Chapter 1

Modulating Role of TTR in Aβ Toxicity, from Health to Disease

Isabel Cardoso, Luis Miguel Santos and Mobina Alemi

Additional information is available at the end of the chapter

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Abstract

Amyloidosis is a generic term that refers to a wide spectrum of diseases that are characterized by the deposition of proteins in different organs, forming insoluble aggregates. Examples include islet amyloid polypeptide (IAPP) associated with diabetes type 2, prion protein (PrP) related with spongiform encephalopathies, (TTR) associated with familial amyloidotic polyneuropathy (FAP), and amyloid-beta (Aβ) peptide linked to Alzheimer's disease (AD), the most common form of dementia. Aβ peptide, thought to be the causative agent in AD, is generated upon sequential cleavage of the amyloid precursor protein (APP), by beta- and gamma-secretases, and it is believed that an imbalance between Aβ production and clearance results in its accumulation in the brain. TTR is a 55 kDa homotetrameric protein synthesized by the liver and choroid plexus of the brain and is involved in the transport of thyroid hormones and retinol. TTR protects against Aβ toxicity by binding the peptide, thus inhibiting its aggregation. Also, increased Aβ levels are found in both brain and plasma of AD mice with only one copy of the TTR gene, when compared to animals with two copies of the gene, suggesting a role for TTR in Aβ clearance. Growing evidence also suggests a wider role for TTR in central nervous system neuroprotection, including in the cases of ischemia, regeneration, and memory.

Keywords: Alzheimer's disease (AD), Aβ peptide, transthyretin (TTR), neuroprotection, blood–brain barrier (BBB)

1. Amyloidosis

1.1. Amyloidosis definition

Amyloidosis has long been used as a general term referring to a wide spectrum of protein-misfolding diseases [1], which are characterized by the extracellular deposition of those proteins in different organs, consequently forming insoluble aggregates called amyloid, a term popularized by Virchow in 1854 [2]. According to the Nomenclature Committee of the International Society of Amyloidosis (ISA), 31 identified proteins form extracellular amyloid fibrils in humans [3]. Amyloid fibrils are characterized by certain tinctorial properties, independently of the precursor protein forming the deposits, that for a long time were the only diagnosis available. These include apple-green birefringence under polarized light after staining with Congo red and yellow-green fluorescence after staining with thioflavin S and thioflavin T; thioflavin T has also been shown to interact with amyloid in suspension producing a specific fluorescent signal with a new excitation maximum at 450 nm. Ultrastructural studies by transmission electron microscopy (TEM) revealed that the amyloid material is fibrillary appearing as bundles of straight or coiled fibrils, non-branched, 7–10 nm wide and variable in length; in most cases, they seem to be helically twisted. Amyloid fibrils present a high content in β-pleated sheet as demonstrated by x-ray diffraction analysis and extensive antiparallel β-sheet strands with their axes running perpendicularly to the axis of the growing fibril (cross-β pattern).

Amyloid deposits are not entirely composed of the amyloid precursor protein. Several components have been found associated with all amyloid fibrils. These include serum amyloid P component (SAP), sulphonated glycosaminoglycans (GAGs), apoliproteins E and J, α1-antichymiotrypsin, several basement membrane components such as fibronectin, laminin and collagen type IV, complement proteins, and metal ions.

The extracellular deposition of fibrillary proteins leads to cell damage, organ dysfunction and death, and thus, these proteins are associated with a unique clinical syndrome, as seen in the case of the islet amyloid polypeptide (IAPP) associated with diabetes type 2 [4], prion protein (PrP) associated with the spongiform encephalopathies [5], transthyretin (TTR) associated with familial amyloidotic polyneuropathy [6] (FAP), and amyloid-beta (Aβ) peptide associated with Alzheimer's disease [7] (AD), among others.

Amyloid disorders are usually divided into two categories depending on the distribution of the amyloid deposits: localized and systemic amyloidosis. In localized amyloidosis, amyloid is restricted to a single tissue or organ, usually in the surroundings of the cells responsible for the synthesis of the precursor protein; in systemic amyloidosis, the amyloidogenic proteins are usually derived from circulating precursors that are either in excess, abnormal or both. Amyloidosis can also be hereditary or non-hereditary.

This chapter will focus in AD, a form of localized amyloidosis affecting the central nervous system, and the most common form of dementia. In particularly, we will discuss the neuroprotective role of TTR in AD, in addition to its amyloidogenic role in FAP, an example of systemic amyloidosis with a special involvement of the peripheral nerve.

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2. Overview of AD

AD was firstly described by Alois Alzheimer in 1906 and is characterized by progressive loss of cognitive functions, ultimately leading to death [8]. This condition highly affects not only the life of patients but also the life of their caregivers. Pathologically, the disease is characterized by the presence of extraneuronal amyloid plaques consisting of aggregates of the Aβ peptide, and neurofibrillary tangles (NFTs) which are intracellular aggregates of abnormally hyperphosphorylated tau protein [9]. Aβ peptide is generated upon sequential cleavage of the amyloid precursor protein (APP), by beta- and gamma-secretases, and it is believed that an imbalance between Aβ production and clearance results in its accumulation in the brain.

2.1. From the first description to the confirmation

Alzheimer's disease was first described in the 1907's paper entitled "Uber eine eigenartige Erkankung der Hirnrinde," by Alois Alzheimer [10], in which he reported the behavior of a 51-year-old female patient (Auguste Deter) of the insane asylum of Frankfurt am Main. The patient presented several symptoms that caught Alzheimer's attention, apart from the central nervous system anatomical characteristics. Among others, time and space disorientation, rapid loss of memory, and mood swings were the most prominent symptoms [10]. In relation to pathological features, the observation of something that looked like "thick bundles" [10] of fibrils, later known as senile/amyloid plaques and NFTs [11], transformed AD into a unique condition, distinguishing it from the other neurological conditions known to date.

2.1.1. Symptoms and Diagnosis

Since 1907, clinicians have been trying their best to accurately identify AD-related symptoms and to divide and organize these symptoms in the simplest form. Burns et al. came out in 2002 with three different categories: (1) cognitive deficits that affect memory (amnesia and agnosia), speech (aphasia), and motor behavior; (2) psychiatric symptoms and behavioral disturbances, including depression, anxiety, delusions, and misidentification; (3) difficulties with the daily living activities, such as driving, using the telephone, dealing with money and, later in the disease, all the basic needs (feeding, dressing, toileting) [12–14]. As expected in such a complex condition, a huge symptomatic variation is found in AD patients, although a positive correlation between symptom severity and disease evolution is observed.

Although AD is seen as an elderly disease due to its higher prevalence in the older population (approximately 5.3 million people solely in the US, in 2015) [15], it is also the most frequent form of dementia under the age of 65, with up to 5% of all cases [16]. Of curiosity, every 67 s, one more person is diagnosed with AD, and, by 2050, one new case of AD is expected to develop every 33 s [15]. Due to this disease complexity, diagnosis guidelines had to be established, and for a long time, the main criteria adopted was the one decided at the 1984 consortium, by the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA). These criteria divided AD in three possible diagnosis scenarios, which were possible, probable, or definite AD [17]. More recently, some minor alterations have been proposed in order to

comprise also the stages prior to the clinical observation of symptoms [18, 19], thus prompting three renewed stages: (1) preclinical Alzheimer's disease; (2) mild cognitive impairment (MCI) due to Alzheimer's disease; and (3) dementia due to Alzheimer's disease [20].

Despite all the attentions directed to the establishment of proper diagnostic criteria and guidelines available, the diagnosis of AD is still not an easy task. Actually, a recent meta-analysis showed that the sensitivity and specificity of the clinical diagnosis ranged from 53 to 99% and 55 to 99%, respectively [21]. Although alone it is considered a low value, when combined with other characterizing techniques (as neuroimaging and biomarkers—see Biomarkers section), it is possible to predict/diagnose AD with a high confidence.

Genetically, AD is usually divided in two forms: autosomal dominant familial AD (FAD; predominantly of early-onset—under the age of 65) and sporadic AD (also called and usually associated to the late-onset AD—more than 65 years) [22]. Although extensively used, it is important to point out that this classification is far too simplistic.

Despite all of the effort put into research, the primary event triggering AD remains yet a mystery. Nonetheless, for FAD, several mutations capable of triggering the disease have been identified, especially in three distinct genes: the amyloid precursor protein (APP) [23], the presenilin 1 (PSEN1), and the presenilin 2 (PSEN2) genes [24], in chromosomes 21q, 14q, and 1q, respectively. Although these three genes comprise approximately 55% of all mutations, they are only responsible for less than 1% of all cases of AD (http://www.molgen.vib-ua.be/ADMutations/). Contrary to FAD, sporadic AD does not exhibit autosomal-dominant inheritance but up to 60–80% of this form of AD is genetically determined [22].

2.2. The biochemical basis of AD

In spite of its multifactorial etiology, AD is characterized by two specific brain lesions, the amyloid plaques, and the NFTs, considered the hallmarks of AD. Also, associated with these abnormalities, it is often observed severe neural loss and reactive gliosis.

NFTs are filamentous inclusions (intracellular lesions), preferentially observed in pyramidal neurons, which are composed of filamentous aggregates of abnormally hyperphosphorylated microtubule-associated protein tau [25]. Even though NFTs are a hallmark of AD, they are also observed for other neurodegenerative disorders termed tauopathies (e.g., sporadic corticobasal degeneration, palsy, and Pick's disease, progressive supranuclear palsy, Seattle family A, parkinsonism–dementia complex of Guam, and some frontotemporal dementias) [26, 27].

As for amyloid plaques, they can be distinguished in different plaques subtypes, depending on their composition, and being the neuritic and diffuse plaques the two major subtypes in AD. Neuritic plaques are constituted by the 40- and 42-amino acids (aa) Aβ peptides [28] (Aβ40 and Aβ42, respectively), surrounded by dystrophic neurites (axons and dendrites), microglia (monocyte- or macrophage-derived cells that reside in the brain), and reactive astrocytes [29]. Diffuse deposits are mainly composed of Aβ42 [28] and lack the neuritic and glial components [29], but evolve over time with formation of discrete niduses that eventually become neuritic amyloid plaques [30].

2.3. Amyloid-β precursor protein and Aβ formation

The amyloid-β precursor protein (APP) is a transmembrane receptor expressed ubiquitously in both neuronal cells and extra-neuronal tissues [31]. In humans, the APP gene is located in the chromosome 21, explaining partially the increased risk for Down syndrome patients to develop AD, and is composed of 18 exons [32]. Three major isoforms are expressed by alternative splicing: APP770 (full length), APP751 (lacking exon 8), and APP695 (lacking exon 7 and exon 8) [23, 31, 33]. APP belongs to a highly conserved family of type 1 transmembrane glycoproteins that extends also to invertebrate species, including the homologous: APL-1 (*Caenorhabditis elegans*), APPL (*Drosophila*), APLP1, and APLP2 (in mammals, besides APP) [34], and appa and appb (zebrafish) [35]. Following translation, APP is trafficked through the endoplasmic reticulum (ER), Golgi and trans-Golgi network (TGN), where it suffers specific endoproteolytic cleavages [33] that will originate several APP metabolites, among them the Aβ peptide. After reaching the membrane surface, APP can still undergo clathrin-mediated endocytosis and then be recycled to the surface again [36], during which Aβ can also be produced [37].

The 4 kDa Aβ peptide was first isolated and sequenced by Glenner and Wong, in 1984 [7] and can be found in the plasma and cerebrospinal fluid (CSF) of healthy humans and other mammals [38]. It was described as a 24 aa peptide but later, sequencing analysis revealed that the peptide could actually comprise 36–43 aa [39], being the two major species Aβ40 and Aβ42. In healthy individuals, these two forms make up about 90 and 10%, respectively, of the Aβ peptides that are normally produced by brain cells [40]. Despite the small difference in size and sequence of the various isoforms, they differ greatly in properties; for example, Aβ42 is more hydrophobic, thus, more prone to aggregation (compared to the less hydrophobic Aβ40). In fact, it readily aggregates in vitro, being considered the more amyloidogenic and hence pathogenic species [41].

2.3.1. Towards amyloid or not?

APP processing can originate different metabolites that bear very different physiological functions, depending on the proteolysis pathway adopted: the amyloidogenic or non-amyloidogenic pathway (**Figure 1**). In the non-amyloidogenic pathway, APP is firstly cleaved by the α-secretase, a zinc metalloproteinase of the ADAM family [42], followed by the action of γ-secretase. The latter is a high molecular weight complex of four proteins: presenilin 1 or 2 (PSEN1, PSEN2), nicastrin (NCT) [43, 44], anterior pharynx-defective 1 (APH1), and presenilin enhancer 2 (PEN2) [45]. The cleavage by the α-secretase (at Lys687 of APP770) [46], within the Aβ domain, abrogates the production of Aβ, resulting in the release of a large soluble ectodomain of APP (sAPPα, ~100 kDa), leaving behind a 83-residue carboxi-terminal fragment (CTFα, of ~10 kDa) [47]. Then, γ-secretase cuts the CTFα, liberating the extracellular p3 peptide and the 50 aa APP intracellular domain (AICD, of ~6 kDa) [48].

On the other hand, as suggested by its name, the amyloidogenic pathway gives rise to the amyloidogenic Aβ peptide, and similar to the previous pathway, it consists of two sequential cleavages, first by the β-secretase (beta-site APP–cleaving enzyme 1–BACE-1), and then by γ-secretase. The first protease cleaves APP at Met671 [49], releasing the large soluble ectodomain

sAPPβ [33]. The remaining 99 aa CTFβ (of ~12 kDa) [50] is then cleaved by the γ-secretase, in the membrane, and originates, as said above, the Aβ peptide and the AICD [48]. This process generates different Aβ species, with variable hydrophobic C-termini (related to the γ-secretase cleaving site), that present different propensity to oligomerize [51] and, consequently, to form the amyloid plaques. Noteworthy, AD-linked mutations in the PSEN1 and PSEN2 proteins, particularly important in the case of FAD, influence γ-secretase-mediated processing of APP, and selectively enhance Aβ42 production compared to Aβ40 [52].

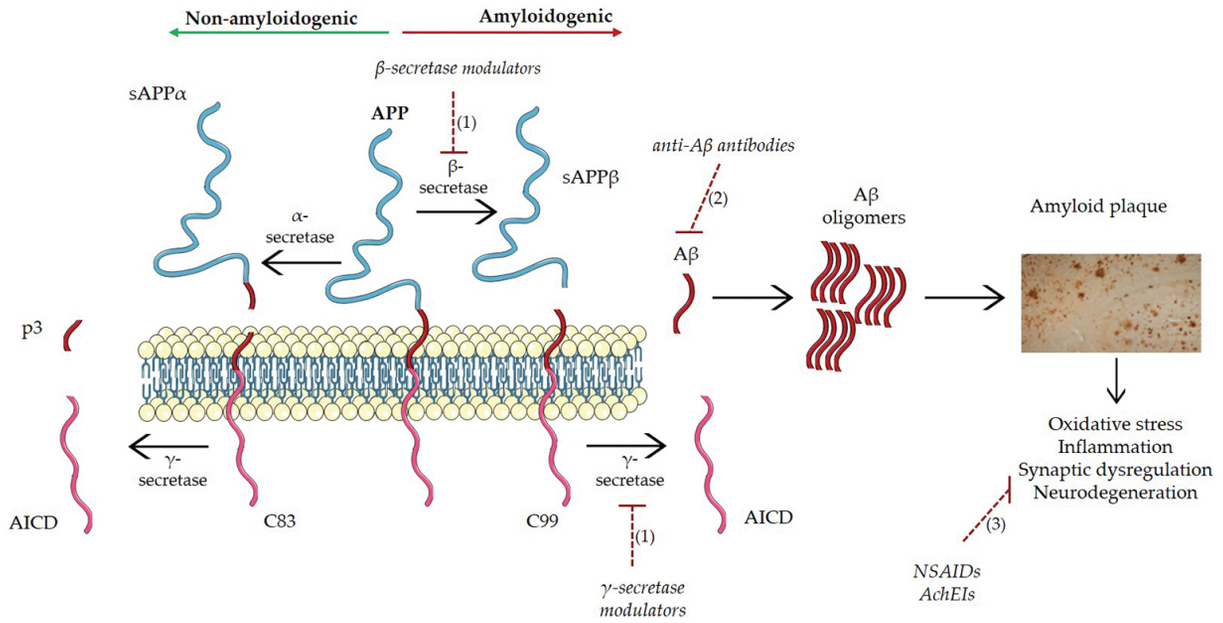

Figure 1. The amyloidogenic and non-amyloidogenic pathways of APP. In the non-amyloidogenic pathway, APP is cleaved by the α-secretase releasing the sAPPα neuroprotective N-terminal fragment, which contains part of the Aβ sequence. The 83 aa APP fragment (C83) then suffers the action of the γ-secretase, liberating the p3 peptide and the AICD fragment. The amyloidogenic pathway involves the sequential cleavage of APP by the β-secretase which releases the soluble ectodomain sAPPβ. The remaining C99 fragment of APP is then cleaved by the γ-secretase, resulting in the formation of the Aβ peptide. Due to its high propensity to aggregate, Aβ peptide oligomerizes, accumulates and forms amyloid senile plaques, in turn leading to the described alterations of AD. Current therapeutic approaches in AD include: (1) inhibition of β- and γ-secretases, (2) improving Aβ clearance, and (3) amelioration of inflammation and synaptic dysregulation.

Although tightly related to AD onset, APP processing is a normal metabolic event and Aβ is a normal product of cellular metabolism throughout life, circulating as a soluble peptide in biological fluids [53]. Plus, Aβ deposition can also be found, together with NFTs, in the brain of non-demented elderly people [54].

2.4. Alzheimer's disease hypotheses

AD is one of the human diseases with the highest number of hypothesis formulated trying to explain its pathogenesis, with very different and plausible molecular mechanism to back them up. Within the list, the amyloid cascade hypothesis stands out, together with the so-called "tau

and tangle" hypothesis, strengthened by the fact that they are based in the two hallmarks of AD.

2.4.1. Amyloid cascade hypothesis

Since its formalization, in 1992 by Hardy and Higgins, the amyloid cascade hypothesis has had a prominent role in explaining the etiology and pathogenesis of AD. They suggested that amyloid deposition was the primary influence driving AD pathogenesis [55], due to two key observations: the detection of Aβ as the main constituent of amyloid plaques, and the discovery of mutations in the APP, PSEN1 and PSEN2 genes associated with FAD [56]. This hypothesis stated that a dysregulation in APP processing or Aβ clearance would provoke an increase in the Aβ42/Aβ40 ratio, which would promote aggregation, accumulation, and plaque formation. In turn, this would be responsible for the subsequent pathology (including tau aggregation, phosphorylation, neuronal attrition, and clinical dementia) [57]. Due to its inability to entirely explain AD pathogenesis, especially by the lack of correlation between plaque burden and clinical manifestations [58], this hypothesis has been upgraded over the past few years. Scientists started to divert their attention from the effects of the amyloid deposits, to study the other forms (monomers, oligomers, and protofibrils—usually shorter and thinner then mature fibrils) [59] of Aβ peptide-induced neurotoxicity. Some studies suggest that Aβ toxicity functions in a plaque-independent manner, indicating that oligomeric intermediates present higher toxicity to the cells [60] and that activation of signaling pathways due to intraneuronal accumulation of Aβ oligomers is responsible for tau hyperphosphorylation and subsequent deposition [61]. Several explanations have been proposed, with some defending that oligomeric toxicity is related to a greater capacity for diffusion and a larger collective surface area for interacting with neuronal and glial cells [60], while other proposed that it is not related to a specific prefibrillar aggregate (dimer, trimer, and so on) but rather to the propensity that each species has to grow and undergo fibril formation [62].

A more consensual vision about Aβ is that it possesses a dual role: On one hand, it can be a neurotrophic agent or a neuroprotector against excitotoxicity (by activating the phosphatidylinositol-3-kinase (PI-3K) pathway); on the other hand, an inducer of neuronal degeneration (at high concentrations) in mature neurons [63].

Other interesting and amyloid cascade-opposite hypotheses have been proposed, stating that Aβ should not be seen as the initiating factor for neurodegeneration in AD, but instead, its deposition is nothing more than a protective mechanism to neuronal insult, in which Aβ binds and removes harmful substances by blocking them in plaques [64, 65].

2.4.2. Tau hypothesis

"Tauists" defend a collection of ideas that maintain the primacy of NFTs formation as the AD-causing event, which Mudher and Lovestone designated as the "tau and tangle hypothesis" [57]. It started to emerge due to solid evidence that amyloid plaques do not account for the complex pathophysiology of AD [66], opposed to the observed highly positive correlation between NFTs and cognitive deficits [67]. It argues that in AD the normal role of tau (micro-

tubules stabilization) is impaired and that NFTs accumulate and occupy much of the neuron, resulting in neuronal death. This was supported by the visualization of the extracellular tangles in the shape of neurons, abundant in the late stages of disease [57]. Also, the discovery of mutations within the tau gene that cause fronto-temporal dementia and parkinsonism linked to chromosome 17 (FTDP-17), demonstrating that tau dysfunction, in the absence of amyloid pathology, was enough to cause neuronal loss and clinical dementia [68], further strengthened this hypothesis. However, tau mutations do not originate amyloid plaques, whereas APP and presenilin gene mutations give rise to amyloid and tau depositions, strongly evidencing that amyloid pathology is upstream of tau pathology [57]. More recently, a more embracing tau hypothesis was proposed, in which a series of damage signals (Aβ oligomers, oxygen-free radicals, iron overload, cholesterol levels in neuronal rafts, low-density lipoprotein (LDL) species and homocysteine, among other) trigger, by innate immunity, the activation of microglial cells with the consequent release of pro-inflammatory cytokines that modify neuronal behavior through anomalous signaling cascades, which finally, promote tau hyperphosphorylation [66]. In turn, tau hyperphosphorylation will contribute to further activation of microglial cells and stimulation of the deleterious cycle, which will lead to progressive neuronal degeneration [66]. The degree of tau phosphorylation in the AD brain is reasonably well correlated with the severity of AD symptoms; however, fetal tau, a much more phosphorylated form of tau than adult tau, does not induce AD-like pathology [69]. In summary, there is no direct evidence for the neurotoxicity of hyperphosphorylated tau (as in the case of Aβ toxicity).

2.4.3. "Other" hypothesis

2.4.3.1. GSK-3 hypothesis

Glycogen synthase kinase-3 (GSK-3), a multi-tasking kinase with major roles in brain signaling, has been recently proposed as a central player in AD pathology. This was supported by observing that the deregulation of this protein is responsible for many of the pathological hallmarks of the disease, in both sporadic and familial AD cases [70]. It was suggested that the hyperactivity of GSK-3β, the most abundant of two isoforms (GSK-3α and GSK-3β) expressed in neurons, is intimately involved with cognitive impairment [71], Aβ production [72], tau hyperphosphorylation [73], acetylcholine synthesis [74], neuronal death [75], and neuroinflammation [76] in AD. Furthermore, regarding Aβ interaction, it was observed that Aβ also regulates GSK-3β activity [77, 78] making it difficult to establish which event is located upstream. Thus, this hypothesis is seen as an integration and extension of the amyloid cascade hypothesis, still conferring to Aβ a central role in AD pathology. Although GSK-3 modulation appears to be an excellent therapeutic approach, no effective result has been observed in trials, perhaps due to its activity in multiple targets.

2.4.3.2. Oxidative stress/mitochondrial hypothesis

The brain is especially vulnerable to free radical damage as a result of its high oxygen consumption rate, abundant fatty acids content, and the relative low levels of antioxidant enzymes

[79]. The most appealing feature of the oxidative stress hypothesis is its slow and cumulative damaging nature that could, over time, account for the late life onset and slowly progressive nature of AD, and neurodegeneration in general [80]. Also supporting this hypothesis is the suggested unbalanced levels of heavy metals in the brain, among others, iron (Fe), copper (Cu), aluminum, and mercury, which function as catalysts for oxygen free radical generation [80]. There has been high controversy in the measurement of these elements, especially Fe, but most studies reveal an apparent unbalance in AD brains compared to controls. In a recent study, Fe was found significantly increased in patients with severe AD [81] (as previously reported [82]). Nonetheless, some consider that this "accepted" elevation, even if significant in some studies of AD pathology, does not account for brain degeneration, and so, presents itself as a misleading therapeutic target with considerable risks for patients (reviewed in [83]). As for Cu, it has been shown to be decreased in AD brains [81], which at first goes against the oxidation hypothesis. More recently, and also in the presence of some contradictory results [84], copper was found to bind strongly to Aβ aggregates, inhibiting in vitro amyloid fibril formation [85]. In addition, and when bound to the aggregates, copper exhibited a redox role, by degrading hydrogen peroxide [86]. Protein and DNA oxidation (in particular mitochondrial DNA), and lipid peroxidation (which affects the phospholipid-rich membrane) were also found to be increased in AD brains [80].

Another hypothesis intimately related to the brain redox status is the mitochondrial cascade hypothesis [87]. Mitochondria are considered the cell "powerhouses"; however, when in a non-physiological energy production, they can provoke severe damage by increasing the reactive oxygen species (ROS). Curiously, mitochondria are the first target of ROS, suffering DNA oxidation, which may lead to a further increase of ROS production, generating a vicious cycle [88]. The authors of this hypothesis state that sporadic and autosomal dominant AD are not etiologically homogeneous and that mitochondrial dysfunction works as a link for both. Very briefly, in autosomal dominant forms, Aβ-induced mitochondrial dysfunction leads to the other AD-characteristic histopathologies, while in sporadic AD, mitochondrial malfunction induces the AD pathologies, including processing of APP to Aβ [89].

2.4.3.3. The cholinergic hypothesis

The cholinergic hypothesis was proposed after the observation of a decrease of choline acetyltransferase (ChAT) in AD patients [90]. It states that the loss of cholinergic cells in the septal nuclei and basal forebrain (described in patients with advanced AD [91]) compromises the innervation of the cerebral cortex and related structures, which play an important role in cognitive functions, especially memory [92]. More recent studies have suggested a bidirectional pathway linking Aβ toxicity in cholinergic dysfunction and the interaction of cholinergic regulatory mechanisms in the processing of APP [93]. Discrediting this hypothesis, later studies showed that the cholinergic degeneration [94] and the decrease in ChAT enzyme activity [95] are not observable in the early stages of disease. This was accompanied by the fact that treatment with acetylcholinesterase inhibitors does not offer long term cure, although it has shown consistent, despite modest, benefits in symptoms improvement [96]. Nonetheless,

some studies point out that compensatory mechanisms could overcome the cholinergic defects, disguising its effects in early stages of AD or mild cognitive impairment [97].

2.4.3.4. Calcium hypothesis

The calcium hypothesis was first introduced by Khachaturian in 1982, stating that cellular mechanisms which maintain the homeostasis of cytosolic Ca^{2+} play a key role in brain aging and that sustained changes in Ca^{2+} homeostasis could provide the final common pathway for age-associated brain changes [98], or in this case, be the proximal cause of neurodegeneration in AD. Out of curiosity, this proposal was purely speculative at the time, only sustained on circumstantial evidence from a handful of studies [98]. It was observed that the persistent elevation of the levels of Ca^{2+} leads to the constant elimination of newly acquired memories, due to a stimulation of long-term depression mechanisms [99]. In fact, calcium signaling dysfunctions occur during the initial phases of the disease, and even before the development of pronounced symptoms [100]. However, whether it is calcium dyshomeostasis that provokes Aβ production and accumulation [101], or vice-versa [102], remains to be elucidated. Either way, in addition to the Aβ unbalance, increased levels of Ca^{2+} promoted protein tau hyper-phosphorylation [103]. It was also suggested that amyloid oligomers induce membrane permeabilization, leading to increased intracellular Ca^{2+} concentration [104]. Nevertheless, there is some disagreement as to the mechanism by which amyloid oligomers increase intracellular calcium.

2.5. Biomarkers and risk factor

Despite of the extensive Knowledge on the causative gene mutations responsible for familial AD, the sporadic (non-genetic) form of this disease, which results from the diverse interactions between genetic and environmental factors, is still lacking characterization. Thus, researchers are continuously looking for specific molecules that should be altered exclusively in AD, and preferentially in the asymptomatic period. This, combined with the meticulous description of the patient risk factors, may give the opportunity for an early action and increase the success rate of therapeutics.

2.5.1. Biomarkers

By definition, and according to the International Programme on Chemical Safety, biomarker is "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [105]. The search for early AD biomarkers has been highly targeted over the last years, as investigators believe that the generation of an effective treatment for AD is only possible if the disease is detected at very early stages. Thus, the discovery of biomarkers is of extreme importance for the early diagnosis of AD and even for predicting the conversion of MCI into AD patients.

The search for solid AD biomarkers started with those who seem to be altered in this condition when compared to normality. Several studies showed that the combination of CSF total- and phospho-tau, and CSF and plasma Aβ42 is able to predict with increased sensitivity

and specificity the development of AD in patients with MCI [21, 106, 107], in addition to the already established role of the ApoE-ε4 isoform [108] (major susceptibility gene—see Section 2.5.2. Risk Factors). Studies in FAD-causing mutations carriers showed increased levels of CSF total-tau and plasma Aβ42, although having reduced CSF Aβ42 levels, at least 10 years before the symptoms establishment [106]. Also, the ratio of Aβ42/Aβ40 in CSF and plasma was found decreased, respectively, for non-demented mutation carriers [109] and cognitively normal elders (which evolved to MCI or AD) [110]. Other CSF biomarkers, such as BACE-1 [111] and sAPPα/β [112], are also suggested; however, independent studies were not consistent [106], possibly due to technical difficulties in the biomarkers quantification [113]. Our group has also proposed TTR as a biomarker in plasma, demonstrating a negative correlation with AD severity [114], which supported prior observations for CSF levels. In that study, the authors also considered TTR a selective biomarker for AD [115]. Other studies contradict this idea suggesting that TTR potential as biomarker raises some doubt since its levels appear to fluctuate substantially within a single individual over a 2-week interval [116].

In addition to the so-called fluid biomarkers, physicians have at their disposal powerful imaging technology. With the improvement in PET (positron emission tomography) and MRI (magnetic resonance imaging) spectroscopy resolution, neuroimaging has been gaining importance and increasing the confidence in AD diagnosis. Due to the possibility of using specific tracers, such as a derivate of thioflavin T that crosses the blood–brain barrier (BBB) and binds selectively to Aβ (C11-labeled Pittsburgh Compound B–PiB), it is possible to identify amyloid deposition in the brain in vivo [117]. In 2004, Klunk and colleagues performed the first study of brain amyloid imaging (BAI) using the PiB compound, in which they showed a robust relationship between amyloid deposition and PiB retention [118]. The combination of increased BAI signal, low CSF Aβ42, and high CSF p-tau in a subject with dementia is seen as a "definite" diagnosis of AD. Furthermore, BAI and CSF profiles can be used to predict patients with MCI who will progress to frank dementia with high degree of confidence [119]. Despite the value of this compound, the resulting data should be subjected to careful analysis since healthy subjects also present amyloid deposition, hindering the differentiation between symptomatic AD and asymptomatic controls with amyloid plaques [120]. MRI (structural evidence) is also a common technique often used, alone or in combination with CSF tau and Aβ42, to predict development of AD [121]. Curiously, a recent study showed that the MRI together with PiB-PET makes the best combination of biomarkers, thus showing the best AD predictive value [119].

2.5.2. Risk factors

2.5.2.1. Environmental factors

As Stephen King wrote in The Gunslinger: "Time's the thief of memory," and so, the most worldwide accepted (and intuitive) risk factor is aging. In every species, age brings a slowing of brain function [122], thus preventing the brain to properly respond/recover from insults. The increasing of life expectancy, in addition to the increasing of population (attributed to the

postwar "baby boom"), turned aging in a major risk factor. Also gender appears to play a role in disease development, since data show that women are more prone to this disease than men. Although women present higher life expectancy, also in younger study groups (60–80 years), where differences in death rate are insignificant, women present higher incidence of cases [123]. The specific mechanism is unknown; however, several factors have been proposed to influence, such as: age-related sex hormone reduction, risks of other diseases (diabetes, depression, cardiovascular disease), and differences in brain anatomy and metabolism [124].

The cardiovascular risk factors, which appear sometimes as a distinct group of factors, include diabetes mellitus [125], overweight [126], hypertension [127], and high cholesterol levels [128]. Individually or in cooperation, these factors increase the predisposition for cognitive decline. The midlife control of the above cardiovascular factors has been associated with a reduction in white matter lesions in late life [127]. As for cholesterol, results appear to be inconsistent; however, lipid-lowering treatments present benefits against white matter lesions [126].

Contrasting with the previous risk factors, wine consumption, coffee consumption, the use of non-steroidal anti-inflammatory drugs (NSAIDs), and physical activity are associated with reduced risks, thus showing some protective effects [129].

2.5.2.2. Genetic factors

ApoE exists as three isoforms ε2, ε3 and ε4, with ε3 having the highest prevalence. As Corder and colleagues stated, in 1993, ApoE plays an important role in AD, with the risk of developing disease increased in carriers of the ApoE-ε4 allele, such that a double dose of this allele was nearly enough to cause AD by the age of 80 [130]. Despite the broad molecular evidence about ApoE role in AD, its genetic variation is also present in other kinds of neurological disorders, including Parkinson's disease and multiple sclerosis [22]. In 2009, three novel AD genes were identified, presenting high degree of association: CLU (clusterin or apolipoprotein J), CR1 (complement component (3b/4b) receptor 1), and PICALM (phosphatidylinositol-binding clathrin assembly protein). Down's syndrome (or trisomy 21) is also considered a genetic risk factor. This condition is the result of a third copy of the chromosome 21, which coincides with the location of the APP gene [131], giving rise to an increased accumulation of Aβ.

2.6. Drugs and treatments

Due to the complexity of AD, a vast number of targets and pathways may be chosen to intervene. Cholinergic degradation inhibitors, immunotherapy, secretase inhibitors, anti-inflammatory drugs, and tau- and Aβ-deposition interfering drugs (**Figure 1**) are but a few examples of the many classes of drugs that are being tested at the moment.

The first drugs developed for AD, acetylcholinesterase inhibitors (AchEI), aimed at increasing acetylcholine levels (see "Cholinergic hypothesis"). Currently, FDA has five drugs approved for the "treatment" of AD in the initial stages: 4 AchEI (Donepezil, Rivastigmine, Galantamine, and Tacrine) and 1 NMDA receptor antagonist (Memantine) (http://www.alzforum.org). As referred above, they treat and ameliorate the symptoms, but do not cure.

In 2010, Rinne and colleagues showed for the first time target engagement from a disease-modifying drug in humans, using the monoclonal anti-Aβ antibody bapineuzumab [132, 133]. This study showed a reduction of fibrillar amyloid in the brain of AD individuals, but did not improve cognition and stopped at phase 3 [133]. Crenezumab, another anti-Aβ, was selected for pre-symptomatic treatment trials of Colombian mutant PSEN1 kindred [133], however, showed extensive cross-reaction with non-Aβ related proteins [134]. Intravenous immunoglobulins (IVIG) have also been proposed as a potential treatment, based on the hypothesis That IVIG contain naturally occurring antibodies that specifically promote clearance of Aβ peptides from the brain [135].

β- and γ-secretase modulators [133], Aβ and tau deposition modulators (e.g., scylloinositol and methylene blue, respectively [39]), and molecules addressing oxidative damage (resveratrol) are also potential drugs under study. Recently, deep brain stimulation (DBS) performed by the group of Dr Lozano in AD patients showed promising results, with improvements and/or slowing in the rate of cognitive decline [136], increased hippocampus volume and glucose metabolism [137]. The authors propose that DBS is able to influence the structure of the brain and that hippocampal atrophy can potentially be slowed, suggesting restorative properties to DBS [137].

A general recommended therapy is a good diet and a healthy lifestyle, in order to control cardiovascular risk factors, decreasing cerebrovascular events. No effective treatment has been found, thus increasing the interest for the early diagnose of AD, to allow a more effective and early stage intervention.

3. Blood–brain barrier and Alzheimer's disease

The BBB is a profoundly specialized brain endothelial structure of the differentiated neurovascular system. These specialized endothelial cells interacting with astrocytes, microglia, and pericytes, confine components of the circulating blood from neurons. Furthermore, the BBB controls the chemical composition of the neuronal environment which is required for the functioning of neuronal circuits, synaptic transmission, synaptic remodeling, angiogenesis, and neurogenesis. BBB malfunction, through the disruption of the tight junctions (TJs) and alteration of the transport of molecules between blood and brain, brain hypoperfusion, and inflammatory responses, may begin or contribute to the process of different diseases such as AD, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis. These data support developments of new therapeutic strategies for the neurodegenerative disorders focused at the BBB [138].

3.1. The blood–brain barrier: the earliest findings

In 1885, when scientist Paul Ehrlich injected trypan blue dye into the bloodstream of mice, he noted that the stain colored all of the animal organs, except the brain [139]. In 1913, one of the Ehrlich's students, Edwin Goldmann, performed a follow-up experiment by injecting the same dye into the brain of mice. He observed that injection of trypan blue directly into the CSF

stained all cell types in the brain but failed to penetrate into the periphery [140]. Although aiming at finding new compounds that could attack disease-causing microbes, these experiments suggested a physical barrier between the brain and the blood, becoming the stepping stones on BBB research. Lewandowsky was the first to use the term blood–brain barrier while studying the limited permeation of potassium ferrocyanate into the brain [141]. However, it took until the 1960s for the specific anatomy of the network of brain–blood vessels comprising the BBB to be glimpsed [142]. Using electron microscopy, in 1967, Reese and Karnovsky showed that the BBB is localized at the level of TJs between adjacent brain endothelial cells [143, 144].

It is now known that the brain of mammals is separated from the blood by the BBB, localized to the brain capillaries and pia-subarachnoid membranes, and by the blood-CSF barrier, confined to the choroid plexus [145]. At the junctional complex formed by the TJs and adherens junctions (AJs), brain endothelial cells are connected to each other [142]. Transcellular transport at the BBB occurs through several mechanisms including passive and active transport through different receptors and transporters. Interestingly, endothelial cells, pericytes, and astrocytes present at the BBB also express several enzymes such as cholinesterase, aminopeptidases and endopeptidases that alter endogenous and exogenous molecules, which can negatively influence neuronal function. This produces a metabolic barrier which protects the central nervous system (CNS) [146].

3.2. The neurovascular unit at the BBB; endothelial cells, pericytes, and glial cells

3.2.1. Endothelial cells and pericytes

A cerebral capillary lumen is enclosed by a single endothelial cell. Anatomically, the BBB endothelial cells are distinguished from those at the periphery by enhanced mitochondrial content [147], loss of fenestrations [148], least pinocytotic activity [149], and the presence of TJs [150]. The BBB tightly sealed monolayer of endothelial cells usually prevents the free exchange of solutes between blood and brain [151] except for the lipid-soluble molecules smaller than 400 Da with less than nine hydrogen bonds, which can cross the BBB without any support, via lipid-mediated diffusion [146].

As for pericytes (granular or filamentous), they are connected to the abluminal membrane of the endothelial cells [152]. Pericytes and endothelial cells are ensheathed by the basal lamina, composed of collagen type IV and other extracellular matrix proteins [153]. However, there is not much known about the involvement of pericytes at the BBB, but the addition of pericytes to co-cultures of endothelial cells and astrocytes has been shown to stabilize capillary-like structures [154]. In conditions associated with increased BBB permeability such as hypoxia or traumatic brain injury, pericytes have also been shown to migrate away from brain microvessels [155, 156]. Moreover, pericyte-derived angiopoietin provokes expression of TJs such as occludin in endothelial cells [157], confirming that pericytes are involved in the maintenance of the barrier properties in the cerebral endothelium [142].

3.2.2. Glial cells

Astrocytes are another cell type present in the neurovascular unit. These cells are usually located between neurons, pericytes, and capillary endothelium, and communicate with these cells via their several foot processes [138]. It is long believed that astrocytes are crucial in the development of the BBB properties [158]. It has been shown that co-culture of brain endothelial cells with astrocytes can enhance TJs of the brain endothelium [159]. Furthermore, endothelial cultures incubated with astrocyte-conditioned media have shown to improve BBB characteristics in vitro by de novo protein synthesis of γGTP in cerebral microvessel endothelial cells, which indicate that the glial cells may induce the cerebral capillary endothelial cells to express differentiated properties which allow the endothelium to function as the blood–brain barrier [160]. Also, astrocytes have been shown to regulate cerebral microvascular permeability [161], via dynamic calcium signaling between astrocytes and the endothelium [162]. This has been explained by Zonta et al., whereby an increase in neuron-induced astrocyte calcium promotes secretion of vasodilatory substances from perivascular astrocyte endfeet, resulting in improved local blood flow [163]. This work constituted a breakthrough in the knowledge of both astrocyte function and regulation of the activity-dependent cerebral blood flow [164].

Finally, another glial cell type present in the BBB is microglia which migrates from the yolk sac into the CNS parenchyma during embryogenesis [165]. Microglia performs critical functions in innate and adaptive brain immune responses. Microglia, when activated, transforms from “ramified” to an "ameboid" and ultimately to a “phagocytic” form. This evolution is correlated with alterations in expression of surface antigens and cytokines release [138]. Studies have shown that perivascular microglial cells are derived from bone marrow [166].

In vivo studies demonstrated that resident microglia cells in the brain parenchyma also communicate with CNS microvessels. This may suggest that microglia plays an important role in regulating BBB features during embryogenesis and diseases [167], besides an indisputable function in CNS development and homeostasis [168]. Furthermore, it has been shown that during embryonic stages of CNS vascularization, stabilization, and fusion of brain endothelial cells are mediated by resident microglial cells [167]. Also, interestingly, it has been shown that specific depletion of microglia results in reduced vessel density in a mouse model of choroidal neo-vascularization [169]. There are studies which suggest that microglial activation may be related to BBB disruption [170] apparently by producing ROS, through NADPH oxidase, which in turn impairs BBB function. Furthermore, TNF-α released from activated microglia has shown to affect BBB integrity, as permeability of endothelial cells co-cultured with microglia, was increased when microglial cells were activated by LPS and it was blocked by a neutralizing antibody against TNF-α, indicating that TNF-α contributes to BBB dysfunction [171]. The post-traumatic inflammatory response is shown to be associated with expression of cytokines such as IL-1β or metalloproteinases particularly MMP2, 3 and 5 produced mainly by microglia at the lesion site. It has also been demonstrated that these proteins can cause disruption of the basal lamina and/or redistribution and degradation of the TJs complexes [172, 173] resulting in BBB breakdown and neurological disorders after traumatic injury [174].

3.3. Junctional complexes and cytoskeleton linked proteins at the BBB

The presence of junctional complexes is one of the main characteristics of inter-endothelial space of the cerebral microvasculature which include TJs, AJs, and possibly gap junctions. Both TJs and AJs restrict permeability across the endothelium, whereas gap junctions (if present) mediate intercellular communication [175].

3.3.1. Tight junction proteins

Occludin, the first integral membrane protein within the TJ family identified [176], is a 60–65 kDa protein with four transmembrane domains which is highly expressed in the cerebral endothelium [177, 178], whereas it is much less present in non-neural endothelial cells [179]. A construct with a deletion in the N-terminal of occludin showed a considerable effect on the TJ integrity [180]. Deletion of occludin in mice has been shown to cause a complex phenotype and postnatal growth retardation [181]. However, occludin functions are not limited to its role as a TJ protein. For instance, there are studies demonstrating that occludin can regulate epithelial cell differentiation [182] and control cell apoptosis in mouse hepatocytes [183]. Also, it has been shown, in a mouse model of multiple sclerosis, that occludin dephosphorylation leads to noticeable signs of disease which occur just prior to apparent changes in the BBB permeability [184]. In cerebral ischemia, occludin and other TJs were shown to be vulnerable to attack by matrix metalloproteinases [185].

The claudin family, 20–24 kDa proteins, includes more than 20 members that build TJ strands through homophilic interactions [186]. Claudin 3, 5, and 12 play important roles at the BBB [187, 188]. Each claudin regulates the diffusion of a group of molecules of a certain size. For instance, mice neonates with deletion of claudin-5 die due to a size-selective loosening of the BBB for molecules smaller than 800 Da [188]. It is speculated that claudins determine the primary "seal" of the TJ and occludin functions as an additional support structure [142]. It has been demonstrated that overexpression of claudin species can induce development of TJ-like strands, while expression of occludin does not lead to the formation of TJ; rather, occludin only localizes to TJ in cells that have already been transfected with claudins [189].

In addition to the claudin/occluding proteins, junctional adhesion molecules (JAMs) perform an important role in the organization of TJ assembly as it has been reported that these proteins can reduce cell permeability and enhance resistance to macromolecules [190]. JAM-1, a 40-kDa member of the IgG superfamily composed of a single membrane-spanning chain with a large extracellular domain [191], is postulated to mediate the attachment of neighboring cell membranes via homophilic interactions [192]. However, there is not much known about JAMs exact function in the mature BBB, but in the rat cortical cold injury model, a characterized in vivo model of BBB breakdown it has been shown that endothelial JAM-1 is significantly reduced which strengthens the idea that JAM-1 contributes to TJs integrity [193].

3.3.2. Adherens junction proteins

The first component of AJs is the vascular endothelial (VE)-cadherin, an endothelial-specific integral membrane protein which is linked to the cytoskeleton via catenins [175] and mediates

cell-cell adhesion via homophilic interactions between the extracellular domains of proteins expressed in adjacent cells [194]. In vitro and in vivo studies have shown that VE-cadherin is required for the cells to regulate vessel maintenance, that is, for the correct organization of the new vessels and for the endothelial integrity in the quiescent vessels [195]. Various mechanisms have been suggested for how VE-cadherin regulates endothelial functions; such as direct activation of signaling molecules with a role in survival and organization of the actin cytoskeleton and regulation of gene transcription cofactors and formation of complexes with growth factor receptors [138].

Even though AJs are required at the BBB to decrease the endothelial permeability [196], it is principally the TJs that present the low paracellular permeability and high electrical resistance [142, 197].

Another component of AJs is Platelet endothelial cell adhesion molecule 1 (PECAM-1), an integral membrane protein of the Ig superfamily with six extracellular domains, a short transmembrane, and a large cytoplasmic domain which is highly expressed in blood and vascular cells especially endothelial cells [198]. PECAM-1 plays a major role in the migration of leukocytes across endothelium [138] and contributes to the steady-state barrier function of endothelial cells. It also functions as a mechano-sensor and stimulates reconstruction of the barrier integrity following perturbations, including the BBB [199, 200]; all physiologic processes that rely on the junctional integrity and signaling [198].

3.3.3. Associated proteins

There are various proteins in the cytoplasm that associate with the transmembrane components of the TJ [142]. Multi-domain scaffolding proteins of membrane-associated guanylate kinase-like homolog family, including zonulae occludentes (ZO) proteins, such as ZO-1 and ZO-2, are characterized for their contributing to the cytoskeletal anchorage for the transmembrane TJ proteins, binding to the claudins, occludin, and actin [201, 202] and controlling the correct distribution of claudins [203].

ZO-1, a 220-kDa protein, is one of the first and best-studied proteins in TJs [204]. It is expressed in epithelial and endothelial cells and even other cells which do not have TJs [205]. It has also been observed to be associated with AJs [206] and gap junction proteins [207]. ZO-1 is located just below the TJ membrane contact points and has been found to be important for both function and stability of TJs. ZO-1 dissociation from the junctional complex is shown to be correlated with increased permeability in the BBB in vitro model [208]. ZO-2, a 160-kDa protein with a high sequence homology to ZO-1 [209], interestingly, has been demonstrated to function redundantly with ZO-1, replacing it and promoting the formation of TJ in the cells lacking ZO-1 [210].

Another component present in the junctional complexes is actin. Although actin has been basically considered structural in function, it is now obvious that the anchorage of AJs and TJs to the actin cytoskeleton is critical for both barrier stability and also for the regulation of cell polarity, cellular movement, fluid sensing, and cell-contact inhibition [211]. Studies using mice lacking the actin-binding protein dystrophin have demonstrated increased brain vascular

permeability due to disorganized α-actin cytoskeleton in endothelial cells and astrocytes [212]. These findings demonstrate that properly arranged actin filaments and their binding to the TJ and/or AJ proteins are critical for normal barrier function. Studies have also shown that HIV-1 gp120 and alcohol are able to alter the cytoskeleton and induce stress fiber actin formation, causing increased permeability of the human BBB endothelium [213]. It has been suggested that alcohol-mediated changes in the brain endothelial cells (BEC) monolayers may increase diffusion of plasma components and viral penetration across the BBB, and therefore, especially at levels attained in heavy drinkers, accelerate HIV-1 penetration into the brain [138].

3.4. Transport at the BBB

Transcellular transport at the BBB happens through several mechanisms. Small lipophilic molecules can access the brain by passive diffusion. Brain CSF bulk flow mediates transport of molecules with different sizes into the CSF at a slow rate [214]. For potentially toxic molecules and metabolic waste products, the CSF works as a sink. These molecules are then eliminated from the CSF back into the blood by active transport or facilitated diffusion across the choroid plexus epithelium, or by vacuolar transport across the epithelial arachnoid granulations [138]. Efflux pumps return many undesired molecules back to the blood to regulate passive transport into the brain [215]. The flow of the plasma oxygen and carbon dioxide across the BBB is diffusive. Therefore, oxygen supply and carbon dioxide elimination are blood-flow dependent, so the gas transport is sufficient as long as cerebral blood flow is within physiological limits [216].

Small polar molecules, such as glucose, amino acids and nucleosides, can pass the BBB by carrier-mediated transport. These carriers can be multi-ligands or specific to only one molecule, such as GLUT1 glucose transporter, the L1 large neutral amino acid transporter, and the CNT2 adenosine transporter which have been cloned from BBB-specific cDNA libraries [146]. The direction of the concentration gradient is usually from blood to brain, regulated by brain demands and the concentration of metabolites in plasma [138]. Ion transporters such as the sodium pump localized on the abluminal membrane are important to sustain the high-concentration gradient for sodium at the BBB, so that sodium-dependent transport can happen. Also, sodium–hydrogen exchanger expressed at the luminal membrane and chloride–bicarbonate exchanger expressed at both sides [217] play significant roles in regulating intracellular pH in the endothelium.

Moreover, large solutes, such as proteins and peptides, are transported across the BBB by receptor-mediated or adsorption-mediated endocytic transport [218, 219]. Analysis of the rat BBB transcriptome has shown that 10–15% of all proteins in the neurovascular unit are transporters which emphasize the critical role of these molecules at the BBB [215, 220]. As a consequence of this controlled transport, the concentration of amino acids and proteins can suffer considerable variations, whereas relatively small differences exist in the concentration of ions between blood and CSF [221].

In the BBB, the ABC transporters for efflux are permeability glycoprotein (P-gp, multidrug resistance protein, ABCB1), the multidrug resistance-associated proteins and breast cancer resistance protein [222, 223] whose major role is to operate as active efflux pumps, transporting

a diverse range of lipid-soluble compounds out of the brain capillary endothelium and the CNS, eliminating potentially neurotoxic endogenous or xenobiotic molecules [216, 224]. P-gp is expressed at the luminal and abluminal membrane, as well as in pericytes and astrocytes [225], and is distributed along the nuclear envelope, in caveolae, cytoplasmic vesicles, Golgi complex, and rough ER [138]. The endothelial cells at the BBB also express several transporters for hormones, some cytokines, and chemokines [226]. Large proteins, such as transferrin, LDL, leptin, immunoglobulin G (IgG), insulin, and insulin-like growth factor also use receptor-mediated transport systems to pass through the BBB [146].

Internalization of ligands and receptors from the plasma membrane is cholesterol sensitive [227] and comprises endocytosis of caveolae, vesicles enriched in caveolin-1 [228]. The caveolar membranes carry several receptors including those for insulin, albumin, receptor for advance glycation endproducts (RAGE), LDL, HDL [229] and are also closely associated with P-gp. Moreover, caveolin-1 can affect the levels of TJs in endothelial cells of the BBB [230]. Interestingly, study of the ultrastructure of the BBB in young and aged mice during ischemia has demonstrated that permeability is associated with a remarkable increase in endothelial caveolae and vacuoles although TJs were generally intact [231].

3.5. Aβ clearance at the BBB

Increase in either total Aβ levels or the relative concentration of both Aβ40 and Aβ42 (where the former is more concentrated in cerebrovascular plaques and the latter in neuritic plaques) have been implicated in the pathogenesis of both familial and sporadic AD.

There are several identified pathways for the removal of the Aβ from the brain. Aβ peptides mainly produced in neurons are degraded by peptidases. Through efflux transporters located in cerebral vessels, Aβ flows out from brain parenchyma into the plasma. Aβ is also removed through perivascular pathways into the cervical lymph nodes as Aβ within ISF diffuses in the extracellular spaces of the brain parenchyma entering basement membranes of capillaries, passing into the tunica media of arteries, and draining out of the brain. Aβ can also be taken up by different cells in the brain [232]. A few AD cases are familial AD, associated with genetic mutations which promote an increase in the production of Aβ [233]. On the other hand, the cause of the sporadic AD, the majority of the AD cases, is considered to be the impaired clearance of Aβ from the brain [234, 235]. In this viewpoint, AD is associated with cerebrovascular disorder, which drives the accumulation of Aβ at the blood vessels (cerebral amyloid angiapothy, CAA) and in the brain parenchyma, extracellularly [138, 236], and intraneuronal lesions—NTFs [237].

In the healthy brain, Aβ concentration is accurately regulated by its rate of production, its enzymatic degradation [238], its rapid clearance across the BBB through LRP1 [239, 240], and influx back into the brain by RAGE [241]. These receptors are multi-ligand cell surface receptors that, in addition to Aβ, mediate the clearance of a large number of proteins. P-gp, belonging to the superfamily of ATP-binding cassette (ABC) transporters, is also involved in effluxing Aβ out of the brain. While LRP1 and P-gp appear to mediate the efflux of Aβ from the brain to the periphery, RAGE has been strongly implicated in Aβ influx back into the CNS. With increasing age and also in AD pathology, the expression of the Aβ efflux transporters is

decreased and the Aβ influx transporter expression is increased at the BBB, adding to the amyloid burden in the brain and its gradual neurotoxic oligomerization [242]. Thus, continuous Aβ elimination by transport across the BBB and/or metabolism is essential to prevent its potentially neurotoxic accumulations in the brain [234]. Studies have demonstrated several transport proteins such as α2-macroglobulin, TTR, apolipoprotein E (apoE), and apolipoprotein J (apoJ), which bind to Aβ and control its clearance, metabolism, and aggregation [243]. It has been shown that apoJ can increase the BBB clearance of Aβ42 [244], while apoE disrupts the clearance of free Aβ across the mouse BBB, in an isoform-specific manner (apoE4>apoE3 or apoE2), by driving Aβ transport from LRP1 to VLDLR which internalizes Aβ-apoE complex at a slower rate than LRP1 [245]. Another transport pathway is the bulk flow of the ISF into the CSF through the perivascular Virchow-Robin arterial spaces, which is followed by drainage into the plasma across the arachnoid villi [243].

3.5.1. Aβ transport by LRP1

LRP1, the major efflux transporter for Aβ across the BBB [239] and a member of the LDL receptor family, acts as both a multifunctional scavenger and a signaling receptor. LRP1 is synthesized as a precursor molecule (600 kDa) in the ER. Then in the Golgi network, a cleavage generates an 85 kDa transmembrane beta-subunit (containing two intracellular NPxY motifs) that remains non-covalently connected to the extracellular 515 kDa alpha-subunit (containing 4 ligand-binding domains for more than 30 ligands) [246].

Transcytosis of Aβ across the BBB starts with its binding to LRP1 at the abluminal side of the cerebral endothelium [239, 240]. However, this has been controversial due to the studies that failed to demonstrate a role for LRP-mediated transcytosis, but rather showed a role for LRP receptors in endocytosis and degradation of Aβ [247]. Anyway, the significant function of LRP1 in AD is not only portrayed by LRP1-mediated endocytosis of Aβ but also by data showing that the cytoplasmic domain of LRP1 has been involved in APP processing [248]. Cleavage of the extracellular domain of LRP1 by beta-secretase (BACE1) releases soluble LRP1 (sLRP1) in plasma [249]. Reduced expression of LRP1 has been described during aging in animal models and in AD individuals [240, 249]. In astrocytes, LRP1 also mediates degradation of amyloid deposits via apoE [250].

3.5.2. Aβ transport by RAGE

RAGE, a multiligand receptor in the immunoglobulin superfamily, which can bind to various ligands including Aβ and advanced glycation end products (AGE proteins) [251], is the most influential influx transporter for Aβ across the BBB [241]. Interestingly, and unlike many receptors (including LRP1), RAGE expression is triggered by the accumulation of RAGE ligands, meaning that the levels of RAGE expression are determined by the levels of its ligands. In the healthy brain, RAGE is expressed at minimal levels at the BBB, except at the endothelium of bigger microvessels of the brain. However, when RAGE ligands increase in the AD brain, RAGE expression rises in the affected cerebral vessels, neurons or microglia [251]. This mechanism worsens the cellular dysfunction due to RAGE-Aβ interactions. Circulating Aβ can enter the brain by a special receptor-mediated transport mechanism that is dependent on

RAGE expression on the luminal surface of brain vessels [252]. Following Aβ binding to RAGE at the luminal membrane of the BBB, transcytosis of circulating Aβ across the BBB into the brain parenchyma and its binding to neurons occurs. Moreover, activation of NF-kB in the endothelial cell leads to proinflammatory cytokines secretion and cerebral blood flow suppression [241]. Aβ-RAGE interaction not only generates oxidative damage to RAGE-expressing neurons, which results in neuronal degeneration, but also activates microglial cells, indirectly leading to inflammation [251]. Therefore, repression of Aβ-RAGE interaction in the BBB can inhibit Aβ influx, oxidant stress, and cytokine production. The inhibitors of Aβ/RAGE interaction have been shown to improve the BBB function and the cerebral blood flow responses to the brain activation, and to reduce neuroinflammation. Some RAGE/Aβ blockers are currently being tested in AD patients [138]. While RAGE is involved in the influx of Aβ into the brain, the soluble isoform of RAGE (sRAGE) has been detected in the plasma. It seems that sRAGE competes with cell-surface RAGE for ligand binding, thus increasing the elimination of circulating Aβ [145].

3.6. BBB dysfunction in AD

Failure in the BBB function is an outstanding event in the development and progression of several CNS diseases including multiple sclerosis [253], ischemia [254], Parkinson's disease, and AD [255]. While in some of the diseases increased BBB permeability is an outcome and consequence of the pathology (such as ischemic stroke), in other cases, BBB failure may be a causative event for the disease (such as multiple sclerosis). Furthermore, BBB dysfunction can be mild with temporary opening of TJs, or it can be a chronic breakdown [256], with changes in transporters and enzymes happening at the same time [216].

Although cerebrovascular abnormalities have been noted in AD, the starting point between BBB failure and AD pathology is not clear yet [142, 257]. Nevertheless, BBB homeostasis is altered in the initial stages of AD leading to the production of proinflammatory cytokines and suppressors of the cerebral blood flow by endothelial cells; then, amyloid deposits are observed in cerebral capillaries and vessels in the later stages of AD [258]. A large number of alterations in the structure and function of the BBB were shown to occur in AD. For instance: decreased LRP1 expression in human brain microvasculature [239], increased LRP1 oxidative damage [259], impaired microvascular P-gp [260], increased expression of RAGE in the cerebral vessels, neurons and microglia [251]. Moreover, it has been shown to occur: decreased glucose consumption by the brain, thus predicting a decay in cognitive function [261], which has been described by the reduction in the GLUT-1 transporters expression (protein levels) in AD hippocampus microvessels [262] but not a decrease in GLUT-1 mRNA levels [263]. Other alterations have been described, such as: decreased endothelial mitochondrial density, increased endothelial vacuolization, accumulation of collagen and perlecans in the basement capillary membrane [264], and increased pinocytotic vesicles [265].Also, it has been reported a reduced number and smaller diameter of capillaries in CNS implying the diminished overall surface for LRP1-mediated transcytosis of Aβ across the BBB, and also a decreased length of brain capillaries [266] which lowers transport of energy substrates and nutrients across the BBB, and reduces the clearance of neurotoxins from the brain [138]; Overexpression and

accumulation of occludin in frontal cortex and basal ganglia of AD brains [267] have also been described as well as lower expression of genes such as the homeobox gene MEOX2 (or GAX), a regulator of vascular differentiation [268]. Finally, activated microglia [269] and astrocytes, the resident brain immune cells present in neurovascular unit of the BBB, have been observed surrounding Aβ plaques in AD brains, releasing inflammatory cytokines, such as IL-1 and IL-6, TNF-α, and transforming growth factor-β [174].

4. Periphery and Alzheimer's disease

4.1. Aβ levels at the periphery

Although studies in rodents have shown an increase in plasma Aβ levels, data in human AD patients have been contradictory; while some demonstrate increased levels of circulating Aβ [270], others report decreased [271] or unchanged [272] levels. Nevertheless, the significant role of circulating Aβ in AD pathology cannot be neglected. Interestingly, Aβ is also produced outside the brain in considerable amounts by the platelets, skeletal muscle, vascular walls, kidney, heart, liver, and by other non-neural tissues [273–275]. These pools may also provide a dynamic exchange of Aβ between the brain and periphery. However, Aβ peptides in the periphery cannot form filamentous structures, probably due to the presence of multiple circulating molecules that bind Aβ and thereby change its free-plasma levels [276].

4.1.1. Plasma proteins involved in peripheral sink and clearance of Aβ

Continuous removal of Aβ, not only from the brain but also from blood and from the entire organism, is essential for preventing its accumulation in the brain. Aβ in the plasma is bound to a number of proteins such as albumin [277], apoE and apoJ [278], TTR and a soluble form of LRP1 (sLRP1). In healthy human plasma, sLRP1 is a major endogenous brain Aβ sinker that sequesters 70–90% of plasma Aβ. sLRP1 has been demonstrated to bind the majority of circulating Aβ preventing RAGE-dependent influx of Aβ back to the brain, while improving its systemic clearance [279]. These data confirm the peripheral sink hypothesis, which imply systemic clearance of Aβ via binding proteins in serum and preventing its uptake through RAGE. Accordingly, this explains how some therapeutics, such as peripheral administration of sLRP1 or antibodies against Aβ [280], decrease Aβ levels in the brain. Moreover, AD patients show decreased plasma sLRP1 levels and increased oxidative damage to sLRP1, reducing its binding capacity for Aβ. This, in turn, increases the free Aβ fraction in plasma [279], that is, accessible for RAGE-dependent influx back to the brain [281].

Higher sRAGE levels are linked to reduced risk of developing several disorders such as cardiovascular disease and AD. Levels of sRAGE are decreased in AD and in vascular dementia, which may confer a target for therapeutic purposes [282]. It has been demonstrated that systemic administration of a truncated form of RAGE decreases Aβ load in a transgenic mouse model [241]. Moreover, sRAGE has an inverse relationship with cholesterol, presenting another modulatory impact on Aβ metabolism due to the role of cholesterol as a mediator of inflammation and APP processing [145].

4.1.2. Alterations in other plasma proteins in AD

Few proteins are synthesized solely by the brain or are present in higher concentrations in CSF compared with the blood. In conditions of the BBB failure, these CSF markers can appear or be increased in the plasma [283]. Hence, estimating levels of CSF proteins in the plasma may be a reliable method to control BBB integrity. For instance, S-100 is primarily produced in the brain by astrocytes and when the BBB is disturbed it is quickly released from the brain and appears in the blood [284].

4.2. Liver and Aβ elimination

At the periphery, the liver play important roles not only in the storage and in the release of nutrients and proteins but also in the neutralization and elimination of a variety of toxic substances such as Aβ from the plasma.

The receptor for LDL (LDLr), LRP1, and megalin/LRP2 play important roles in endocytosis of lipoproteins and systemic lipid homeostasis. Moreover, LRP1 mediates the clearance of a multitude of extracellular ligands such as Aβ and regulates diverse signaling processes such as growth factor signaling, inflammatory signaling pathways, apoptosis, and phagocytosis in liver.

In the liver, LRP1, which can be blocked by the receptor-associated protein (RAP), has been shown to be the predominant transporter that mediates systemic clearance of Aβ [285]. LRP1 localized to hepatic cells binds to and systemically clears circulating Aβ. Reduced hepatic LRP1 levels are associated with decreased peripheral Aβ clearance in aged rats. In aged squirrel monkeys, systemic clearance of Aβ is also reduced and associated with increased Aβ levels in the brain. In addition to the liver, sLRP1-Aβ complexes and free Aβ are removed through the kidneys [279]. Liver was also demonstrated to be the major source of Aβ and to be able to regulate brain Aβ levels [286].

The half-life of circulating Aβ is short, in the range of minutes [287, 288]. This also suggests that rapid systemic clearance of Aβ prevents reuptake by RAGE after efflux.

It has been shown that some proteins such as insulin [289] and TTR [290] increase LRP1 levels in hepatic plasma membrane, and in turn enhance peripheral Aβ clearance. Insulin-degrading enzyme, a zinc metalloendopeptidase that hydrolyzes numerous peptides, including Aβ [238], insulin [291] and the AICD, has been purified from several mammalian tissues including liver, brain and blood cells [292]. Furthermore, experiments in rats demonstrated that after 3.5 min post-infusion of radiolabelled Aβ into the lateral ventricle, 40% of the injected radioactivity was already in the blood and urine, and internalized by the liver and the kidneys, indicating not only a quick clearance mechanism but also the involvement of systemic organs in the elimination and catabolism of Aβ [293]. Therefore, the capacity of the liver to internalize, catabolize, and eliminate large doses of Aβ, may explain not only the low plasma Aβ levels but also its small variation noted with age and disease stages.

In some cases, both parenchymal and non-parenchymal liver cells take up proteins, whereas in other cases this is done mostly by hepatocytes, as happens for TTR, apoA-I, SAP and Aβ. In

vivo and in vitro experiments showed that hepatocytes are the main cells involved in Aβ uptake (about 90%) and in its catabolism [288].

5. Transthyretin

Transthyretin (TTR), formerly called prealbumin due to its electrophoretic characteristics, located just in front of the albumin band, is a plasma protein secreted mainly by the liver and choroid plexus (CP) [294]. The name "transthyretin" discloses its dual physiological role as a carrier for thyroid hormones [295] and retinol, the latter through the binding to retinol-binding protein (RBP) [296]. TTR was first described in the CSF [297] and shortly after in the plasma [298].

Although the involvement of TTR in the transport of thyroid hormones and RBP, as well as in FAP, is very well established, its involvement in neuroprotection is part of a very recent knowledge and constantly evolving.

5.1. TTR gene structure and expression

TTR is codified by a single copy gene localized in the long arm of chromosome 18 [299]. The entire nucleotide sequence including the 5′ (transcription initiating site) and the 3′ (untranslated region) flanking regions was determined [300, 301] and attributed to the region 18q11.2–q12.1 [302]. The full gene is 7.6 kilobase (kb) long comprising 4 exons and 3 introns [300, 301]. Exon 1 contains 95 basepairs (bp), including 26 bp 5′ untranslated, and codes for a 20 aa residue leader peptide and aa 1–3 of the mature protein; exon 2 (131 bp), 3 (136 bp), and 4 (253 bp) hold the coding sequences for aa residues 4–47, 48–92 and 93–127, respectively. The introns (A, B and C) are 934, 2090 and 3308 bp long, respectively. Introns A and C contain two open reading frames (orf) of unknown significance [301].

The TTR mRNA spans ~0.7 kb and contains a 5′-untranslated region (26–27 nucleotides), a coding region (441 nucleotides), and a 3′-untranslated region (145–148 nucleotides) preceding the poly(A) tail [294, 303]. Human [299], rat [304, 305] and mouse [306] coding regions exhibit a considerable degree of homology (~85%), suggesting a phylogenetically preserved modulating role in gene expression.

TTR is predominantly synthesized by the liver where more than 90% of the protein is produced. The remaining is produced by the CP and the retina. TTR is detected in the fetal blood very early during development, as soon as eight weeks after conception [307]. TTR plasma concentration is age dependent, and in healthy newborns, it is about half of that in adults [308, 309]. TTR values vary from 20 to 40 mg/dL. In spite of the low TTR levels in CSF (~2 mg/dL), the CP is presented as the major site of TTR expression, expressed as a ratio of TTR/mass of tissue, corresponding to a ~30-fold higher than that found in plasma [310]. TTR represents 20% of the total CSF proteins [310].

With respect to the regulation of TTR expression, several studies showed that liver and CP TTR expression are increased in response to sex hormones, as demonstrated in mice [311,

312]. In rats, hydrocortisone and psychosocial stress are also inducers of TTR in the CP [313]. Others studies indicate that TTR can be expressed by brain cells, for instance in response to the heat shock factor 1 (HSF1) and to the AICD fragment of APP, as we will discuss further ahead in the context of TTR protection in AD.

5.2. TTR protein structure

The TTR mRNA codifies for the TTR-monomer; the polypeptide of 147 aa residues whose N-terminal region is a hydrophobic signal peptide of 20 aa residues. The TTR monomer is subjected to a cleaving process, during its migration through the ER, giving rise to the native TTR monomer after breaking of the signal peptide [294]. Assembly of four identical subunits (13,745 Da) occurs yielding the mature tetrameric protein, with a molecular mass of 54,980 Da [314].

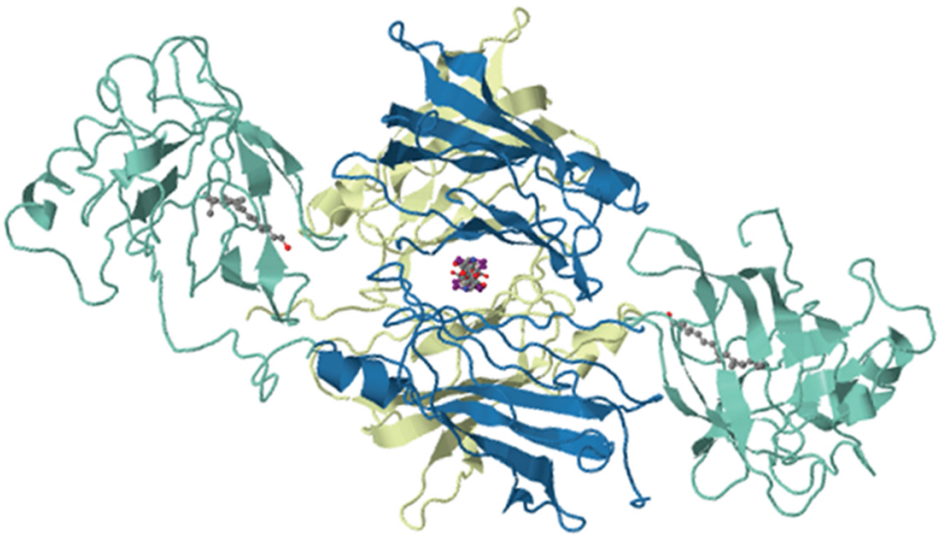

Figure 2. The human TTR tetrameric structure complexed with two molecules of RBP (light green), which in turn is bound to retinol, and with T4 (binding in the central channel).

The tridimensional structure of TTR was made available at 1.8 Å resolution by X-ray diffraction studies on the crystallized protein [315]. Each monomer contains two β-sheets formed by strands DAGH and CBEF. All, except strands A and G, display an antiparallel orientation, and are arranged in a topology similar to the classic Greek key barrel. The strands are 7–8 residues long, except strand D, which, is only 3 residues in length. Only about 5% of the aa residues are located in one segment of α-helix comprising residues 75–83, at the end of strand E. Two monomers associate forming the dimer by interactions between chains F and H of each monomer. Fixing the chains of one monomer as A–H and the ones of the other monomer as A ′–H′, the arrangement within a dimer is DAGHH′G ′A ′D ′ and CBEFF′E ′B ′C ′. The tetramer consists of two dimers with connecting edges occurring between the AB loop of one dimer with the H strand of the other dimer (**Figure 2**). The quaternary structure of TTR has the shape of a globular protein with an overall size of 70 Å × 55 Å × 50 Å [315]. The two dimers are slightly rotated in relation to each other along the y axis.

5.3. Physiology and Metabolism

5.3.1. Transport of thyroxine (T_4)

Thyroid hormones are transported in blood circulation and delivered to the target tissues, with an incredible high amount of the hormones (99%) bound to serum proteins. In human plasma, TTR, thyroxine-binding protein (TBG) and albumin are responsible for the delivery of T_4 into the target tissues. Although TBG is much less concentrated in the plasma than TTR, it presents the highest affinity constant for T_4 ($Ka = 1 \times 10^{10}\ M^{-1}$) and transports about 70% of the plasma T_4. TTR has an intermediate affinity for T_4 ($Ka = 7 \times 10^{7}\ M^{-1}$) and transports about 15% of the hormone, and finally, albumin presents the lowest binding affinity ($Ka = 7 \times 10^{5}\ M^{-1}$) [316].

The four monomers of the TTR tetramer, demarcate an open channel through the molecule where two binding sites for thyroid hormones are located (**Figure 2**). These two binding sites present negative cooperativity [317] implying that when the first thyroid hormone molecule occupies the first site, the affinity for the second molecule is highly reduced.

T_4 transport into the brain is of particular interest and has raised much controversy [318]. Although TTR is the major T_4-binding protein in the CSF, studies done in *ttr* knock-out mice ($TTR^{-/-}$) [319] revealed that TTR is not essential for thyroxine to reach the brain and other tissues [320]. In addition, measurement of several parameters of thyroid hormone function indicates that these mice are euthyroid despite strongly reduced total T_4 plasma levels [321]. No other protein was found to replace TTR in the transport of T_4 in the CSF, and T_4 levels were normal in the cortex, cerebellum, and hippocampus while strongly reduced in the CP of the $TTR^{-/-}$ mice [322].

Therefore, it was suggested that TTR might be a reservoir for T_4 both in the plasma and in the CP and CSF, which might become important under conditions of increased hormone demand.

5.3.2. Transport of vitamin A

TTR is also responsible for retinol transport through binding to retinol-binding protein (RBP, **Figure 2**). RBP is a 21 kDa monomeric protein comprising 182 aa residues [323]. The conformational structure of RBP bound to retinol was determined by x-ray crystallography [324]. In most instances, RBP is transported in the bloodstream in the form of a saturated holo-RBP protein equimolarly attached to TTR [325]. Although TTR has four binding sites for RBP [326, 327], under physiological conditions only one RBP molecule is bound to TTR due to the RBP limiting concentration.

5.3.3. TTR metabolism

The biological half-life of TTR is about 2–3 days in humans [328, 329], 22–23 h in monkeys [330] and 10–13 h in rats [331]. The major sites of TTR degradation in the rat are liver (36–38%), followed by the muscle (12–15%) and skin (8–10%) [332].

The normal physiology of TTR is not completely understood, in particular, its cellular uptake. Nevertheless, several observations suggest that TTR internalization is receptor-mediated, both

in human hepatoma cells (HepG2) [333] and in chicken oocytes [334]. Megalin, a receptor implicated in the renal re-uptake of plasma proteins carriers of lipophilic compounds, was shown to play a role in renal uptake of TTR [335]. Furthermore, different TTR mutations presented different levels of cell association and degradation, suggesting that the structure of TTR is important for megalin recognition. TTR internalization was further explored by studying TTR uptake using hepatomas and primary hepatocytes [336]. This work showed direct evidence for TTR internalization by a specific receptor, forming a ~90 kDa complex. TTR internalization was inhibited by RBP (70% decrease) and T_4 (20% decrease) and TTR mutants revealed differences in uptake, indicating again that the recognition by receptor is structure dependent. Internalization was also inhibited by lipoproteins and RAP, a ligand for all members of the low-density lipoprotein receptor family (LDLr). All together, these results suggest a common pathway for TTR and lipoprotein metabolism and the existence of a RAP-sensitive receptor for TTR internalization. TTR is also internalized by several other cells types in cell culture, such as astrocytoma cells [337], cardiomiocytes [338, 339], neurons [340, 341], endothelial cells and others. More recently, TTR was also shown to be uptaken by sensory neurons, an event also mediated by megalin [340].

5.3.4. Proteolytic activity

TTR was also found to act as a cryptic protease and the first substrate described was apoA-I [342, 343]. TTR is also able to cleave lipidated apoA-I (mainly in the lipid poor pre β-HDL subpopulation) which can be relevant in the lipoprotein metabolism [343]. Moreover, apoA-I cleaved by TTR presents less ability to promote cholesterol efflux [343] and shows increased amyloidogenecity and propensity to form aggregates. Liz et al. also described that TTR was able to cleave amidated neuropeptide Y (NPY) and that its proteolytic activity affects axonal growth, leading to the conclusion that TTR has natural substrates in the nervous system [344]. Newly, the same authors described TTR as a metallopeptidase [345], and this result was supported by another study that showed the involvement of a carboxylate and an ammonium group, possibly from a lysine side chain, in the TTR hydrolytic activity [346]. Costa et al. showed for the first time that TTR can cleave Aβ peptide in vitro [347] and implications in Aβ clearance in AD will be addressed further ahead.

5.4. TTR as a cause of disease

TTR is associated with the most prevalent type of hereditary systemic amyloidosis. The pathologic conditions include FAP and familial amyloidotic cardiomyopathy (FAC). A non-hereditary condition is also related to TTR, the systemic senile amyloidosis (SSA), and affects about 25% of people over 80 years of age. In SAA, the deposits occur in the heart and are composed of wild-type (WT) TTR.

FAP is related to a peculiar form of hereditary autosomal dominant polyneuropathy. Corino de Andrade first described the disease in 1952 [6] in the Portuguese population mainly from the northern part of the country. Characterized by systemic deposition of amyloid and with a special involvement of the peripheral nerves, the age of onset of the disease is usually between 20 and 35 years of age, with a fast progression to death within 10–15 years.

Clinically, FAP is characterized by early impairment of temperature and pain sensation in the feet, and autonomic dysfunction leading to paresis, malabsorption, and emaciation. Painless injury to the feet complicated by ulcers, cellulitis, osteomyelitis, and Charcots joints may also occur [348]. Motor involvement occurs with disease development causing wasting and weakness, and there is a progressive loss of reflexes. The amyloid deposits can occur in any part of the peripheral nervous system, including the nerve trunks, plexus, and sensory and autonomic ganglia. The other organ frequently involved in FAP, which is the heart. Clinically, the cardiomyopathy may present as an arrhythmia, heart block, or heat failure.

The first report relating immunologically TTR as the main protein in FAP fibrils was in 1978 by Costa and colleagues [349]. In 1984, the Val30Met mutation was identified in the protein isolated from Portuguese FAP patients. This variant was shown to be a biochemical marker for FAP [350] that resulted from a point mutation in the exon 2 of the TTR gene [351]. Since the identification of the Val30Met variant, many others aa substitutions were identified in the TTR protein and now over 100 mutations are described (http://amyloidosismutations.com/mut-attr.php); these variants are associated with different clinical phenotypes and a considerable number of non-pathogenic TTR mutations have been identified, including a T119M variant described to be protective.

TTR tetramer dissociation is believed to be on the basis of a series of events leading to TTR amyloid formation. In fact, the amyloidogenic potential of the TTR variants relates inversely with its tetrameric stability, and it is thought that upon dissociation of the tetramer, non-native TTR monomers are formed which can assemble, forming amyloidogenic intermediate species, such as oligomers and aggregates. Similarly to other amyloidogenic proteins, it is now believed that cellular toxicity is derived from the initial intermediate species occurring in the initial stages of FAP [352]. TTR stabilization has been proposed as a key step for the inhibition of TTR fibril formation and has been the basis for FAP therapeutic strategies. Such stabilization can be achieved through the use of small compounds sharing molecular structural similarities with T_4 and binding in the T_4 central-binding channel. Most of such compounds belong to the class of NSAIDs as it is the case of diflunisal and tafamidis, currently being administrated to FAP patients. For instance, in FAP patients, diflunisal was shown to stabilize TTR, increasing its serum concentration [353], and to reduce the rate of progression of neurological impairment and to preserve quality of life [354].

6. TTR and neuroprotection

Several lines of evidence indicate that TTR possesses neuroprotective properties in multiple contexts. Studies with $TTR^{-/-}$ mice revealed that these animals show reduced signs of depressive-like behavior, probably due to the modulation of noradrenergic system by the increase of noradrenaline in the limbic forebrain [355]. Additionally, increased levels of NPY, known as an antidepressant neurotransmitter [356], were reported in dorsal root ganglia (DRG), sciatic nerve, spinal cord, hippocampus, cortex, and CSF of $TTR^{-/-}$ mice [357], supporting the importance of TTR in the modulation of depressive behavior. Furthermore,

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Sousa and co-workers also described that $TTR^{-/-}$ mice present memory impairment compared with wild-type ($TTR^{+/+}$) animals, indicating that the absence of TTR accelerates cognitive deficits usually associated with aging [358].

In addition, TTR was associated with nerve regeneration. Fleming et al. revealed, for the first time, that TTR acts as an enhancer of nerve regeneration, following the observation that $TTR^{-/-}$ mice have decreased ability to regenerate from a sciatic crushed nerve [359]. Later, the same authors showed that the absence of TTR leads to impaired retrograde transport and decreased axonal growth, and also that the effect of TTR in neurite outgrowth and nerve regeneration is mediated by megalin-dependent internalization [340].

It was also established a relationship between TTR and ischemia, one of the major causes of brain injuries in world. Santos and co-workers proposed that in a compromised heat-shock response, CSF TTR contributes to control neuronal cell death, edema, and inflammation, influencing the survival endangered neurons [360].

More recently, a new neuroprotective role in the CNS was attributed to TTR, as a transcription inducer of insulin-like growth factor receptor I (IGF-IR), known as a protective receptor against apoptosis [361]. Vieira and colleagues described, for the first time, that TTR induces increased levels of IGF-IR, showing that TTR triggers IGF-IR nuclear translocation in cultured neurons [341].

6.1. TTR protection in Alzheimer's Disease

There are several reports suggesting a relevant protective role of TTR in AD. However, the precise mechanism(s) is not entirely understood.

In 1993, Wisniewski described that Aβ40 fibril formation was inhibited upon incubation with human CSF [362], which was explained by the sequestration of Aβ by extracellular proteins circulating in CSF such as apoE and apoJ [362–364]. The earliest description of TTR protection in AD was presented by Schwarzman and colleagues in 1994 when they observed that, in CSF and contrarily to the expectations, apoE was not the major protein binding to Aβ, but TTR [365], proposing the sequestration hypothesis 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 [365, 366], which could be related either with an Aβ overproduction, a reduction in the levels of the sequestering proteins, inability of those proteins to interact with the peptide, deficient clearance mechanisms, or a combination of all the events above stated.

Supporting a protective role for TTR in AD are its decreased levels observed both in the CSF [367] and in the plasma [114, 368] of AD patients as compared to age-matched subjects. Serot and coworkers suggested that the decrease in TTR levels in the CSF of AD brains was possibly related with an epithelial atrophy in the CP [367]. Interestingly, very recently, studies in an AD transgenic mouse model reported CP dysfunction and revealed a specific increase only of the Aβ42 isoform in epithelial cytosol and in stroma surrounding choroidal capillaries, accompanied by a thickening of the epithelial basal membrane, greater collagen-IV deposition

around capillaries in CP that probably restrain solute exchanges, and attenuated expression of epithelial aquaporin-1 and TTR protein compared to non-transgenic mice [369].

Modulation of Aβ aggregation and toxicity was also investigated in brain vascular smooth muscle cells, isolated from dogs and AD patients, containing intracytoplasmatic granules of Aβ produced in the presence of apoE. In this model, TTR was able to rescue the cells from this accumulation and the positive thioflavin S staining, initially observed, was no longer detected [370].

Although not fully consensual, several authors reported the presence of TTR in amyloid plaques both in AD patients [371–373] and in AD transgenic models [372], strengthening a role for TTR in Aβ deposition and in AD. TTR was also identified as a survival gene, and its differential overexpression in mice hippocampus was suggested to be responsible for the lack of neurodegeneration observed in the Tg2576 transgenic mice overexpressing the mutant form of human amyloid precursor protein with the Swedish mutation (APP_{Sw}) [374]. Up-regulation of TTR and other survival genes was induced by the sAPPα, a neuroprotective fragment resulting from APP processing by α-secretase [373]. TTR up-regulation was also reported in situations of exposure of AD transgenic mice to an "enriched environment," also resulting in pronounced reductions in cerebral Aβ levels and amyloid deposits, compared to animals raised under "standard housing" conditions [375]. Later on, AD transgenic models with genetic reduction of TTR and/or overexpression of human TTR [372] further showed the ability of TTR to modulate Aβ aggregation and toxicity. While the overexpression of human TTR ameliorated AD features in APP transgenic mice [372], the ablation of the mouse TTR gene resulted in accelerated amyloid deposition and increased Aβ brain levels [372, 376, 377]. In vitro studies further demonstrated that a direct interaction between TTR and Aβ abrogated the noxious properties of Aβ oligomers [378].

Animal models, in particular mice models, also provided evidence for a gender-associated modulation of brain Aβ levels [377] as elevated brain levels of Aβ42 were observed in AD female mice with only one copy of TTR when compared to female with the two copies of the TTR gene, while no significant differences were observed in males. Additionally, this work also indicated that reduced levels of brain testosterone and 17β-estradiol in female mice with TTR genetic reduction might underlie their increased AD-like neuropathology [377]. Interestingly, estradiol was found to be decreased in female AD patients when compared to healthy age and gender-matched controls [114], which in conjunction with TTR regulation by sex hormones, as already described, can account for TTR lowering in AD and the prevalence of this disease in women. In fact, plasma TTR levels were found decreased in AD women, as compared to healthy age- and gender-matched controls, whereas plasma TTR levels in AD men were not significantly different from their respective controls [114], further confirming the gender modulation by TTR.

6.1.1. TTR and Aβ interaction

TTR and Aβ binding was initially demonstrated by adding radiolabelled Aβ1-28 synthetic peptide to human CSF and subsequent analysis by SDS-PAGE [365]. Later on, the TTR/Aβ

complex was also demonstrated in plasma [379], although the details of the interaction as well as the effects in Aβ fibrillogenesis and toxicity were not known.

In 2008, Costa and colleagues characterized the TTR/Aβ interaction by competition-binding assays using synthetic Aβ42, which revealed that the WT TTR binds to different Aβ peptide species: soluble (with a Kd of 28 ± 5 nM), oligomers, and fibrils [378]. Other studies showed that TTR drastically decreased the rate of aggregation without affecting the fraction of Aβ in the aggregate pool and an estimated apparent KS of 2300 M^{-1} was calculated [380]. These data support a hypothesis, wherein TTR preferentially binds to aggregated rather than monomeric Aβ and arrests further growth of the aggregates. Recent work indicates that the intensity of TTR binding to Aβ peptide is highest for partially aggregated materials and decreased for freshly prepared or heavily aggregated Aβ, suggesting that TTR binds selectively to soluble toxic Aβ aggregates [381]. Although Schwarzman and colleagues had shown in 1994 that TTR is capable of inhibiting Aβ1-28 aggregation [365], Costa and co-workers showed by transmission electron microscopy (TEM) analysis that TTR is capable of interfering with Aβ fibrillization, both at inhibiting its aggregation and at disrupting pre-formed Aβ fibrils [378]. Thus, new and innovative studies are necessary to clarify the details of this interaction.

Another point of controversy refers to the TTR species involved in Aβ binding. Some studies support that the TTR monomer rather than the tetramer binds more strongly to Aβ [382], and it is even suggested that while the TTR monomer arrests Aβ aggregate growth, the tetramer modestly enhances the peptide aggregation [382]. Another study performed using diverse natural TTR mutants showed that different TTR variants bind differentially to Aβ in the following manner: T119M>WT>V30M^{3}Y78F>L55P TTR [378], indicating the lower the amyloidogenic potential of TTR, the stronger the affinity toward the peptide. Since the amyloidogenic potential correlates inversely with TTR tetrameric stability, authors concluded that the TTR tetramer is the species binding to Aβ peptide. Previous work had already shown that amyloidogenic TTR mutants such as L55P and E42G, the only ones able to form TTR amyloid fibrils at pH 6.8 amongst the forty-seven variants tested, were unable to bind Aβ [383].

Given the above considerations, it is therefore conceivable that mutations of the TTR gene could alter the TI'R/Aβ sequestration properties. However, the screening study in AD patients found no correlation between TTR variants and AD [384], and therefore other factors, namely conformational changes resulting from aging, should be affecting TTR levels and its binding properties towards Aβ.

Computer-assisted modelling was developed to determine the possible key residues participating in the interaction and the data suggested that residues 38–42, Asparagine 62 (E62) and E66 of each TTR monomer had a central role in the interaction [365]. Later studies have confirmed that only the residues 38–42 of TTR were important for the interaction [366]. More recently, Du and Murphy, identified the A strand, in the inner β-sheet of TTR, as well as the EF helix, as regions of TTR that are involved with Aβ [382] association. New data from the same group now indicates the involvement of the G strand of TTR with the particular involvement of L82A and L110A, suggesting that Aβ binding to TTR is mediated through these bulky hydrophobic leucines [385].

6.1.2. Effects of TTR stabilization in AD

The decrease in TTR levels in the context of AD is found early in disease development as indicated by the lower levels also found in plasma TTR in MCI patients [114]. TTR levels continue to decrease as disease progresses correlating negatively with disease severity, both in CSF [115] and plasma [114], and with senile plaque burden [386]. Similar results were found in AD transgenic mice as TTR was decreased as early as 3 months of age [377], well before amyloid deposition. Mice, however, seem to be able to compensate and female showed restored TTR levels at the age of 10 months [377].

The reason for TTR decrease is not known, but its tetrameric stability seems to play an important role. An unstable TTR can result in accelerated clearance, accounting for the lower levels observed. Further, such instability can also affect the Aβ sequestration properties of TTR. Supporting this stability hypothesis is the observation that plasma TTR from AD patients presents impaired ability to carry T_4. This also supports that it is the TTR tetramer that binds Aβ, since T_4 binding to TTR implies that the tetramer is assembled. Very interestingly, TTR genetic stabilization, that is, the presence of the T119M allele, was associated to decreased cerebrovascular disease and increased life expectancy [387]. In the context of AD, and arguing in favor of the stability hypothesis is the observation that the TTR/Aβ interaction can be improved in vitro in the presence of small chemical molecules known to bind to the T_4-binding channel and to stabilize its tetrameric fold [388]. Importantly, in vivo studies using one of such stabilizer—iododiflunisal, known to be a very potent TTR stabilizer [389] and shown to improve TTR/Aβ interaction [388]—administrated to AD transgenic mice resulted in amelioration of AD features such as cognitive function and Aβ brain deposition [390]. In addition, plasma levels of Aβ42 were decreased upon iododiflunisal administration. These results opened the possibility for the use of TTR stabilizers in AD therapeutic drug development.

6.1.3. Mechanisms of TTR protection in AD

Aβ sequestration by TTR and other extracellular proteins was the first hypothesis proposed to explain why CSF is able to inhibit Aβ amyloid formation, implying that, when sequestered, Aβ cannot aggregate to form amyloid [362, 365]. However, the precise mechanism leading to final Aβ removal is not yet elucidated.

Following the identification of the TTR proteolytic activity, it was also described that Aβ peptide is cleaved by TTR, in vitro, with consequent generation of non-amyloidogenic fragments or fragments with amyloidogenic potential inferior to the full-length peptide [347]. Since clearance of Aβ from the brain can occur via proteolytic degradation of the peptide by several enzymes, such as neprilysin (NEP), insulin-degrading enzyme (IDE), Endothelin-converting enzyme (ECE), angiotensin-converting enzyme (ACE), uPA/tPA-plasmin system, cathepsin D, and matrix metalloendopeptidase 9 [391, 392], lower levels of TTR or inhibition of its proteolytic activity would result in less Aβ peptide eliminated by the cells, and therefore in its accumulation and amyloid formation. Interestingly, several sites of Aβ cleavage by TTR are common to several of the proteases mentioned [347]. Further, NEP is known to cleave not only monomeric but also oligomeric forms of Aβ localized intra and extracellularly, as determined in vitro and in vivo [393, 394]; similarly, TTR was shown to degrade

both monomers and aggregates of Aβ in vitro. Nevertheless, Aβ degradation by TTR was not yet shown in vivo. It is also not known if binding/sequestration of Aβ and its degradation by TTR are part of the same mechanism or are independent processes.

In addition to the peptidolytic removal of Aβ, clearance of the peptide from the brain also occurs via active transport at the BBB and BCSFB, as already discussed. The receptors for Aβ at the BBB bind Aβ directly, or bind to one of its carrier proteins, and transport it across the endothelial cell. The first hint pointing to the involvement of TTR in Aβ transport and clearance came from the analysis of brain and plasma levels of the peptide in mice with different TTR genetic backgrounds. Results showed that AD transgenic mice with just one copy of TTR had lower brain and plasma Aβ levels, as compared to animals with two TTR gene copies, raising the hypothesis that TTR might be involved both in Aβ brain efflux and in its peripheral removal at the liver. Very recently, it has been showed that TTR promotes Aβ internalization and efflux in hCMEC/D3 cells, a BBB cellular model widely used. Importantly, TTR stimulated brain-to-blood Aβ permeability in hCMEC/D3 which in turn can be explained because TTR itself can only cross the BBB only in the brain-to-blood direction [290, 332]. Thus, TTR can transport Aβ from, but not into the brain, acting as a neuroprotective molecule.

The presence of TTR in brain areas other than its site of synthesis and secretion—CP and CSF, respectively—has been already shown. In situations of injury, such as ischemia, TTR was detected at the local of infarct and shown to derive from CSF TTR [360]. However, other studies demonstrated TTR synthesis by cortical [395] or hippocampal neurons both in vitro [396], and in vivo [397] showing that TTR expression in the brain can be regulated [396]. For instance, Kerridge and colleagues showed that TTR expressed in SH-SY5Y neuroblastoma cell line is up-regulated by the AICD fragment of amyloid precursor protein (APP), specifically derived from the APP695 isoform [396]. Induced accumulation of functional AICD resulted in TTR up-regulation with concomitant Aβ decreased levels. Wang and colleagues reported that TTR expression in SH-SY5Y cells, primary hippocampal neurons, and the hippocampus of APP23 mice is significantly enhanced by HSF1 [397]. In any case, TTR is available in the brain and might participate in brain Aβ efflux by promoting BBB permeability to the peptide. It is also possible that TTR contributes to Aβ clearance from the brain through the BCSFB.

TTR was also able to increase Aβ internalization by hepatocytes prompting TTR as an Aβ transporter both in the brain and at periphery. Previous work showed that TTR is internalized by hepatocytes using a RAP-sensitive receptor, which together with the knowledge that as follows: (1) LRP1 is the main Aβ receptor both at the BBB and at the liver, (2) LRP1 is preferentially expressed at the basolateral membrane of the endothelial cells of the BBB, and (3) TTR can only cross the BBB in the brain-to-blood direction, indicates this receptor is involved in TTR-assisted Aβ transport. So far, it has been shown that mice with TTR genetic ablation present decrease levels of brain and liver LRP1 and that TTR added to hCMEC/D3 cells results in increased LRP1 expression. These findings open new perspectives for TTR/LRP-related therapeutic interventions in AD. However, a direct interaction between LRP1 and TTR is yet to be demonstrated. TTR has also been suggested to act in a chaperone-like manner by binding toxic or pretoxic Aβ aggregates in both the intracellular and extracellular environment [372].

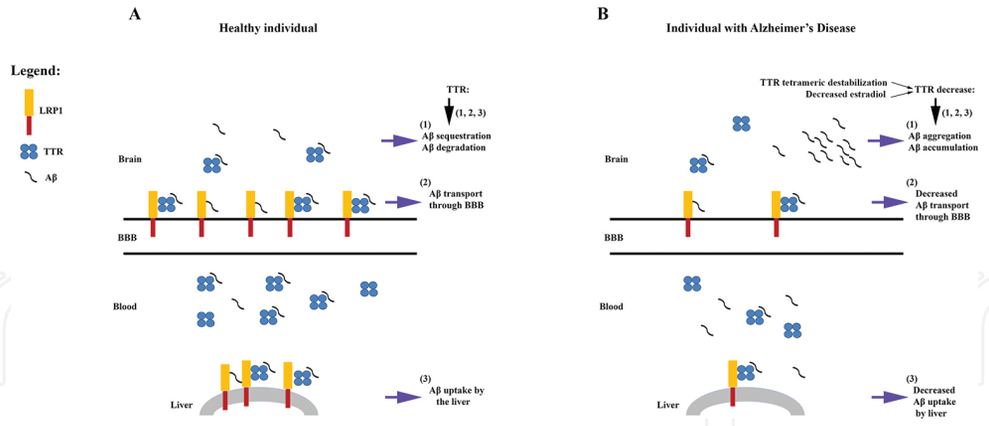

Figure 3. Schematic representation of the proposed mechanisms underlying TTR Protection in AD. (A) In a healthy individual, TTR binds Aβ peptide in the brain at the CSF promoting its degradation and elimination through the epithelial cells of the CP. At the BBB, Aβ effluxes through LRP1 to the blood, a process in which TTR also participates. At the periphery, TTR and other Aβ bindable substances create a peripheral sink avoiding the return of Aβ to the brain, and transport the peptide to the liver, where it will be internalized by liver LRP1 for final degradation. (B) In AD patients, TTR decreased tetrameric stability for yet unknown reasons, and decreased expression due to decreased sex hormones, result in lower protein levels in the brain and plasma. In turn, this contributes to failure in Aβ sequestration, efflux at the BBB, and peripheral transport to the liver. Impaired Aβ transport at the BBB and internalization/degradation by the liver is aggravated by LRP1 decreased expression, modulated by TTR.

Therefore, more studies are necessary to unravel the mechanism(s) underlying TTR protection in AD, and to clarify how the hypothesis presented so far fit together (**Figure 3**).

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Author details

Isabel Cardoso[1,2*], Luis Miguel Santos[1,2] and Mobina Alemi[1,2,3]

*Address all correspondence to: icardoso@ibmc.up.pt

1 IBMC-Instituto de Biologia Molecular e Celular, Porto, Portugal

2 i3S-Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

3 Faculdade de Medicina, Universidade do Porto, Porto, Portugal

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