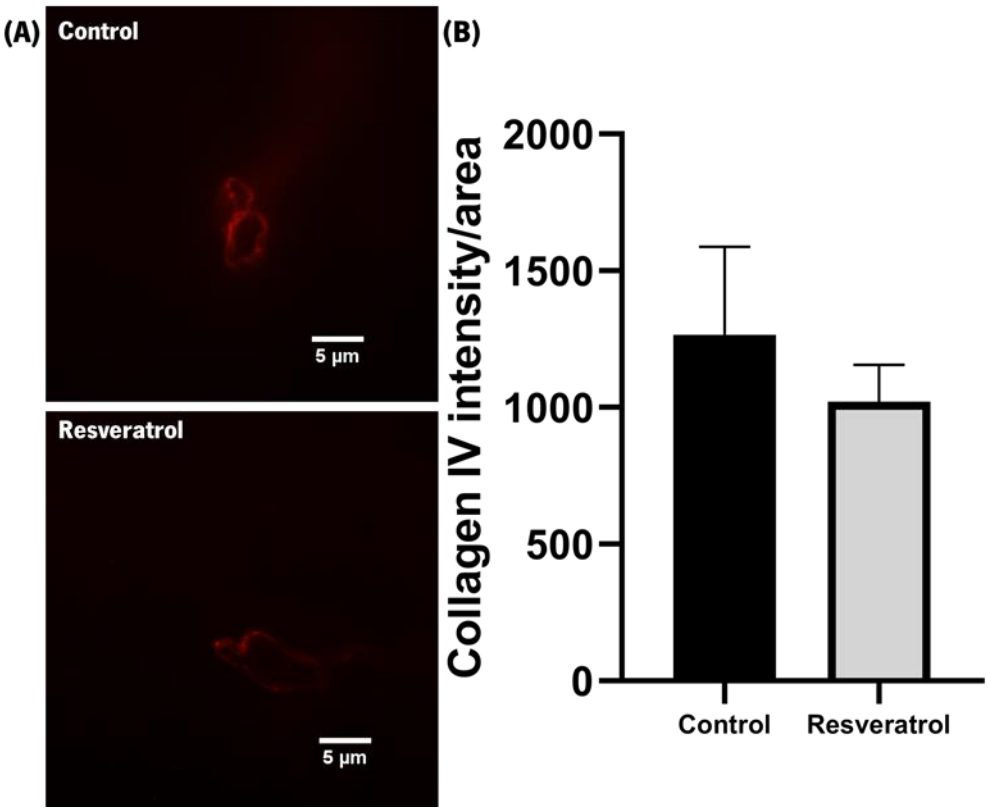

Figure 18. Impact of TTR stabilization on collagen IV levels in brain microvessels. (A) Representative images of collagen IV (red) immunostaining depicting cortical microvessels of AD/TTR+/- 7-10-month old mice treated (n=4) or non-treated with resveratrol (n=3). **(B)** Quantification of collagen IV intensity/area ratio in cortical brain microvessels from tested groups did not show any differences in collagen IV expression. Data are expressed as mean ± SEM. Scale bar = 5 µm.

2.1.3.8 Impact of TTR and Aβ species on collagen IV levels in bEnd.3 cell line

The thickening of the BM, with an associated increase in collagen IV content, is observed in AD. However, the reasons leading to these features are not known. Aβ accumulation in the brain, namely around the walls of the vessels can result from impairment of its clearance mechanisms, and from an increase of the thickness of the BM in brain vessels, which leads presumably to the formation of a barrier, preventing Aβ from reaching the blood. Although this phenomenon occurs during normal ageing, in AD collagen IV levels are higher than age-matched controls, suggesting a role for Aβ in this increment. To confirm the hypothesis that Aβ peptide has the ability to induce collagen IV expression, bEnd.3 cells, a mouse model of the BBB, were treated for 24 h with different species of Aβ peptide: soluble, oligomers and fibrils.

Initially, to accomplish this task the presence of different Aβ species was confirmed by TEM. As revealed in Figure 19, the preparations were heterogenous, containing several Aβ species, and thus we were not fully successful at producing homogenous samples of soluble, oligomeric or fibrillar Aβ. Nevertheless, the preparations seemed to be enriched in the respective species and thus, we proceeded to the incubations with cells.

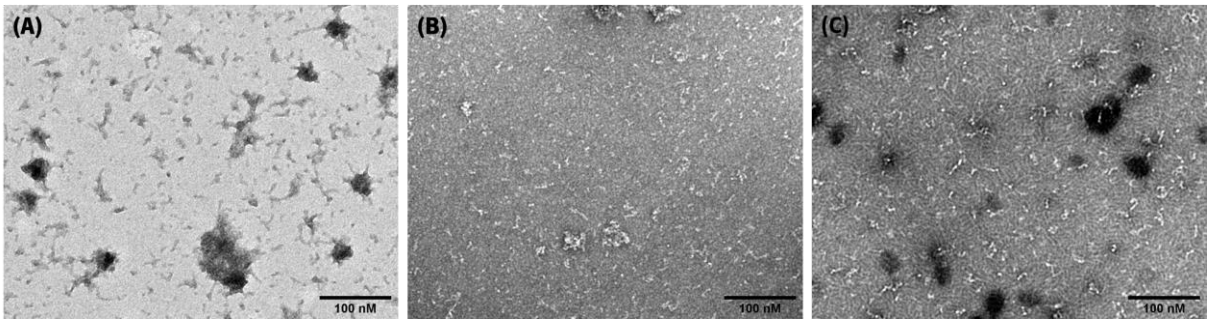

Figure 19. Qualitative evaluation of Aβ species using TEM. Representative images of Aβ species provided by TEM: **(A)** soluble; **(B)** oligomers; **(C)** fibrils. To confirm the formation of oligomers and fibrils from its soluble form, negative staining was performed, using uranyl acetate to counterstain. Scale bar = 200 nm.

As illustrated in Figure 20B, treatment with Aβ oligomers and Aβ fibrils resulted in a significant increase in collagen IV expression, when compared with control, with oligomers producing the highest effect. No differences were observed between soluble Aβ and control.

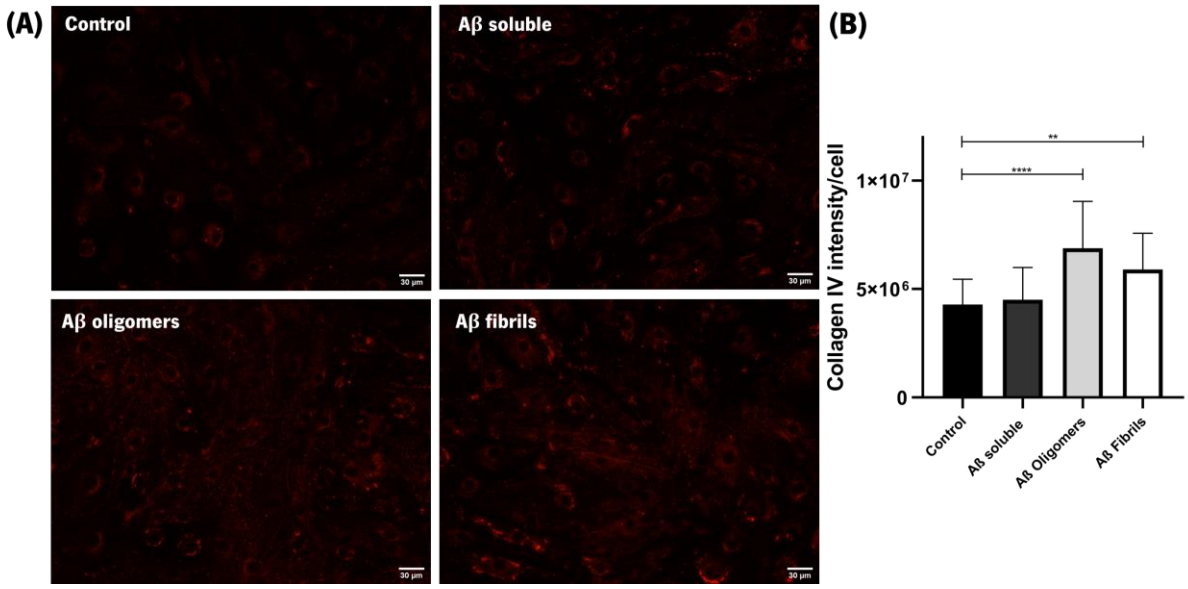

Figure 20. Evaluation of collagen IV levels in bEnd.3 treated with Aβ species. (A) Collagen IV (red) immunostaining in bEnd.3 cells non-treated (control) or treated with soluble, oligomeric or fibrillar Aβ. **(B)** Quantification of collagen IV intensity/cell ratio revealed an increase in collagen IV content in cells treated with Aβ oligomers and fibrils, as compared to the control group. Data are expressed as mean ± SEM. **p<0.01; **** p<0.0001. Scale bar = 30 µm.

In order to confirm the ability of TTR to modulate collagen IV content, in physiological in pathological conditions, another set of experiments was conducted. TTR alone or previously co-incubated with Aβ for 48 h, and then added to cells for 24 h, were tested. Unexpectedly, and as displayed in Figure 21, TTR alone resulted in levels of collagen IV significantly higher than in control cells, and comparable to the ones induced by Aβ oligomers, while TTR pre-incubation with Aβ (Figure 21A, lower left panel) reduced collagen IV levels, which remained similar to control cells.

Alternative incubations of TTR/Aβ were assayed, namely simultaneous addition of TTR and Aβ oligomers to cells, preceded or not by an ON incubation of cells with TTR (Figure 21A, lower left and middle panel). Again, in both cases, collagen IV levels were decreased, as compared to Aβ oligomers and TTR alone, and similar to control cells.

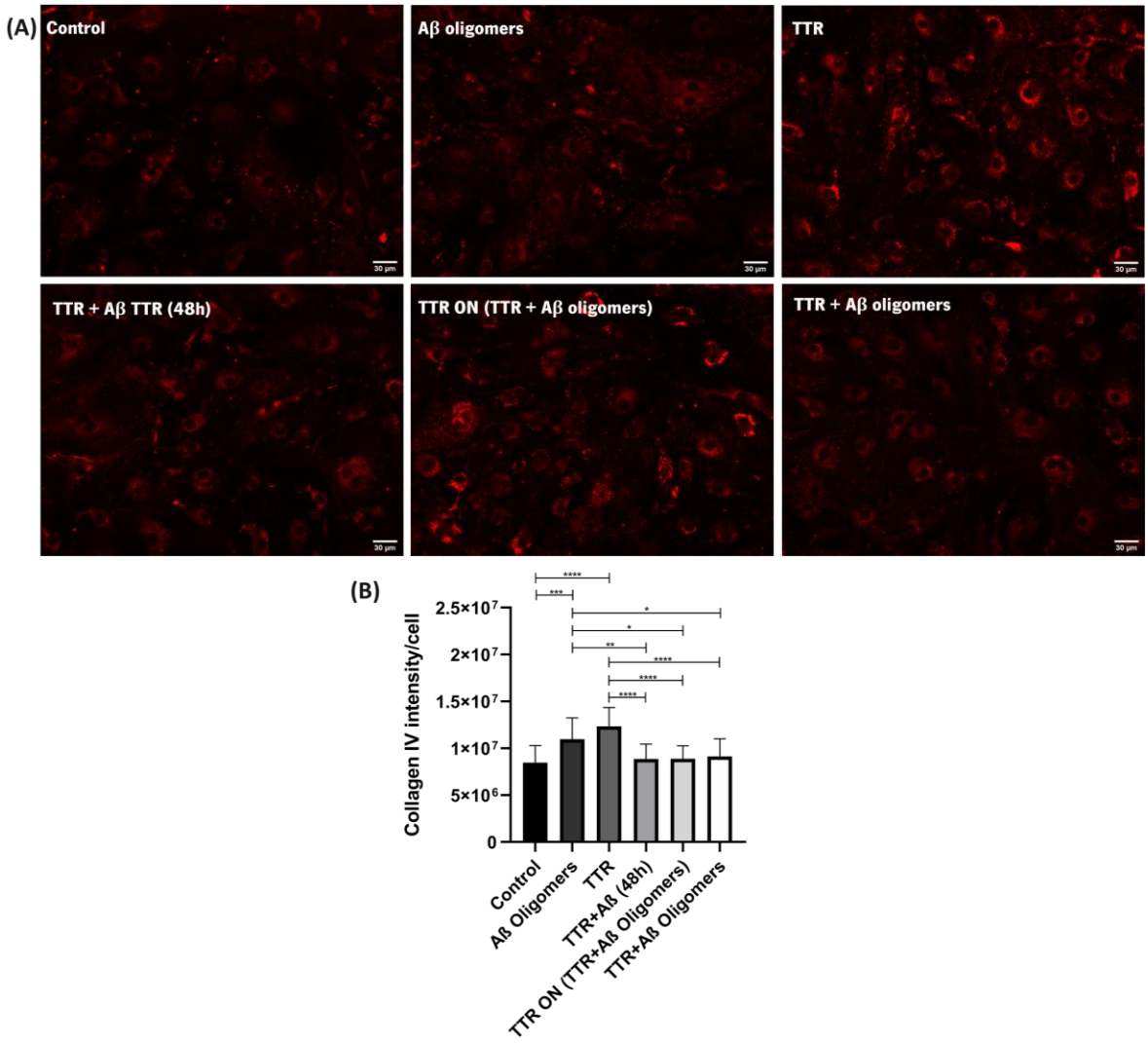

Figure 21. Evaluation of collagen IV levels in bEnd.3 cells treated with Aβ oligomers and TTR. **(A)** Images illustrate collagen IV (red) immunostaining of bEnd.3 cells treated in different conditions. Cells were incubated with media alone (control), or with Aβ oligomers or TTR alone (TTR). Alternatively, cells were treated with TTR previously incubated with Aβ for 48 h, at 4°C (TTR+Aβ (48h)), with TTR and Aβ oligomers, simultaneously, preceded (TTR ON (TTR+Aβ oligomers)) or not (TTR+Aβ oligomers) by an ON treatment with TTR. **B).** Quantification of collagen IV intensity/cell ratio revealed that both Aβ oligomers and TTR induced a significant increase in collagen IV, as compared to control cells; in opposition, in all Aβ/TTR treatments, collagen IV content was similar to control cells. Data are expressed as mean ± SEM. *p<0.05; **p<0.01; ***p<0.001; **** p<0.0001. Scale bar = 30 µm.

Of notice, collagen IV localization varied depending on whether cells were incubated with Aβ oligomers or TTR. In fact, and as can be observed in Figure 21, cells incubated with Aβ oligomers, showed collagen IV mainly localized in the extracellular space, whereas in cells incubated with TTR, collagen IV seemed to appear in cytoplasm. Moreover, among treatments of cells with TTR+Aβ, the one in which

cells were pre-treated with TTR ON, also resulted in apparent cytoplasmatic collagen IV localization. These observations may have implications in alterations occurring in AD.

2.1.4 Discussion

TTR is a homotetrameric protein typically known as a carrier of T4 and retinol in plasma and CSF. During the last years, several functions have been appointed to this protein, in particular, its role as neuroprotector in physiologic and in disease contexts. In AD, TTR binds to Aβ avoiding its aggregation, accumulation and toxicity, and facilitating its efflux across the BBB. This barrier is essential to maintain brain homeostasis however, during normal ageing and AD, BBB becomes dysfunctional contributing to disease processes. TTR was found capable of positively modulating the BBB, so it is possible that it can regulate neurovasculature in other ways, namely in angiogenesis and vascular alterations. In fact, available studies correlate TTR and angiogenesis but its possible role as an angiogenic protein was never addressed *in vivo*.

Nunes and co-workers demonstrated that TTR stability has a regulatory role in angiogenesis, using umbilical ECs and comparing the effect of the unstable mutant TTR V30M with the WT counterpart. TTR V30M promoted a decrease in survival, migration and expression of several angiogenesis-related genes in ECs relatively to WT [126]. However, authors did not evaluate the angiogenic potential of TTR *per se*, i.e. compared to a negative control.

Here, using *in vivo* CAM assay, we demonstrate that TTR influences angiogenesis by promoting the formation of new vessels. Although it is clear that TTR stability is important for its performance, co-incubation of TTR with IDIF, a potent TTR stabilizer, did not magnify the effect produced by the protein alone, in the CAM assay. This result suggests that, in this scenario, stability does not influence the angiogenic potential of WT TTR or that WT TTR is stable enough to produce the maximum of TTR angiogenic activity. Thus, to fully understand the importance of TTR stability in angiogenesis, it would be interesting to use an unstable mutant of TTR, such as the highly unstable L55P TTR, and test the effect of IDIF.

We next set up the tube formation assay using hCMEC/D3 cells, which are ECs derived from human brain microvessels, that are also widely used as a BBB model. This assay has been previously employed to study the participation of TTR in related events but usually under pathological conditions and using other types of cells. For instance, Lee and collaborators, using lung ECs demonstrated that TTR influences the angiogenic ability of these cells by increasing tube formation. Furthermore, using other assays, authors showed that TTR increases EC migration, proliferation and permeability, implicating TTR in the development of lung cancer. [129]. In a different work, retinal ECs treated with TTR showed an increment in the formation of capillary-like structures and the promotion of EC migration and proliferation. Interestingly, under simulated diabetic retinopathy conditions, characterized by hypoxia and high glucose

levels, TTR treatment inhibited the angiogenic features already mentioned, suggesting that TTR suppresses the development of diabetic retinopathy [127]. In these works, TTR seems to contribute to disease development depending on the context in which it is inserted, both increasing and diminishing EC angiogenic function. Thus, in the future, it is important to study the permeability of the new vessels formed in the presence of TTR, in the CAM assay, to understand if TTR angiogenic activity is physiologic or pathologic [181].

We next used the supernatants of the hCMEC/D3 cells grown under tube-formation conditions, in the presence or absence of TTR, to study the molecular pathways affected by TTR, and found that VEGF, Ang-2, IL-6 and Il-8 were significantly up-regulated in the presence of the protein.

VEGF is a well-studied and the major driver of angiogenesis playing a role in most of the steps of the process. Apart from the potent vascular permeability factor role [182], it regulates multiple processes in EC such as migration, proliferation, differentiation and survival [183]. It's also implicated in tubular fusion and vascular network formation [184]. VEGF generates a chemoattractive gradient directing the sprouting vessels, by the tip cell filopodia.

Ang-2 was previously found to be up-regulated in retinal ECs after treatment with TTR [127] and plays a controversial role in angiogenesis. If, on one hand, it increases migration capacity and tube formation in brain ECs [185], on the other hand, *in vivo* retinal studies showed that Ang-2 promotes EC death and vessel regression if VEGF is absent. However, when in the presence of VEGF it stimulates an increase in capillary diameter, remodeling of basal lamina, proliferation and migration of EC. [186].

Regarding IL-8, enhanced proliferation, survival, migration and tube formation are features promoted by the cytokine [187,188]. The other molecule analyzed, IL-6, induced an increase in EC proliferation, migration and tube formation and this angiogenic capacity was corroborated by a CAM assay [189,190]. Together these data suggest that TTR mediates the expression of angiogenic factors regulating multiple biological activities in brain ECs.

Considering that TTR has a neuroprotector effect, we studied the effect of TTR on changes of cortical microvessels. In the brain, the vascular BM, contributes to the integrity of the BBB, act as a physical barrier, support for the cellular components of the NVU and a modulator of BBB. Brain vessels undergo several alterations during ageing, and it is speculated that these changes may play a role in AD.

BM thickening and increased collagen IV levels are some of the features observed in both ageing and, more severely, in AD. Previous work in our lab, in the AD context, had already demonstrated that the collagen IV layer was increased in brain microvessels of 7-month old AD/TTR+/- and AD/TTR-/- mice, as compared to AD/TTR+/+ animals [191]. However, at the time, it was not known if the differences were

due to a direct or indirect effect of TTR. In our work, collagen IV levels were evaluated in 3-month old mice, in both NT and AD animals, with the two different TTR genetic backgrounds (one or two copies of TTR gene). In the AD model, Aβ deposition begins approximately at 6 months of age [163]. Thus, it allows inter-model comparison of collagen IV levels before the onset of AD deposition. Immunofluorescence analysis revealed that TTR+/- mice exhibited more collagen IV around brain microvessels than TTR+/+ littermates, suggesting a direct effect of TTR. The same pattern was found, as expected, in 3-month old AD mice and thus AD/TTR+/- showed higher levels of collagen IV than AD/TTR+/+. Furthermore, inter-model comparison with the same TTR background showed that AD/TTR+/- had more collagen IV than TTR+/-, around brain vessels. This last result suggests that TTR reduction has a negative impact on this AD feature and that this vascular alteration occurs early, confirming that vascular changes can precede several of AD events.

Our hypothesis to explain the differences obtained between inter-models implicate Aβ peptide as the culprit, since in the AD mice it is overproduced. In fact, it was already demonstrated that our 3-month old AD/TTR+/+ mice display reduced Aβ levels in plasma compared to AD/TTR+/- [123] which can be a reflex of what is happening in the brain. So, TTR reduction seems to disrupt the balance of Aβ produced vs Aβ cleared, as it promotes clearance and regulates its production by previously described mechanisms. This leads to progressive Aβ accumulation in the interstitial fluid that can impact the vessels in different ways, namely by increasing BM thickness and collagen IV content. Interesting, collagen IV has been reported to localize to senile plaques [192] and *in vitro* tests evidence that collagen IV inhibits the formation and promotes disassembly of Aβ fibrils, in a dose-dependent manner [86,87]. Take into account these facts we theorize that secretion of these proteins may be for counterattack fibrilization and toxicity of Aβ, and simultaneously strengthening the vessel wall. Actually, some studies correlate BM thickening with increased collagen IV content [81,84], however, it seems too early to state that collagen IV acts alone in this thickening taking into account that other BM molecules had its abundance changed in AD.

As mentioned in a study, AD BM thickening occurs prior to the deposition of Aβ fibrils around vessels (CAA) [80]. Nevertheless, it is likely that BM thickening creates a physical barrier that with disease development leads to a progressive increment in collagen IV expression in response to increasing Aβ accumulation. This prevents the transport of Aβ out of the brain, both through the BBB and by perivascular drainage, leading to CAA and a consequent increase in collagen IV expression, in a "snowball effect". Moreover, it is known that Aβ has affinity to proteoglycans present in the BM [132,193] that might sequester Aβ from the interstitial fluid, contributing to the accumulation of Aβ in vessels [194]. Despite this,

TTR seems to act with its neuroprotective effect on AD, preventing Aβ from exerting its toxic effect on vessels and reducing collagen IV levels.

Interesting, TTR gene silencing at the liver decreased matrix metalloprotease-2 (MMP-2) protein levels in plasma, inhibit Mmp-2 gene expression and downregulate matrix metalloprotease-9 (MMP-9) activity both on dorsal root ganglia [195]. MMP-9 and MMP-2 are also associated with collagen IV proteolytic degradation. [196]. Thus, lower TTR levels induced by genetic alterations in our mice, may have led to decreased metalloproteases expression, resulting in turn, in increased collagen IV in brain microvessels. Moreover, the literature indicates that Ang-2 [197] and VEGF [198] increase MMP-9 activity, while IL-6 stimulates its secretion [199] and IL-8 boosts the production of both MMP-2 and MMP9 [188]. VEGF, in turn, stimulates podosome-mediated collagen IV proteolysis in microvascular ECs [200]. Thus, it is possible that TTR is directly or indirectly mediating collagen IV levels through the modulation of metalloproteases.

Next, we test if TTR stabilization by resveratrol could improve the vascular alterations measured in our AD model. AD/TTR+/- mice treated with resveratrol, as part of a previous project [163], were analyzed for the levels of collagen IV in brain microvessels and compared to non-treated animals. Unexpectedly, we found no differences. Previous work with these animals showed less brain Aβ deposition and increased plasma TTR levels [163], thus anticipating improvements also at the vascular level. Moreover, the treatment of AD/TTR+/- animals with another stabilizer, IDIF, resulted not only in decreased brain Aβ deposition and amelioration of cognition [159] but also in a thinner layer of collagen IV, as compared to non-treated animals [201]. Furthermore, its described that TTR stabilization seems important to prevent pathological changes to the brain vasculature, as heterozygous individuals for TTR T119M have reduced risk of cerebrovascular disease compared to homozygotes for WT TTR [152]. Although resveratrol retains the ability to stabilize TTR, IDIF is a much potent stabilizer [151]. Additionally, resveratrol produces other beneficial effects, besides TTR stabilization, which can provide the basis for the improvements described but without affecting the vascular alterations[162].

The cause for BM thickening is unknown and although it was already referred here that, in AD, thickening occurs prior to the deposition of Aβ around vessels, Aβ may still be involved. In our work, oligomers and fibrils but not soluble Aβ induced up-regulation of collagen IV by bEnd.3 cells, maybe to counteract the toxic effect of oligomers and to inhibit fibrillization. To confirm the ability of TTR to modulate collagen IV content, its expression by bEnd.3 cells was also investigated in the presence of TTR, and contrarily to the expectations, TTR also induced collagen IV expression. However, collagen IV localized differently in cells, more in the extracellular space in cells incubated with oligomers, and more intracellular when incubated with TTR. This may reflect differences occurring in pathological and physiological

conditions, respectively, and should be explored. Further studies are necessary to understand if there are differences between collagen IV species (collagen IV induced by Aβ oligomers versus collagen IV induced by TTR) or if other molecules are altered. Interestingly, ON treatment of TTR followed by simultaneous incubation of Aβ/TTR resulted in decreased levels of collagen IV, localized in the cytoplasm.

It is not yet clear what leads to increased collagen IV levels in neurovasculature, but these changes are also found in rats suffering from chronic cerebral hypoperfusion [202], suggesting that decreased blood flow in the brain leads to high collagen IV content around the vessels. Indeed, diminished cerebral blood flow is an early impact event during AD development [77]. The thickened and rigid vascular wall may slow down nutrient supply and waste elimination, and possibly disturb perivascular drainage. This because it is theorized that perivascular transport is driven by pulsations of the blood vessel walls, that are not so flexible in the latter situation leading to the consequent accumulation of Aβ in the blood vessel [193]. This event along with the formed barrier will potentially contribute to progressive endothelial dysfunction and to an increasing accumulation of the peptide in the brain. However, TTR reduces the collagen IV layer by mechanisms that lack elucidation, suggesting that it could positively affect some harmful features in AD.

It's inevitable not to try to establish a link between this collagen IV increase and its possible effects on angiogenesis. The BM is a dynamic structure, undergoing continuous remodeling, as its composition and structure vary during various physiological and pathological processes. In addition, it performs functions such as mechanical cell support and signaling cascade modulation in a promiscuous interaction with the ECs, coordinating cell response to extracellular stimuli. During BM remodeling, it can release matrix-sequestered growth factors that promote angiogenic events. However, BM remodeling does not only mean destroying the matrix but rather exposing cryptic protein that plays a role in angiogenesis. Proteolytic cleavage of collagen type IV by MMP-2 results in the exposure of a functionally cryptic site that stimulates angiogenesis [203]. Conversely, collagen IV fragments derived from proteolysis such as tumstatin, arresten and canstatin evidenced anti-angiogenic activity. It's unclear whether is the MMP involved in the cleavage of these fragments, but some evidence suggests MMP-9 as the mediator of tumstatin formation [204]. In turn, intact collagen IV was shown to play an important role in angiogenesis in a study where umbilical ECs with a silenced collagen IV chain gene had inhibited their ability to form tubes [205] In regards to *in vivo* studies, collagen IV knockout mice demonstrated lower brain capillary density than control [206]. Furthermore, exercise promoted an increase in collagen IV levels simultaneously with increased vascular density, in the brain of stroke mice model. MMP-9 inhibition has been shown to be involved in the process contributing to this increment [207]. However, the relevance of each of these molecules is unknown, as there is no date available studying all molecules at once.

In summary, this work showed that TTR has pro-angiogenic properties, up-regulating molecules such as IL-6, IL-8, Ang-2 and VEGF. TTR is also involved in the early vascular alterations occurring in AD, which may be used as a target for therapeutic intervention in AD.

2.2. Chapter II

Effect of small molecules chaperones on the Transthyretin/Aβ peptide interaction - Longitudinal study of the treatment with iododiflunisal and tolcapone

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2.1.5 Summary

A number of physiological studies indicate that TTR plays a role in AD pathogenesis as a neuroprotective protein. TTR binds Aβ, thought to be the causative agent in AD, avoiding its aggregation, accumulation and toxicity, and facilitating its efflux across the BBB. Studies in humans found decreased TTR levels in the cerebrospinal fluid CSF and plasma of AD patients, compromising its ability to protect in AD.

Previous work by the Marató consortia, of which our group is part of, demonstrated, *in vitro*, that TTR levels can be raised and the TTR/Aβ interaction enhanced by a set of small molecules, one of them IDIF, an iodinated analog of the NSAID diflunisal. This improvement is believed to be the result of TTR stabilization, as most of these compounds bind at the TTR central channel and stabilize the tetramer. By *in vivo* studies, it was found that when administered to an AD mouse model, IDIF decreases brain Aβ levels and deposition, and improves cognition in these mice. The consortia settled a drug discovery program focused on the discovery of small compounds enhancers (chaperones) of the TTR/Aβ interaction. To efficiently search for new small molecule chaperones, the consortia developed and validated a robust high-throughput ternary screening assay that allowed to select the best 10 compounds.

Also as part of the Marató project, the effect of IDIF and another potential chaperone of the TTR/Aβ interaction, tolcapone, was investigated *in vivo,* in the same AD model aforementioned. For that, studies *in vivo* by PET using a specific Aβ marker were applied, allowing to quantitatively assess the amyloid burden in the brain in a longitudinal fashion, from 5 to 15 months of age.

The work performed in the context of this thesis and here presented, involved only the final evaluation of amyloid burden in the brain, after the sacrifice of the animals, by immunohistochemistry. No differences were detected between the control (non-treated) and the treated groups. The pattern of amyloid deposition in this AD model reveals that deposition starts around 6 months of age, very discretely, and evolves with age to burst at 10-12 months of age, in particular in females, the only gender used in the study. It is possible that IDIF and tolcapone exerted a positive effect, such as the decrease of Aβ deposition, at an earlier age, which was overcomed, at the later stages of the study, by the great capacity of Aβ to aggregate and deposit. The conclusion on the therapeutic effect of IDIF and tolcapone will be known after the analysis of the PET results, which allows comparisons between groups, at different ages.

2.1.6 Material and Methods

2.1.6.1 Animals

The AD mouse model AβPPswe/PS1A246E/TTR transgenic mice was generated by crossing the AD mouse model AβPPswe/PS1A246E [177] (B6/C3H background) purchased from The Jackson laboratory with TTR-null mice (TTR-/-) (SV129 background) [178] as previously described [179]. F1 animals AβPPswe/TTR+/− and PS1A246E/TTR+/− were crossed to obtain AβPPswe/PS1A246E/ TTR+/+, AβPPswe/PS1A246E/TTR+/−, AβPPswe/ PS1A246E/TTR−/− and non-transgenic controls TTR+/+, TTR+/−, and TTR−/−. The colony was maintained on a B6/C3H/SV129 genetic background. Hereafter, the AβPPswe/PS1A246E/TTR colony will be referred to as AD/TTR, and the different genotypes AβPPswe/PS1A246E/TTR+/+, AβPPswe/PS1A246E/TTR+/−, and AβPPswe/PS1A 246E/TTR−/− referred to as AD/TTR+/+, AD/ TTR+/−, and AD/TTR−/−, respectively.

Animals were housed in a controlled environment (12 h light/dark cycles, temperature between 22-24 °C, humidity between 45–65% and 15-20 air changes/h), with freely available food and water. All the above experiments were approved by the Institute for Research and Innovation in Health Sciences (i3s) Animal Ethics Committee and in agreement with the animal ethics regulation from Directive 2010/63/EU.

Littermate cohorts of AD/TTR+/- female animals were used in this study. Animals were bred at IBMC and, at the age of 2 months, transported to the laboratory of Dr. Jordi Llop, BiomaGUNE, San Sebastian, Spain.

2.1.6.2 IDIF and tolcapone administration and PET studies

IDIF and tolcapone, provided by Dr. Gemma Arsequell (Department of Biological Chemistry, IQAC-CSIC, Barcelona, Spain) were administrated in the drinking water (N=6-7 mice/group), starting at the age of 5 months until sacrifice, at 15 months. *In vivo* PET studies using a specific Aβ marker, [18F]Florbetaben, routinely used at CIC BiomaGUNE to evaluate Aβ in mouse models of AD, were carried out at 2-months intervals to quantitatively assess the amyloid burden in the brain in a longitudinal fashion.

2.1.6.3 Tissue processing

After sacrifice, brains were were removed and bisected longitudinally; each half was either immediately frozen for biochemical analyses, or fixed for 24h at 4◦C in 10% neutral buffered formalin and then transferred to a 30% sucrose solution for cryoprotection, and sent to IBMC for cryostat sectioning and immunohystochemical analysis.

2.1.6.4 A β immunohistochemistry

Aβ plaque burden was evaluated by using a monoclonal biotinylated $A\beta_{1-16}$ antibody (6E10) (Covance Research Products) to perform free-floating immunohistochemistry of 30 µm-thick cryostat coronal brain sections. For partial amyloid denaturation, 70% formic acid was used for 15 min at RT, with gentle agitation. After washing in dH_2O and then PBS, endogenous peroxidase activity was inhibited with 1% hydrogen peroxide (H2O2) in PBS for 20 minutes. Following PBS washes, sections were blocked in blocking solution (10% FBS and 0.5% Triton X-100) for 1 h at RT and then incubated with biotinylated 6E10 primary antibody (diluted 1/750 in blocking buffer) ON at 4°C, with gentle agitation. Sections were washed with PBS and incubated in Vectastain Elite ABC Reagent (Vector Laboratories), washed again in PBS followed by development with diaminobenzidine (Sigma-Aldrich), mounted on 0.1% gelatin-coated slides and then left to dry ON at RT. After dehydration, slides were coverslipped under Entellan (Merck & Co.). Sections were examined with an Olympus BX63 microscope, at 2x magnification. Aβ plaque burden was evaluated using Image-Pro Plus software, by analyzing the immunostained area fraction in the hippocampus and cortex (expressed as a percentage of the analyzed area) of five sections per animal.

2.1.6.5 Statistical Analysis

All quantitative data were expressed as mean ± SEM. Initially, data was assessed whether it followed a Gaussian distribution. When found to follow a Gaussian distribution, differences among conditions or groups were analyzed by one-way ANOVA with Dunnett's post hoc pairwise tests for multiple comparisons tests.

2.1.7 Results

TTR stabilization has been reported as critical to TTR–Aβ interaction. To increase the ability of TTR to bind Aβ, small compounds have been used to modulate this interaction. Considering this, AD/TTR+/- female mice were treated from 5 to 15 months of age with two stabilizers. After sacrifice, Aβ plaque load was evaluated by immunohistochemistry in the hyppocampus and cortex. Figure 22A displays representative images of the results obtained for each of the groups, control (non-treated), IDIF-treated and tolcapone-treated. The statistical analysis did not show any significant difference between them (Figure 22A, plot). A comparison in hyppocampus and cortex, separately, also did not reveal significant differences (Figure 22B).

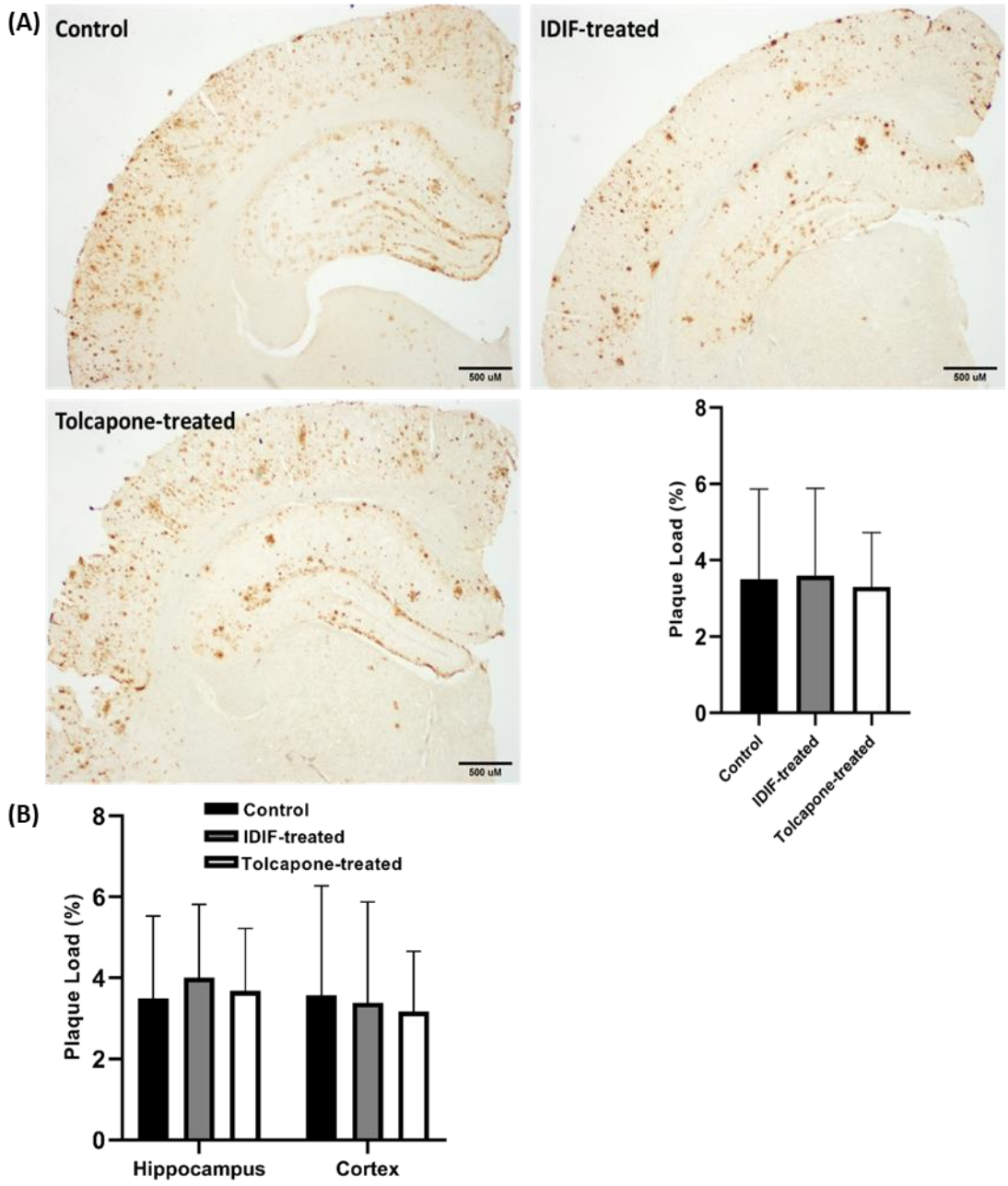

Figure 22. Assessing the effect of TTR stabilizers on plaque load. Evaluation of Aβ plaque burden in 15-month old female AD/TTR+/- mice treated with IDIF (IDIF-treated) (n=7) or with tolcapone (Tolcapone-treated) (n=7) since 5 months of age, and without treatment (Control) (n=7). (**A)** Photomicrographs illustrate the immunohistochemical analysis of brain Aβ plaques (in brown) using the 6E10 antibody, and the plot shows plaque burden estimation, showing no significant differences. **(B)** Quantification of plaque load separately in the hyppocampus and cortex demonstrated no significant differences in both brain areas. Data are expressed as mean ± SEM. Scale bar=500 μm.

Previous work by Ribeiro and co-workers reported beneficial effects of IDIF administration to the same AD animal model, including decreased amyloid burden and brain Aβ levels, and improved cognition

[159]. In this work, animals were also aged 5 months when treatment started. However, drug administration lasted only 2 months, thus mice were 7-months old when they were evaluated. As previously reported, in this AD model, amyloid deposition starts around the age of 6 months[163], very discretely, and evolves with age to burst at 10-12 months [179]. Females, the only gender used in the study, are particularly affected. From our study, we can not exclude a positive effect of IDIF and tolcapone that is most likely to occur at earlier ages, before or around the age of the large increase in Aβ deposition. Thus, it is expected that at 5 months of age, the capacity of Aβ to aggregate and deposit zes the therapeutic effect of IDIF and other compounds.

2.1.8 Discussion

TTR is mostly known for its transport functions of T4 and retinol. Besides this, it is a key protein in FAP, a systemic amyloidosis with a special involvement of the peripheral nerve. Amyloidogenic mutations in this protein cause the dissociation of the homotetramer, forming altered monomers which are prone to aggregate and deposit as amyloid fibrils.

TTR stabilization has been previously proposed as a key step for the inhibition of TTR fibril formation and has been the basis for FAP therapeutic strategies. Such stabilization has mainly been achieved through the use of small compounds sharing molecular structural similarities with T4, mostly belonging to the class of the non-steroid anti-inflammatory drugs (NSAIDs), and binding in TTR central channel [208]. Currently, FAP treatments include the orphan drug tafamidis and tolcapone, a drug for the treatment of Parkinson's disease recently repositioned for FAP, both acting as TTR tetrameric stabilizers [209].

TTR is recognized as a neuroprotective protein in AD and it is now well established that TTR binds Aβ, avoiding its aggregation and toxicity. The specific mechanism is not unraveled but it may involve the degradation of the peptide and its elimination at the BBB [115,150,151]. In the context of TTR neuroprotection in AD, there is no consensus on the TTR species that provides the best effect and thus, whether TTR instability demonstrated in AD [131,156] is a negative consequence or is a protective mechanism to deal with the excess of Aβ, is not known, yet. While some authors reported that a TTR monomeric mutant binds strongly to Aβ and is stronger at avoiding its aggregation and toxicity [164–166], our group has raised the stability hypothesis. This postulates that TTR stabilization by small chemical compounds would restore its levels, known to be reduced in AD. With this, the TTR/Aβ interaction is improved, thus avoiding disease progression [151,159]. Administration of IDIF, a potent TTR tetrameric stabilizer, to AD mice resulted in decreased Aβ brain levels and its deposition. Furthermore, cognitive performance was improved [159], providing the basis for the Marató consortia, aiming at finding new molecules enhancers of the TTR/Aβ interaction, to advance towards the identification of an effective therapy for AD.

Taking IDIF as the reference compound, one of the tasks proposed in the Marató project was to test IDIF and tolcapone in our AD mouse model but this time, in an *in vivo* longitudinal study by PET, using a specific amyloid marker. Tolcapone was selected because being a drug in the market, approved for the treatment of Parkinson's Disease, it could then be directly repurposed or repositioned as a possible AD therapeutic, once the animal assays show their activity and after being tested in AD patients in clinical Phase II programs.

The work here presented refers to the final evaluation of Aβ deposition in the brain, after the sacrifice of the animals by immunohistochemistry. No significant differences were found between the control and the treated groups, even when considering the hyppocampus and the cortex, separately. In this transgenic model, APP and PSEN are overexpressed and, consequently, Aβ peptide is overproduced further reenforced by the presence of mutations in the transgenes, characteristic of FAD cases. Thus, AD transgenic mouse models, although undoubtedly a valuable tool in research, have limitations concerning the simulation of the disease in humans.

The mouse model here used, in besides to carry the APP and PSEN human mutated genes, carries only one allele of the TTR mouse gene (AD/TTR+/-). As referred, previous work with this model showed that IDIF produced positive effects in these animals. However, after IDIF administration, animals carrying the two copies of the TTR gene (AD/TTR+/+) presenting a less severe AD-like neuropathology, did not improve in any of the assays used [159]. TTR instability is certainly induced by the presence of mutations, as in the case of FAP but other factors such as failure of the folding system. The interaction with various components such as metal ions and carbohydrates [153–156], and protein concentration can add to instability could also affect TTR stability. In fact, previous work by Quintas and colleagues, showed that TTR can dissociate to an unfolded non-native monomer, at low protein concentrations [94,210]. It is, therefore, possible that the AD/TTR+/- animals present unstable TTR and this may represent the negative result of having lower TTR levels. So, when treated with IDIF, these mice can partially recover due to TTR stabilization and consequently improve the performance of the protein concerning its interaction with Aβ peptide.

AD is a very complex disorder with several factors playing a role. AD/TTR+/+ mice should not have a negative contribution from TTR since they carry both copies of the gene. The AD-like neuropathology is most likely the result of the overexpression of mutated APP and PSEN, and consequences in several mechanisms. As such, IDIF administration to these animals does not have a positive impact as in the AD/TTR+/- mice. However, TTR lower levels and predicted instability is not responsible for the full AD-like neuropathology in AD/TTR+/- mice. Thus, it is possible that at older ages, especially around 10-12 months, when Aβ burden is massive [179], TTR negative contribution is overcomed by other mechanisms contributing to disease. At this point, TTR stabilization by IDIF and by other molecules is no longer enough to counteract disease progression. Supporting this idea it is noteworthy that amelioration of IDIF treated AD/TTR+/− mice at both biochemical and behavioral levels did not go beyond the disease extent found in AD/TTR+/+ animals [159].

In summary, the final conclusion on the effect of IDIF and tolcapone in the AD-like neuropathology in the AD/TTR+/- mice as well as the validation of the study by Ribeiro and co-workers, will be clarified after the analysis of the PET results. This will allow to evaluate the effect of these two compounds at the several time points in which PET was performed, from 5 to 15 months.

3 FUTURE PERSPECTIVES

With this work, the angiogenic activity of TTR began to be revealed *in vivo* but a long road has yet to be covered to characterized this new TTR function.

Future work should:

1. Address the importance of TTR stability by evaluating the behavior of mutants of TTR known to be tetrameric unstable, such as the L55P TTR, in the CAM assay. If the angiogenic activity is reduced, compared to the WT, then the use of stabilizers should restore that activity.

2. It would also be interesting to evaluate the importance of the TTR proteolytic activity in its angiogenic response, by using a proteolytically inactive mutant of TTR in the CAM assay.

3. Measure the molecules found altered by TTR (IL-6; IL-8; Ang2 and VEGF) in mice with different TTR genetic backgrounds, as well as levels of metalloproteases -2 and -9.

4. To contribute to a detailed characterization of the angiogenic activity of TTR, and in order to specify the processes in which TTR participates, EC migration, proliferation and survival should also be addressed, namely in *in vitro* studies.

5. To gain insights into how TTR regulates angiogenesis it would be interesting to measure the levels of key components of important pathways in angiogenesis, in addition to the ones already evaluated and reported in this thesis.

6. Evaluate the brain vascular density in mice with different TTR genetic backgrounds, with or without the AD component.

7. Perform early treatments, starting at the age of 3 months and for 2 months, of AD/TTR+/- mice with TTR stabilizers, namely IDIF, and then measure collagen IV levels and vascular density.

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