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# The role of proteotoxic stress in vascular dysfunction in the pathogenesis of Alzheimer's disease

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**Abstract:** Alzheimer's disease (AD) is the principal cause of dementia in the elderly; however, its prevalence is increasing due to the fact that current pharmaceuticals used to manage the symptoms are not capable of preventing, halting, or reversing disease progression. In the last decade, evidence has accumulated to support the hypothesis that a primary cerebral vascular dysfunction initiates the cascade of events that leads to neuronal injury and the subsequent cognitive decline observed in AD. The mechanisms underlying these vascular defects and their relationship with neurodegeneration are still poorly understood however. It is pathologically known that cerebrovascular dysfunctions can induce the deposition of amyloid-β (Aβ), an amyloidogenic and toxic peptide that in turn causes cerebrovascular degeneration. Mammalian cells regulate proteostasis and the functioning of intracellular organelles through diverse mechanisms such as the Unfolded Protein Response, the Ubiquitin-Proteasome System and autophagy; however, when these mechanisms cannot compensate for perturbations in homeostasis, the cell undergoes programmed death via apoptosis. This review summarizes recent studies that together correlate the deregulation of protein quality control pathways with dysfunction of vascular endothelial cells of the brain in AD, thus supporting the hypothesis that it is the vicious, progressive failure of the proteostatic network and endothelial activation that underlies the cerebrovascular changes that symptomize AD.

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## 1 Alzheimer's disease (AD)

Alzheimer's disease (AD) is a chronic and progressive neurodegenerative disorder that affects the Central Nervous System (CNS). Presently, without efficient strategies to stop disease progression, patient death is seen on average, 4-8 years after diagnosis [1]. In Western countries, the prevalence of AD is approximately 5% in people over 65 years of age increasing to 40% in individuals over 85 years of age [2, 3]. Since the prevalence of AD cases doubles every 5 years after 65 years of age, the economic and social burden will be tremendous in the next decades due to increased lifespans worldwide. Therefore, it is imperative to understand the cellular and molecular mechanisms underlying the disease process to find new therapeutic targets.

Clinically, AD is characterized by a progressive decline in cognitive functions resulting from neurodegenerative processes occurring in the hippocampus and cerebral cortex that begin decades before the first clinical symptoms [4]. Mild cognitive impairment (MCI) is considered an intermediate stage between natural ageing and the onset of AD. Individuals exhibiting MCI are at an increased risk for progression into clinical AD with an estimated rate of conversion being 10-15% per year after observed MCI [5, 6].

The neuropathological hallmarks of AD include extracellular plaques comprised primarily of fibrillar amyloid-β (Aβ) peptide, and intracellular neurofibrillary tangles (NFTs) that are composed of hyper-phosphorylated tau. These extracellular alterations are accompanied by the presence of reactive astrocytes, activated microglia as

well as synaptic and neuronal loss in susceptible brain regions, correlating them with glucose hypometabolism [7].

Ageing is the principal risk factor for AD; however, there are more than 400 AD-associated mutations implicating more than 30 genes [8]. Mutations in the genes encoding for the amyloid precursor protein (APP), presenilin (PS)1 and PS2 cause the early-onset form of the disease known as Familial AD (FAD) that represents up to 5% of all AD cases. Other genetic variants induce an increased risk of developing late-onset sporadic AD (SAD) [8, 9]. The most prevalent genetic risk factor for SAD is the ε4 allele of apolipoprotein E (APOE) [8]; nevertheless, there are many other risk factors for SAD such as: hypercholesterolemia, increased fat intake and obesity, type 2 diabetes, hyperglycaemia, and hypertension amongst others that are lifestyle-associated risk factors for the development of vascular problems [3, 10, 11] thus supporting a close and substantial association between these defects and AD.

## 1.1 The "Amyloid Cascade Hypothesis"

Aβ, the main component of extracellular senile plaques in AD brains, is a 4 kDa peptide resulting from the cleavage of APP, a 695-770 amino acid transmembrane protein. Sequential cleavage of Aβ performed by β- and γ-secretases (amyloidogenic pathway), results in Aβ peptides with different lengths (~38-43 amino acids) [12]. Subject to normal physiological conditions, more than 90% of APP is cleaved by α- and γ-secretases as part of the non-amyloidogenic pathway, which precludes the formation of Aβ [13] (Figure 1). In the brains of AD patients, Aβ levels are increased; however, and more importantly, the ratio of $Aβ_{1-42}$, which is more prone to aggregate and is more toxic to synapses and neurons, to $Aβ_{1-40}$ is higher in comparison with those of asymptomatic controls [14-17]. Succeeding production of its monomeric form, Aβ aggregates into the most synaptotoxic and neurotoxic Aβ species, oligomers. [18, 19]. Oligomers then congregate to form insoluble fibrils that deposit into the plaques that are found in the brains of AD patients [20].

According to the 'Amyloid Cascade Hypothesis', the accumulation of Aβ in the brain, namely oligomeric Aβ, results from an imbalance between the production and clearance or degradation of Aβ and is at least partially involved in the neurodegenerative process of AD [16] (Figure 2). Accordingly, mutations of APP that increase Aβ production cause the onset of FAD; however, on the other hand, such APP mutations that decrease Aβ generation

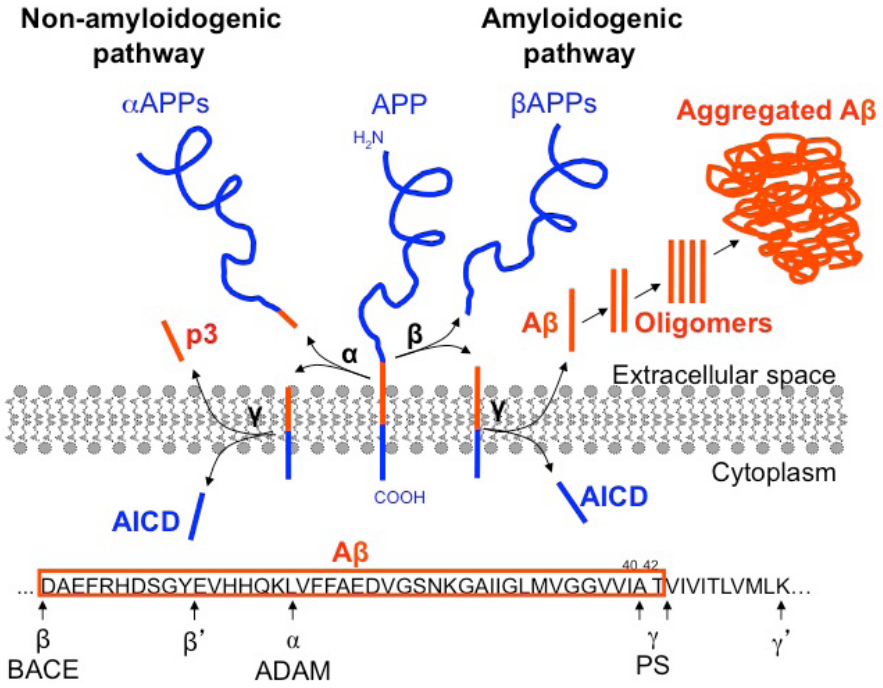


**Figure 1:** Amyloid Precursor Protein (APP) processing. α- or β-secretases may cleave APP through the non-amyloidogenic or amyloidogenic pathway originating αAPPs or βAPPs soluble fragments, respectively and membrane-retained fragments that are then cleaved by a γ-secretase originating the p3 fragment (non-amyloidogenic pathway) or the Aβ peptide (amyloidogenic pathway) and the AICD. Aβ monomers can form toxic soluble oligomers and higher molecular weight insoluble fibrils that aggregate and deposit in susceptible brain regions.

consequently reduce susceptibility to the development of AD [21]. Moreover, all currently identified mutations affiliated with FAD affect the production of Aβ [8]. On a related note, subjects with Down Syndrome (possessing three copies of chromosome 21, which contains the gene for APP) have Aβ plaques, NFTs and glial activation early in life explaining why 70 to 80% of adults with this syndrome develop AD [22, 23]. The cognitive and neuropathological features of human AD have been reproduced in several transgenic murine models that overexpress FAD-associated mutations. These mice accumulate Aβ in the hippocampus and neocortex, present neuritic plaques surrounded by astrocytes and activated microglia in addition to deposits of hyperphosphorylated tau. These alterations are accompanied by impairments in memory and learning as well as in motor function [24, 25, 26]. Importantly, these mice also exhibit amyloid angiopathy followed by weakened vessel walls, aneurysm, vasculitis, and haemorrhage [27, 28]. Numerous *in vitro* studies demonstrate the synaptic and neuronal toxicity induced by Aβ including, but not limited to: evidence of abnormal tau phosphorylation, $Ca^{2+}$ deregulation, oxidative stress, mitochondrial and endoplasmic reticulum (ER) dysfunction, neuroinflammation, alteration of synaptic transmission, excitotoxicity, changes in membrane cholesterol levels and distribution, and activation of cell death pathways [18, 29-35].

## 2 Vascular dysfunction in AD

The blood brain barrier (BBB) is an active and dynamic boundary that allows the selective permeation of molecules into the brain, simultaneously protecting it from blood toxins and bacterial infections. The BBB mediates the transport of oxygen and nutrients from the blood to the parenchyma and conversely of potentially toxic molecules resulting from cellular metabolism outward from the parenchyma to the blood [36]. The BBB is composed of a monolayer of brain endothelial cells (ECs) sealed with 'tight junctions' (TJs) that are proteinaceous transmembrane complexes comprised of members such as: occludin, claudins and junctional molecule-1, and submembrane molecules connecting to the actin network [37]. ECs of the BBB regulate the neuronal environment by governing the transport of several molecules such as Aβ from the blood to the brain parenchyma and vice versa. They also govern such fluctuations through the synthesis of factors able to influence neuronal cell function, such

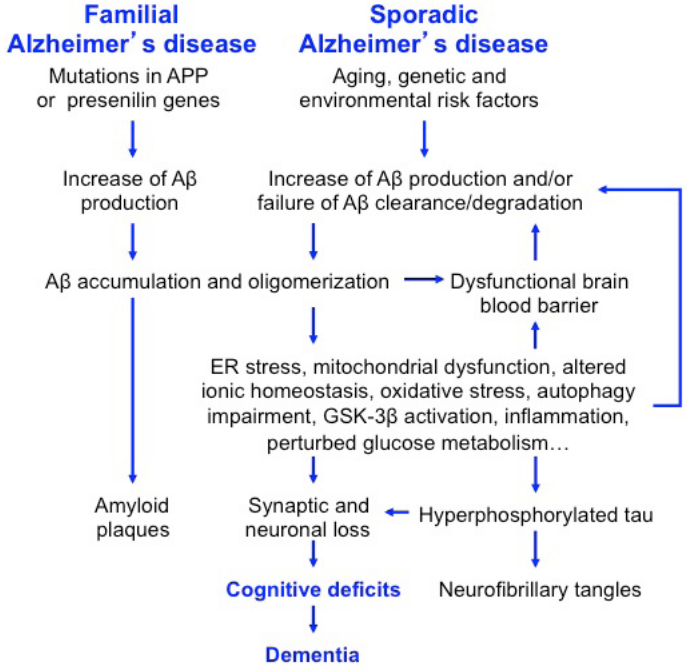


**Figure 2:** Amyloid Cascade Hypothesis. According to this hypothesis, the oligomerization of Aβ, which results from mutations in APP and/or PS genes in FAD, or from the synergistic effect of ageing, genetic (e.g. the presence of APOEε4 allele) and environmental factors in SAD cases, triggers a cascade of intracellular alterations in different types of brain cells (neurons, glia and ECs). These alterations compromise the function of the BBB that, in turn, decreases Aβ clearance from the brain parenchyma and promotes several deleterious events, including hyperphosphorylation of tau and the subsequent formation of NFTs and cytoskeleton deregulation that ultimately lead to synaptic and neuronal dysfunction culminating finally in cognitive deficits and dementia.

as vascular endothelial growth factor (VEGF) [38, 39]. As would be expected, such alterations in the metabolism of ECs can result in neurodegeneration.

The neurovascular unit that strongly affects neuronal cell activity of the brain [40] also integrates pericytes, astrocytes, neurons and microglia [41]. The processes of pericytes wrap around the endothelium, contributing to vascular stability [42] and act as mediators between the vasculature and parenchyma [43, 44]. Astrocytes, through cell projections called astrocytic feet, contact ECs providing support to those cells and therefore regulate BBB maintenance, and are also mediators between BBB and neurons [45].

Neurovascular dysfunction and deregulation of the BBB have been shown to contribute to neurodegeneration and cognitive decline, and thus have an important role in the pathogenesis of AD (Figure 3) [36, 46, 47]. 30-60% of AD patients have also been additionally diagnosed with vascular diseases and 40-80% of Vascular Dementia patients have AD [48]. Risk factors for AD such as diabetes, obesity, hypercholesterolemia, the APOE ε4 genotype, hypertension, atherosclerosis and high homocysteine levels are also risk factors for vascular diseases as well [49-52]. Numerous structural and functional cerebrovascular abnormalities including but not limited to damage of the cerebral vasculature and decreased blood flow have been identified in the brains of AD patients and in animal models [41, 53]. Finally, accumulation of Aβ in the cerebral microvasculature correlates better with early cognitive impairment in transgenic mice than accumulation of Aβ in the parenchyma [54].

Brain imaging and post-mortem studies in MCI subjects demonstrated that glucose uptake is diminished in susceptible brain areas with the expression of GLUT1, the major glucose transporter at the BBB, being decreased [55], to suggest early deficits at the BBB in AD. It was recently demonstrated that a deficiency in GLUT1 in APP-overexpressing mice leads to breakdown and compromise of the BBB [56]. Leakiness of the BBB has been observed in a number of similar transgenic, APP-overexpressing

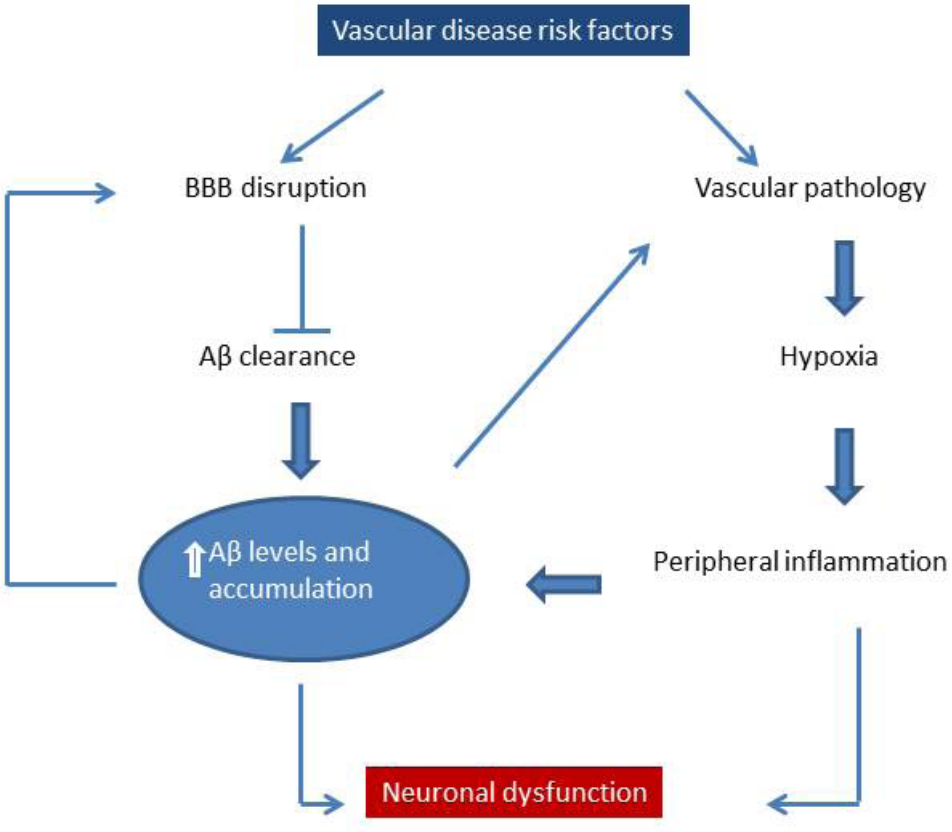


**Figure 3:** Vascular dysfunction in AD. Impaired Aβ clearance due to disruption of the BBB leads to accumulation of Aβ, which in turn perturbs BBB function and induces vascular alterations. Under hypoxia arising from vascular abnormalities, ECs become activated and cause peripheral inflammation to further elevate the Aβ levels, not only by decreasing the clearance but also increasing its production. The increased levels and accumulation of Aβ, as well as the activation of ECs all contribute to the neuronal dysfunction that occurs in AD.

AD murine models before the appearance of other disease pathology [57, 58]. Aβ immunization reversed BBB pathology in Tg2576 mice [58] suggesting that Aβ production may be involved in this process. Accordingly, post-mortem studies of patients with extensive capillary cerebral amyloid angiopathy (CAA) also presented Aβ deposition together with decreased levels of TJs [59]. This phenomenon can be observed upon infusion of Aβ into the internal carotid artery of rats, which leads to the accumulation of Aβ in the parenchyma of the brain that damages cerebral endothelium and ultimately disrupts the selective permeability of the BBB [60]. This is in part explained by the binding of Aβ to RAGE that disrupts TJs at the BBB [61, 62]. To show this, Biron and Colleagues [63] used Tg2576 AD mice demonstrating that amyloidogenesis mediates disruption and leakiness of the BBB by promoting neoangiogenesis and hypervascularity, thus resulting in redistribution of the TJs that maintain the barrier.

Combined evidence from neuroimaging and neuropathological studies show that vascular pathology develops early in AD and occurs before a decline in brain function, many years before the disease becomes symptomatic [46, 64]. Moreover, impairment of the cerebral microcirculatory system can lead to neuronal cell loss due to shifts in vessel architecture, decreased cerebral blood flow and altered oxygen utilization [41]. A reduction in cerebral blood flow has been shown to precede dementia [65, 66, 67] and the development of plaques or cognitive abnormalities in AD patients and APP-overexpressing mice [68]. Furthermore, hypoperfusion appears to induce oxidative stress that can initiate mitochondrial failure, which is known as a primary factor in the pathogenesis of AD [69].

Signalling cascades associated with vascular activation and angiogenesis are upregulated in the microvessels of AD brains leading to the expression or release of numerous related factors such as interleukines, nitric oxide, integrins, thrombin, VEGF, tumor necrosis factor-α (TNF-α) and monocyte chemotactic protein-1 [39, 41]. Activated ECs release proteases, inflammatory proteins and other toxic products that can damage or kill neurons [41] as is seen in cortical neurons of rats where high doses of VEGF have been shown to reduce cell survival [39]. To support the hypothesis that vascular activation is a relevant mechanism in AD pathogenesis, P. Grammas and colleagues [70] recently showed that the vascular activation inhibitor, sunitinib, reduces cerebrovascular expression of inflammatory proteins as well as Aβ and improves cognitive function in AD2576APPSwe and 3xTg-AD murine models.

Significant co-localization of VEGF and Aβ has been detected in the brains of AD patients, supporting a deficiency in available VEGF and the consequent proliferative activity of ECs [71]. However, instead of increased vascularity, several studies show a low microvascular density in the AD brain suggesting that antiangiogenic factors are also greatly increased in this neurodegenerative pathology [41]. The direct interaction of Aβ with VEGF receptor 2 and the subsequent inhibition of VEGF-mediated signalling can, in part, explain the anti-angiogenic effect of Aβ [72]. Furthermore, Paris and colleagues [73] demonstrated that Aβ inhibits the formation of capillaries by human ECs in the brain in a manner that is dose-dependent and ultimately induces degeneration at high doses. Adding to this, increased levels of APP compete with Notch-1 for cleavage by PS thus leading to decreased Notch cleavage, and as a consequence, impaired angiogenesis [74]. This is seen in wild type and transgenic Tg2576 mice that overexpress the Swedish double mutation of human APP (K670N and M671L) where the density of the blood vessels in the somatosensory cortex decreases with ageing; however, this decrease is more pronounced in the transgenic mice [75]. Decreased levels of circulating endothelial progenitor cells (EPCs), which repair and maintain the endothelium serving as a cellular reservoir for the replacement of dysfunctional cells, have been shown in AD patients [76]. Moreover, in an experimental model that replicates biomarkers of AD, intravenously transplanted bone-marrow-derived EPCs attenuate Aβ overload to improve deficits in both learning and memory [77].

In SAD, increased Aβ accumulation does not seem to arise from enhanced Aβ production but is rather a consequence of its faulty clearance [78]. In accordance, brain regions affected by AD have increased expression of RAGE [79] and a downregulation of LRP1 that is associated with Aβ deposition in the cerebral vessels of aged animals and AD patients [80]. Moreover, peripheral inflammation increases Aβ levels in the brain via three mechanisms: increased influx through up-regulation of RAGE, decreased efflux through the impairment of LRP activity and increased neuronal production [81].

Deposition of Aβ into capillary walls and along the pericapillary glia is a common feature of the AD brain [82]. Although diffuse and neuritic plaques and pericapillary cells preferentially exhibit $A\beta_{1\text{-}42}$, capillary walls have both $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in the brains of AD patients [82, 83]. Moreover, fibrillar $A\beta_{1\text{-}42}$ enhances the production of $A\beta_{1\text{-}40}$ in human ECs [84] while patients diagnosed with small vessel disease have increased levels of $A\beta_{1\text{-}40}$ but not $A\beta_{1\text{-}42}$ in plasma [85]. Similarly, this is seen in cats and in dogs

where diffuse and neuritic plaques also preferentially exhibit $A\beta_{1-42}$ while vascular deposits have both $A\beta_{1-40}$ and $A\beta_{1-42}$ [83] and in the cerebral microvessels of transgenic, APP-overexpressing mice that contain higher levels of $A\beta_{1-40}$ than $A\beta_{1-42}$ isoforms [86]. Fibrillar deposits of Aβ develop exclusively or mainly in the cerebral microvasculature or in the immediate perivascular regions of transgenic AD murine models due to poor clearance of Aβ across the BBB. These alterations contribute to the age-dependent degeneration of the cerebral vasculature and development of CAA, characterized by dysfunction of the endothelium of brain capillaries [86, 87].

## 3 Aβ-induced impairment of protein quality control mechanisms: a novel hypothesis to explain endothelial dysfunction in AD

The majority of AD patients present Aβ deposits in cerebral veins, arteries and capillaries [82]. Strong evidence exists to support that Aβ accumulation can be triggered by vascular deficits and, in turn, the induction of endothelial damage by Aβ oligomers and fibrils contributes to the impairment of the neurovascular unit. Aβ is toxic to all cell types within the neurovascular unit, as shown by Veszelka and collaborators in a study using primary cultures of ECs, astrocytes, pericytes and neurons from rat brains [88]. Injection of $A\beta_{25-35}$ into the brain of mice disrupts the neurovascular unit leading to deficits in learning and memory [89] displaying the toxic effects of Aβ on ECs, which has been extensively described in cultured cells, in isolated vessels and in whole animals [90, 91, 92, 93, 94]. Aβ accelerates endothelial senescence, affects viability and also induces apoptosis due partially to a decrease in telomerase activity, as seen in zebrafish embryos and cultured human and mice cerebral ECs [94-97]. Furthermore, Aβ reduces regeneration through irreversible morphological and functional alterations in cultured vascular ECs of humans, and also promotes mitochondrial-dependent apoptosis [98, 99, 100]. The induction of EC apoptosis correlates with the presence of Aβ oligomers and protofibrils that precedes fibril formation [101]. Furthermore, $A\beta_{1-40}$ increases the permeability of these cells, compromising their survival and decreasing the expression of various TJs proteins, including occludin [59, 102]. Neuronal damage can occur as a consequence of Aβ-induced endothelial dysfunction; accordingly, co-cultured neural-like PC12 cells with microvascular ECs of the bovine brain showed that Aβ triggers nitric oxide production by the ECs leading to neural cell death via apoptosis [103]. Although the deleterious effect of Aβ on ECs of the brain is widely described, the underlying mechanisms have yet to be fully elucidated. Recent studies support the hypothesis that Aβ-induced endothelial dysfunction involves impairment of proteostasis and is mediated by the chronic activation of the endoplasmic reticulum (ER) stress response, called the Unfolded Protein Response (UPR) [104, 105]. This response can be triggered by several types of cellular stress, such as changes in ROS and $Ca^{2+}$ levels, leading eventually to the accumulation of misfolded proteins in the ER lumen [106-108]. ER stress can activate major signalling pathways in order to increase the capacity of the ER to process the misfolded proteins, inhibit protein translation and enhance degradation via ER-associated degradation (ERAD) or autophagy [109]. Severe and/or chronic ER stress can however, have negative effects leading to apoptosis through different pathways such as: the elevation of ROS and $Ca^{2+}$ levels, activation of the ER-resident caspase-12 (in rodents or caspase-4 in humans), CAAT/enhancer binding protein homologous protein (CHOP) or c-Jun NH2-terminal kinase (JNK) [110]. Canonical mammalian pathways of the UPR pathway involve three specialized ER stress-sensing proteins: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 α (IRE1α) and activation of transcription factor 6 (ATF6) [111]. In cells undergoing ER stress, the ER chaperone, glucose-regulated protein 78 (GRP78) dissociates from the ER transmembrane sensors [112] and promotes their activation, thus inducing phosphorylation and oligomerization of IRE1α and PERK, as well as the translocation of ATF6 to the Golgi where it is cleaved by Site 1 and Site 2 proteases (S1P and S2P) [111]. Active IRE1α processes mRNA encoding for X-box binding protein 1 (XBP1), a transcription factor that upregulates genes that encode mediators of ERAD, organelle biogenesis and protein quality control [113]. PERK activation reduces protein load in the ER by decreasing general protein synthesis through phosphorylation of the α-subunit of eukaryotic translation-initiation factor 2α (eIF2α), which paradoxically increases selective translation of activating transcription factor 4 (ATF4) mRNA [114]. ATF4 is a member of the bZIP family of transcription factors that activates expression of several UPR target genes involved in antioxidant responses (such as the transcription factor Nrf2), apoptosis and autophagy [115, 116]. In cells experiencing ER stress, ATF6 is cleaved at the Golgi apparatus allowing the newly released cytosolic domain to translocate to the nucleus where it regulates ER chaperones, ERAD-related genes, and proteins involved in organelle biogenesis [117].

The levels of ER stress influences the outcome of the cellular response [118]; when ER stress is mild, the cell can recover and adapt. However, when ER stress is prolonged or too severe, these mechanisms fail to restore proteostasis leading eventually to autophagy [119, 120] and apoptosis if the stress cannot be alleviated [121].

## 3.1 The relationship between endoplasmic reticulum stress and Aβ

Recent findings in microvascular ECs of rat brains demonstrated that $A\beta_{1\text{-}40}$ induces ER stress and transiently increases levels of protein markers from the all three UPR branches [104]. It is well known that Aβ can be internalized by ECs of brain capillaries [122, 123] that potentiates its accumulation due to alterations in proteostasis, namely in the ER UPR ($UPR^{ER}$), the Ubiquitin-Proteasome System (UPS) and in autophagy [105]. Because ER stress decreases the traffic of proteins, such as APP, along the secretory pathway, stressful conditions potentiate the contact between APP and β- and γ-secretases, which are more abundant in the ER, Golgi and endosomes than in the plasma membrane. Under these conditions, the amyloidogenic processing of APP is promoted and therefore, the production of Aβ is enhanced [31, 124]. Chronic ER stress in ECs decreases secretion of Aβ leading to the accumulation of Aβ inside the cell [124]. In the AD brain, the ratio between Aβ efflux and influx is decreased compared to that of healthy subjects, indicating that clearance of Aβ from the brain is compromised in the disease [125]. In parallel with the delay seen in the transport of proteins along the secretory pathway, ER stress also inhibits the translocation of proteins and consequently decreases the levels of channels and transporters in the membrane of the cell, thus compromising several signalling pathways. Accordingly, ER stress has been detected concomitantly with changes in the levels of several proteins that regulate $Ca^{2+}$ and redox homeostasis in ECs treated with toxic doses of Aβ [126].

## 3.2 Calcium and reactive oxygen species

$Ca^{2+}$ homeostasis is highly dependent upon $Ca^{2+}$ channels in the membrane of cellular organelles and in the plasma membrane of the cell itself. In the case of ECs, VEGF-induced signalling and cell migration is mediated by $Ca^{2+}$ influx and requires the activity of the sarco/ER $Ca^{2+}$-ATPase (SERCA)2b, ER store-emptying through the ryanodine receptor and store-operated $Ca^{2+}$ entry [127]. An extracellular stimulus, such as ATP, can bind to G protein-coupled receptors that are abundant in the plasma membrane of ECs from the BBB, leading to the production of $IP_3$ that activates $IP_3R$ in the ER membrane allowing the release of $Ca^{2+}$ from this organelle [128]. In brain ECs, this $Ca^{2+}$ rise is transferred from cell to cell in $Ca^{2+}$ waves and plays a pivotal role in the increased permeability of the BBB [128, 129].

Aβ was shown to deregulate $Ca^{2+}$ homeostasis in bovine aortic ECs [130] and more importantly, $A\beta_{1\text{-}40}$ impairs $Ca^{2+}$ homeostasis in brain vascular ECs leading to an increase of cytosolic and mitochondrial $Ca^{2+}$ levels and a reduction of $Ca^{2+}$ within the ER. The decreased capacity of the ER to restore luminal $Ca^{2+}$ levels upon $Ca^{2+}$ depletion, promotes a decrease in the membrane potential of the mitochondria [104, 126]. These alterations likely arise from Aβ-induced ER stress since in brain capillary ECs increased levels of intracellular $Ca^{2+}$ are intimately associated with ER stress markers [108]. Prolonged ER stress in vascular ECs of the brain induces a significant transfer of $Ca^{2+}$ from the ER to the mitochondria that culminates in an overload of mitochondrial $Ca^{2+}$ and activation of mitochondria-dependent apoptosis. This event is rescued by blocking the mitochondrial Bax channel [124]. The alterations in $Ca^{2+}$ homeostasis induced by $A\beta_{1\text{-}40}$ in brain vascular ECs are related with alterations in the levels of proteinaceous regulators of $Ca^{2+}$ in the ER, mitochondria and plasma membrane such as SERCA2, $IP_3R$, voltage-dependent anion channel (VDAC), STIM1 and Orai1 [126]. Rises in the levels of intracellular $Ca^{2+}$ in brain ECs can trigger reorganization of the cytoskeleton that can disrupt the barrier created by ECs and hence, increases BBB permeability [131], as is found in AD patients [132].

In several cell types, namely ECs of the brain, $Ca^{2+}$ dyshomeostasis can increase production of reactive oxygen species (ROS) and in turn, elevated levels of ROS can deregulate $Ca^{2+}$ homeostasis [128, 133]. For instance, depletion of $Ca^{2+}$ in the ER inhibits the activity of $Ca^{2+}$-dependent chaperones leading to the accumulation of unfolded proteins that consequently activates ER oxidoreductin (ERO)1, which enhances the generation of oxidants [134]. Accordingly, the induction of ER stress with tunicamycin increases oxidative stress in coronary ECs [135]. Moreover, ERO1 upregulation under ER stress leads to the activation of $IP_3R$ and, subsequently, $Ca^{2+}$ levels are depleted in the ER and increased in cytosol and mitochondria [136, 137]. Furthermore, oxidation of mitochondrial $Na^+/Ca^{2+}$-exchangers, plasma membrane $Ca^{2+}$-ATPases and SERCA decreases the activity of these $Ca^{2+}$ transporters hence impairing the ability of the ER to re-establish $Ca^{2+}$ levels resulting again in an overload of $Ca^{2+}$ in the mitochondria [138, 139].

Capillary ECs are enriched in mitochondria and are

consequently more prone to oxidative stress than other cell types [41]. ECs also have other sources of oxidative stress like endothelial nitric oxide synthase (eNOS) that leads to nitric oxide production in the presence of high $Ca^{2+}$ levels [140]. Elevated ROS levels and endothelial cell-to-cell ROS transmission are all associated with endothelial dysfunction, apoptosis and disruption of the BBB [141-143]. In cultured human vascular ECs, the expression of numerous genes involved in the response to inflammation, oxidative stress [96] and ROS accumulation [100] has been shown to be induced by Aβ. In 3xTg-AD mice, early oxidative stress is associated with vascular dysfunction [144].

A multitude of evidence exists to support a close relationship between ER and oxidative stress in ECs such as the fact that induction of ER stress enhances oxidative stress and decreases the activity and expression of eNOS in coronary ECs [135]. Furthermore, ER stress-induced UPR activation in ECs can be inhibited by the presence of antioxidants [145]. In rat microvascular brain ECs, $A\beta_{1\text{-}40}$ activates the $UPR^{ER}$ and induces early production of ROS that are neutralized by the subsequent increase in antioxidant defences [126, 146]. Interestingly, lymphocytes from patients with MCI and mild AD also present high levels of ROS and low levels of antioxidant defences, namely superoxide dismutase (SOD), which are not observed in lymphocytes from moderate/severe AD patients [147]. Similar results have been found when comparing both young and old 3xTg-AD mice [147].

## 3.3 Proteasome- and lysosome-dependent degradation

Misfolded proteins that are not repaired in the ER can be retro-translocated to the cytosol to be degraded in the proteasome via the ERAD pathway [148]. However, in brain ECs and neurons, chronic ER stress inhibits the proteasome [105, 149] due most likely to an excessive accumulation of oligomeric or fibrillar proteins that block the proteasome. On the other hand, deregulation of the Ubiquitin Proteasome System (UPS) induces ER stress and activates the UPR [105]. In a parallel manner, Aβ has been shown to inhibit the proteasome in cultured vascular brain ECs and transgenic AD murine models [105, 150,151], as is also described in the brain of AD patients [152].

Dysfunctional parts of the ER, other organelles and aggregated proteins may be degraded through the autophagy-lysosome system [120], which is seen in the robust induction of the UPR driven up-regulation of autophagic genes [120]. Conversely malfunctioning autophagy can activate the UPR in several cell types, such as vascular brain ECs [105] and cultured human brain vascular ECs where Aβ also induces the accumulation of autophagic vacuoles [98], similarly to what is observed in the brains of AD patients [153]. This is probably due to a decrease in autophagic flux induced by Aβ upon sustained ER stress or inhibition of the proteasome as observed in brain ECs [105]. As found in brain ECs treated with $A\beta_{1\text{-}40,}$ excessive activation of the UPR can decrease the levels of proteins within the secretory pathway, such as those involved in lysosome biogenesis, thus leading to lysosomal dysfunction and a decrease in autophagic flux [104, 105]. Similarly, Aβ decreases the autophagic flux in human neuroblastoma SH-SY5Y cells due to disruption of microtubules and consequent inhibition of the transport of autophagosomes to lysosomes [154]. The reduction of autophagic flux can also be due in part to inhibition of the proteasome induced by Aβ since the proteasome itself is involved in the formation and remodelling of microtubules that decrease lysosome biogenesis when experience dysfunction [155].

## 3.4 Apoptosis

When protective protein quality control mechanisms such as the $UPR^{ER}$, UPS and autophagy are not able to cope with the excessive accumulation of abnormal proteins, apoptotic cell death can be triggered [120]. Activation of caspase-3, an apoptosis effector caspase, and increased levels of apoptotic markers have been found in vascular cells from postmortem AD brains [156, 157]. Aβ-induced apoptosis has been reported *in vitro* in neurons, ECs, pericytes, and other brain cells [18, 158, 159]. Moreover, the accumulation of Aβ in the brain vasculature of mice has been shown to induce EC apoptosis [86] and the neurovascular dysfunction arising from the toxic effects of Aβ on brain ECs has been implicated in the cognitive decline in AD [36]. Aβ has recently been found to activate the UPR and to deregulate both the UPS and autophagy in brain ECs while on the other hand these effects have been shown to increase the levels of Aβ leading the cell into a deadly cycle [37]. Severe ER stress has also been reported to induce apoptosis in ECs [160] and $A\beta_{1\text{-}40}$ accordingly activates caspase-12, increases the levels of CHOP and causes apoptotic cell death in brain vascular ECs [104]. Alternately, deletion of CHOP or high levels of GRP78 have been shown to protect from death in ECs [161, 162]. A mitochondria-mediated apoptotic pathway triggered by $Ca^{2+}$ transfer from the ER to the mitochondria is activated by $A\beta_{1\text{–}40}$ in brain vascular ECs [104]. Accordingly, the release of $Ca^{2+}$ from ER can activate pathways that culminate in

apoptosis [163-165]. Lastly, $A\beta_{1-40}$ impairs proliferation of ECs in the brain thus inhibiting endothelial replication resulting in the inability of vessels to repair and regenerate after injury [166].

## 4 Conclusions

Vascular abnormalities seem to precede neuronal degeneration and occur many years before the first cognitive symptoms that hallmark AD. ECs within the brain play an important role in the survival of neurons and glia cells within the neurovascular unit, but become dysfunctional in response to toxic levels of the AD-associated Aβ peptide that deregulates $Ca^{2+}$ homeostasis and induces oxidative stress inside the cell. Recent studies on ECs of the brain support the hypothesis that endothelial dysfunction in AD arises from Aβ-induced impairment of protein quality control mechanisms (Figure 4). Physiologically, misfolded proteins in the lumen of the ER can acquire their correct, native conformation after activation of the $UPR^{ER}$ or they can be translocated to the cytosol, where they are ubiquitinated and degraded in the proteasome. Pathological Aβ oligomers and fibrils inhibit the proteasome activity, thus potentiating and exacerbating the accumulation and aggregation of misfolded proteins. Such aggregated proteins and dysfunctional organelles, such as parts of the ER and mitochondria, can be degraded in lysosomes through autophagy, but lysosome-mediated degradation is impaired by Aβ leading to the accumulation of potentially toxic proteins and dysfunctional organelles resulting finally in the demise of the cell through activation of apoptotic cell death pathways. Concomitantly with the impairment of protein quality control mechanisms and dysregulation of $Ca^{2+}$ and redox homeostasis in ECs of the brain, Aβ also activates protective stress responses and compensatory strategies that are, unfortunately, unable to compensate the irreversible and deleterious effects induced by the chronic presence of Aβ.

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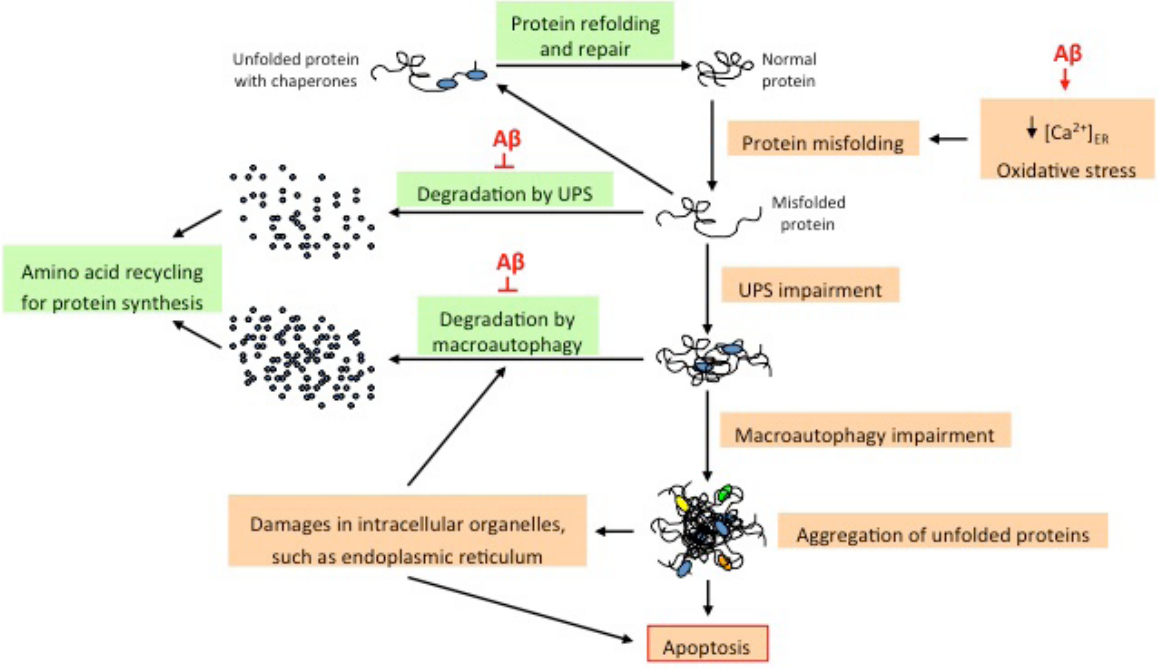


**Figure 4:** Aβ impairs proteostasis in brain endothelial cells. Aβ deregulates ER $Ca^{2+}$ homeostasis and induces oxidative stress in ECs, which promotes the accumulation of misfolded proteins leading to ER stress. Abnormal proteins can be repaired after ER stress-induced UPR activation or can be retrotranslocated to the cytosol, ubiquitinated and degraded by the UPS in the proteasome. However, Aβ oligomers/ fibrils inhibit the proteasome activity leading to the accumulation of misfolded proteins. Aβ also impairs macroautophagy decreasing protein degradation within the lysosome, which leads to the accumulation of aggregated proteins and dysfunctional organelles and, finally, activation of apoptotic cell death.

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