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Lipoprotein-mediated brain cholesterol transport: a potential pharmacological target in Alzheimer's Disease

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Abstract

Alterations of cholesterol homeostasis in the central nervous system (CNS) have been associated to various neurodegenerative disorders, including Alzheimer's disease (AD). CNS cholesterol trafficking is mediated by HDL-like particles present in the cerebrospinal fluid (CSF) and containing mainly apolipoprotein E (apoE) that interact with membrane cholesterol transporters such as ABCA1 and ABCG1 and convey cholesterol from astrocytes to neurons essential for neuronal plasticity maintenance, function and regeneration after injury. The objective of the first part of this study was to establish whether the ability of CSF to promote cell cholesterol efflux trough ABCA1 and ABCG1 (CSF cholesterol efflux capacity, CSF-CEC) was impaired in AD, analysing AD, non-AD dementia patients (non-AD DEM) and control subjects. As expected, AD patients showed reduced CSF $A\beta_{1-42}$, increased total- and phospho-Tau and higher frequency of apoE4 genotype. ABCA1- and ABCG1-mediated CSF-CEC were markedly reduced in AD, but not in non-AD DEM, were a reduced passive diffusion CEC was observed. Only non-AD DEM patients displayed lower CSF apoE concentrations compared to controls. No differences in CSF-CEC were found by stratifying CSF- CEC for apoE4 status. ABCG1 CSF-CEC positively correlated with $A\beta_{1-42}$, while ABCA1 CSF-CEC inversely correlated with total- and phospho-Tau. The CSF-CEC impairment and the correlation with the neurobiochemical markers suggest a pathophysiological link between CSF HDL-like particle dysfunction and neurodegeneration in AD.

The objective of the second part of this study was to investigate the role of the protein PCSK9 in AD focusing on brain cholesterol transport. As already mentioned, cholesterol produced by astrocytes is transported by HDL-like particles and finally incorporated by neurons through the activity of specific receptors targeted by PCSK9, such as the LDL receptor (LDLR) and its family members [very low density lipoprotein receptor (VLDLR), apolipoprotein E receptor 2 (apoER2) and the LDL receptor–related protein-1 (LRP1)]. As in peripheral tissues, brain PCSK9 may modulate lipid metabolism by degrading the receptors involved in brain cholesterol uptake. Consequently, the second objective was to measure PCSK9 levels in CSF of AD patients and controls and looked at possible correlations with CSF total apoE and apoE4. We found higher CSF PCSK9 levels in AD than in control patients. CSF total apoE concentrations did not differ between the two groups, while apoE4 levels were higher in AD subjects. Considering all samples, a significant positive correlation was found

between PCSK9 and apoE4 and PCSK9 levels were higher in APOE ε4 carriers. This observation demonstrated for the first time an alteration in CSF PCSK9 levels in AD and suggest a pathophysiological relationship between PCSK9, apoE4, and AD. Finally, we investigated some possible molecular mechanism by which PCSK9 may exert a pathogenic role in AD and a deleterious effect on brain cells. Previously, we prepared and characterized reconstituted HDL containing apoE (rHDL-apoE) for its capacity to promote cholesterol efflux and to interact with LDL receptors. Successively, we observed that the internalization in differentiated neurons of rHDL-apoE in the presence of PCSK9 was significantly reduced in SHSY5Y overexpressing PCSK9 compared to control cells. Therefore, we used confocal laser scanning microscopy to investigate the interaction between fluorescinated-apoE (apoE-FITC) with neurons overexpressing or not PCSK9. We highlighted a morphological evidence with fluorescent grains distributed along the cell membrane to outline the cells; this phenomenon was markedly less evident in PCSK9 expressing cells Moreover, we observed that the overexpression of PCSK9 lead to a downregulation of the apoER2 and the LDLR. Our preliminary *in vitro* results suggest a possible role of PCSK9 in interfering with cholesterol supply to neurons. Further analyses and in vivo data from peculiar transgenic mice are necessary to better understand other possible mechanisms involved in the pathogenetic role of PCSK9.

Abbreviations

AD, Alzheimer's disease; **Aβ**, amyloid β; **ABCA1**, ATP-binding cassette A1; **ABCG1**, ATP-binding cassette G1; **apoER2,** apoE receptor 2; **apoE**, apolipoprotein E; **apoE4**, apolipoprotein E4; **apoJ**, apolipoprotein J; **APP**, amyloid precursor protein; **BBB**, blood brain barrier; **CEC**, cholesterol efflux capacity; **CHO**, Chinese hamster ovary; **CNS**, Central Nervous System; **CSF**, cerebrospinal fluid; **DEM**, dementia; **HDL**, high density lipoprotein; **LCAT**, lecithin cholesterol acyltransferase; **LDLR**, low-density lipoprotein receptor; **LRP1**, low-density lipoprotein receptor-related protein 1; **LXR**, liver X receptors; **PCSK9,** Proprotein convertase subtilisin/kexin type 9; **RXR**, retinoid X receptors; **VLDLR**, very low-density lipoprotein receptor; **22OHC,** 22(R)-hydroxycholesterol; **9cRA**, 9-cis-retinoic acid;

Introduction

1. Alzheimer's disease: epidemiology, pathophysiology, diagnosis and treatment

Alzheimer's disease (AD) is a neurodegenerative disorder of complex, multifactorial and heterogeneous nature. Today, over 46 million people live with dementia worldwide and this number is estimated to increase in the coming years. AD has been estimated with a prevalence of 10-30% and an incidence of 1-3% in the people over 65 years with a mean of lifespan of 10 years[1]. The great majority of AD suffered from sporadic disease, but a small part of patients is affected of a rare familiar autosomal dominant form of AD. This inherited form has early onset between 30 and 50 years and is caused by mutations in the genes encoding for amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) causing overproduction and formation of an abnormal form of the neurotoxic peptide beta-amyloid (Aβ). For clinical aspects, the sporadic and familiar forms share the same disease progression and biomarker profiles[2].

Alois Alzheimer was the first who reported in 1907 the case of a 50 years old patient affected of a severe disease process of the cerebral cortex at the 37th Meeting of South-West German Psychiatrists in Tubingen. The woman exhibited symptoms including paranoia, progressive sleep and memory disturbance, aggression and confusion. After the autopsy, Alzheimer explored the woman brain and discovered peculiar histological plaques and neurofibrillary tangles[3].

The risk of developing AD can be ascribed to genetics for about 70% of the cases. The most frequently gene mutations associated to the early AD onset are APP, PSEN1 and PSEN2, genes encoding for proteins involved in the generation of β-amyloid, while for late onset AD the presence of apolipoprotein ε4 gene. In addition, other non-modifiable risk factor are represented by age and the female sex[4]. However, some lifestyle habits such as physical activity, smoking, alcohol consumption, obesity, employment and education, moreover, some non-genetic risk factors including the presence of cerebrovascular disease (haemorrhagic infarcts, stroke, small and large ischemic cortical infarcts, vasculopathies), diabetes, hypertension are also associate with increased risk of AD[5].

The two well know hallmarks of AD including amyloid plaques deposition and neurofibrillary tangles (NFTs). The consequences of these pathophysiological processes involved neurodegeneration with synaptic and neuronal loss leading to brain atrophy.

Amyloid plaques are extracellular accumulation of Aβ that derived from the proteolytic cleavage of amyloid precursor protein (APP) by enzymes including α-, β- and γ- secretases. APP processing occur through the non-amyloidogenic mediated by α- secretase and the amyloidogenic pathway mediated by β- and γ- secretase. APP mediated cleavage by α-secretase at plasma membrane leads to the production of sAPPα, a soluble molecule that seems to have a role in neuronal plasticity, survival and neuroprotection against excitotoxicity[6]. On the other side, the cleavage of APP by β- and γ- secretases results in formation of the insoluble and neurotoxic Aβ peptide. The β-secretase, also called BACE1, produces APP soluble fragment, sAPPβ, associated to neuronal death. Both α- and β-cleavage generate also a carboxy-terminal fragments (CTFs), called αCTF and βCTF respectively, that are bound to cell membrane and can be cleaved by γ-secretase. These αCTF and βCTF intermediates are substrate of γ-secretase to produce p83 and Aβ, respectively. The first one is degraded and seems to have no relevant function.

γ-secretase activity occurs through a complex including four proteins: presenilin (PS, PS1 and PS2), nicastrin, anterior pharynx-defective-1 (APH-1), and presenilin enhancer-2 (PEN-2). The cleavage of APP into Aβ includes a variety of peptides differing for length (from 38 to 43 amino acids), the most common are the ones with 40 or 42 amino acids (Aβ40 and Aβ42). The latter one is more abundant since its higher tendency to form fibrils and for its insolubility[7]. Under physiological conditions, there is a balance between amyloidogenic and non- amyloidogenic pathways, while in pathogenic conditions the amyloidogenic pathway overcomes the other one.

Neurofibrillary tangles, the other main clinical feature in AD, are instead composed by paired helical filaments of hyperphosphorylated tau, in particular phosphorylation at residues 181 to discriminate AD from dementia Lewy body while the one at residue 231 to discriminate between AD from frontotemporal dementia[8]. The mechanism of increased tau aggregation in AD is still unknown. Until now, the amyloid hypothesis represents the dominant theory for AD pathogenesis, suggesting that formation and accumulation of Aβ is the driven force that cause AD, due to an imbalance between Aβ production and clearance. In addition, neurofibrillary tangles of hyperphosphorylated tau that also coexist in the disease could represent a consequence of neuronal dysfunction and neurodegeneration mediated by inflammation. Today, the diagnosis is made in accordance

with specific criteria using the Aβ PET imaging and cerebrospinal fluid (CSF) analyses. The discovery of Pittsburgh compound B (PiB), a radioactive analogue of the fluorescent dye amyloid thioflavin- T[62] that is able to cross the BBB and bound Aβ with high affinity, opened the way for the use of Aβ imaging with PET[9]. Aβ PET imaging is a marker useful also in the prodromal phase of the disease since studies showed that Aβ deposition started before clinical symptoms and brain atrophy and so could predict the progression from mild cognitive impairment to AD. According to diagnosis criteria Aβ PET imaging is matched with the analyses of CSF profile. CSF collection by lumbar puncture is a routine clinical procedure in neurology and the most specific biomarkers for the diagnosis including $A\beta_{1-42}$, tau and p-tau. $A\beta_{1-42}$ CSF levels correlate with post-mortem plaques deposition[10] and with positive Aβ PET imaging[11], suggesting that this biomarker depend on peptide deposition in the brain. On the other hand, CSF levels of tau increase not specifically in AD but also in other neurodegenerative disorders such as Creutzfeldt-Jakob disease indicating that this biomarker is associated with neuronal and axonal degeneration overall. Current pharmacological treatments for AD are very limited and mostly based on administration of cholinesterase inhibitors (rivastigmine, donepezil, and galantamine) and/or N-methyl- D-aspartate receptor antagonist, memantine. Newly synthesized compounds and biological drugs may potentially impact multiple AD pathophysiological pathways and can act as inhibitors of monoamine oxidases (MAO-A, MAO-B), inhibitors of cholinesterases (AChE, BuChE), modulators of Aβ binding, modulators of mitochondrial permeability transition pores, alcohol dehydrogenase and antioxidants[12]. In the last twenty years, many efforts have been done to decrease Aβ levels of monomers, oligomers, aggregates and plaques with compounds that specifically decrease the production, antagonized the aggregation or increase Aβ clearance. Unfortunately, all these approaches failed in large clinical trials demonstrating no beneficial effects for patients affected from mild cognitive impairment to AD. In particular, the failure of several biological drugs including solanezumab, crenezumab and gantenerumab, that specifically target the amyloid protein, underscores the difficulty of testing drugs targeting the amyloid plaques, the hallmark of AD. Thus, it can be asserted that no drugs until now has been able to exhibited that preventing or removing the accumulation of amyloid translates into a result maybe because it's the wrong target[13].

2. Cholesterol in the Central Nervous System

Cholesterol is one of the most important molecules in cell physiology. It is a key component of all eukaryotic membranes and plays several essential roles; it is a key regulator of cell fluidity and permeability; it contributes to the structural makeup of the membranes and it is involved in membrane trafficking and transmembrane signalling processes. It is also a precursor for the biosynthesis of molecules such as steroid hormones (e.g., cortisol and aldosterone and adrenal androgens), and sex hormones (e.g., testosterone, estrogens, and progesterone) bile acid and vitamin D[14].

The first who described the importance of cholesterol in the nervous system was the French scientist Couerbe in the 1834, who recognized it as “un element principal” of the central nervous system. Despite concerted efforts in the interim, it is only during the past few decades that the brain has begun to surrender the secrets of the behaviour of one of its most abundant lipids. Although the central nervous system accounts for only 2% of the whole body weight it contains approximately 25% of the total pool of unesterified cholesterol in the human body[15]. Brain cholesterol plays different structural roles: it’s an important component of the myelin sheath surrounding axons produced by oligodendrocytes and an important constituent of neuronal membranes[16]. In particular, cholesterol is the major component of synaptic vesicles, whose formation, shape, and release properties are controlled by the cholesterol content[17]. On the postsynaptic side, cholesterol has an important role in the organization and the correct positioning of neurotransmitter receptors. For this reason, a reduced amount of cholesterol on the postsynaptic surface can impair neurotransmission and induce a loss of dendrite spines and synapses[18].

Ontogenetic studies revealed that in mice the central nervous system grew rapidly during the first 3 weeks after birth about 5% of body weight. The cholesterol pool in this district increased at a rate of 0.26 mg/day and the CNS synthesized sterol at a rate of 0.28 mg/day. On the opposite, in adult mice between 13 and 26 weeks of age, the trend switch with a marked decrease in these parameters including a reduction in the size of the CNS to 1.7% of body weight and in the rate of cholesterol synthesis to 0.035 mg/day[19].

Regulation of cholesterol homeostasis is essential for normal brain functions and development. In neurons, an enzyme 24-hydroxylase (CYP46A1) metabolized the excess of cholesterol in 24-hydroxycholesterol (22S-OH), an oxidized product that is able to cross the BBB and be found in the plasma[20]. In facts, after its synthesis, 24S-OH is present into the systemic circulation both in rats[21] and in humans[22] and its presence derives from brain production and transport across the BBB through diffusion mediated by gradient[23]. While 24S-OH effluxes from the brain to the peripheral circulation, at the same time, 27-hydroxycholesterol (27-OH) is produced in the CNS and flows to the CNS. 27-OH, the most abundant plasma cholesterol metabolite, is produced through the activity of the 27-hydroxylase (CYP27A1), present in different cell types. In non-pathological conditions, 27−OH is present in very low concentration in the CNS since it is efficiently metabolized, however, the 27-OH levels in the brain depend on the integrity of the BBB[24].

Neuros are able to synthetize cholesterol during the prenatal period and progressive they lose this capacity in the postnatal period and become dependent on cholesterol produce from other cell type like astrocytes[25;26], which in turn, display from 2 to 3 higher biosynthetic capacity compared to neurons[27]. Remarkable, brain relies on endogenous local cholesterol since it is isolated from the other body compartment though the blood brain barrier (BBB) that avoid the passage of cholesterol from systemic circulation to the brain[25]. Cholesterol in the CNS display several roles and alteration of its metabolism could have deleterious implications in human beings[17].

2.1 Cholesterol homeostasis and Alzheimer's disease

Dysregulation of cholesterol homeostasis has been implicated in various neurodegenerative disease including AD. Evidence supporting an imbalance in brain cholesterol homeostasis and neurodegenerative disease, including AD, derives for example from recent genomic association studies (GWAS) that have identified several loci involved in lipid metabolism among AD susceptible genes including ABCA7, SORL1, APOE and CLU[28,29]. Nonetheless, human and *in vivo* data on cholesterol levels in serum, plasma and brain are controversial[30].

Mechanistically, cell cholesterol content may influence the metabolism of Aβ. In particular, the well-known amyloidogenic APP processing, that generates toxic Aβ peptides take place

in cholesterol-enriched domains in cell membranes called lipid rafts. These lipid rafts act as platforms to host various membrane proteins implicated in many cell-signalling processes. It has been suggested by some studies that disturbance of lipid rafts domains result in the production of Aβ peptide. Indeed, the enzymes involved in APP processing including α- β- and γ- secretases together with APP have been described to be influenced by lipid rafts composition[31,32]. One of the first data suggesting that brain's Aβ production can be affected by cholesterol derives from the finding that rabbits fed with cholesterol-enriched diet showed increased amyloid accumulation[33]. To further support this previous observation, the effect of a high fat and cholesterol-enriched diet was studied in a mouse AD model of amyloidosis. They found that diet induced hypercholesterolemia resulted in an increased in either Aβ deposition and amyloid plaques formation[34].

In humans, evidence supporting increased cholesterol levels and the risk of developing AD are not compelling. A meta-analysis of 10 studies published between 1986 and 1999 revealed that cholesterol levels are significantly lower in AD patients compared to control subjects[35]. On the contrary, the Framingham study showed that total serum cholesterol levels are not associated with AD risk. In accordance also a cohort of subject have been followed at baseline and for two years and data didn't support a relationship between total cholesterol and the risk of AD[36]. The reason for this controversy may be related to the fact that in all these studies plasma cholesterol has been considered, whose concentrations do not necessary reflect these in the cerebral compartment.

With respect to brain cholesterol, cholesterol concentrations have been measured in several brain regions and in the CSF of AD patients compared to controls. Also in this case conflicting results have been reported indicating both decrease or increase or no change in cholesterol content in AD patients compared to control subjects[37]. For example, Manson et al. found that cholesterol levels were decreased in the temporal gyrus of post-mortem AD patients then control subjects[38]. On the contrary, Sparks et al. showed that cholesterol levels in the frontal cortex grey matter were increased in AD patients carriers of the apoE4 then control subjects with the same genotype[39]. Finally, Heverin et al. described that cholesterol levels were comparable in the cerebral cortex of AD and controls[40].

Thus, until now, data are not sufficient convincing and robust to support the hypothesis that brain cholesterol is a causative factor for AD.

3. Lipoproteins in Alzheimer's disease: clinical evidence and potential mechanism

Cholesterol and triglycerides are insoluble in water and so these lipids need to be conveyed together with proteins. In particular, lipoproteins are particles composed by an hydrophobic core constituted by cholesterol esters and triglycerides, enveloped by free cholesterol, phospholipids and apolipoproteins. Plasma lipoproteins are distinguished into seven classes: chylomicron remnants, VLDL, IDL, LDL, HDL and Lp(a), based on their size, lipid composition and apolipoproteins[41].

High-density lipoproteins (HDL) are an heterogeneous group of particles varying both in composition and size, ranging between 7-20nm with a density between 1.063 and 1.21 g/ml. HDLs are mainly composed by two main proteins, apolipoprotein A1 (apoA-1) and apolipoprotein A2- (apoA-II), but several other minor apoproteins as well as enzymes such as lecithin-cholesterol acyltransferase (LCAT), platelet-activating factor acetylhydrolase (PAF-AH) and serum paraoxonase (PON1) are associated with the HDL particles.

HDL levels are very well known for their inverse association with the cardiovascular risk, at least from the epidemiology[42]. Among the several protective functions of HDL, these particles are well recognized for their capacity to promote cholesterol efflux (CEC), that represents the ability of HDL to accept cholesterol from macrophages, the first and critical step of reverse cholesterol transport (RCT), a process by which the excess of cholesterol is removed from the periphery to the liver for the final excretion in the bile[43]. However, HDLs exert several other biological proprieties including antioxidant, anti-inflammatory, anti-thrombotic, pro-endothelial function and also the capacity to modulate immune system.

Plasma HDL has been widely examined for their protective role in the cardiovascular system. Moreover, recent studies also proposed a beneficial role of HDL in the CNS; in particular growing evidence showed that HDL can modulate cognitive function in aging and age-related neurodegenerative disorders including AD[44].

In this context, some pre-clinical studies highlighted the role of HDL in AD using different transgenic mouse model of AD that have been used to explore human lipoproteins metabolism. For example, APP/PS1 transgenic mice, bearing human Swedish APP and PS1 mutations, two well-known mutations of familiar AD, overexpressing human apoA-1 are characterized by an increase in plasma HDL levels and a parallel improvement of memory

deficit with reduced amyloid deposition[45]. In accordance, the deletion of apoA-1 in the same mice model worsened memory impairment[46].

Albeit observational and in vivo studies support a protective role of plasmatic HDL in cognition and AD, however, the exact mechanism by which HDL positively affect cognitive function remains uncertain. One of the possible mechanisms included the HDL-influence on the APP processing pathway. In particular the hypothesis is that apoA-1 and HDL mediates the cholesterol efflux resulting in enhanced membrane fluidity that increased the non-amyloidogenic cleavage by α-secretase to generate the soluble form of Aβ that undergoes hepatic clearance and does not accumulate in plaques[47]. Alternatively, apoA-1 bind APP on cell surface avoiding APP from undergoing the endocytic process, essential for β- and γ-secretases activities and resulting in decrease production of the neurotoxic insoluble Aβ.

Another mechanism implicated in the neuroprotective effect of HDL could been enhancement of Aβ clearance. At this regards, in vitro evidence suggests that ApoA-1 interacts with Aβ and prevents its aggregation[47]. In addition, apoA-1 and HDL have an high affinity for Aβ and mediate its clearance thought the BBB[48]. Interestingly, a very recent work displayed that HDL lipidation state could affect Aβ clearance from the brain in particular, discoidal HDL had a higher capacity to promote Aβ efflux across BBB in vitro then apoA-1 in other lipidation states. In addition, discoidal HDL can also disturb Aβ conformation by decreasing fibril concentration and extension[49].

To further support the protective role of apoA-1, also AD mice, in which apoA-1 have been knocked down or overexpressed, enhanced or reduced cerebrovascular Aβ deposition have been observed, respectively[46]. Beyond the direct action on Aβ, further mechanisms that contribute to the protective role of HDL could be related to the antioxidant and anti-inflammatory proprieties of HDL. Oxidative stress and neuroinflammation play in facts an important role in the pathogenesis of AD[50]. In this context it has been observed that reconstituted HDL containing apoA-1, if administered in a rat model of stroke, ameliorated the neuronal impairment through an antioxidant mechanism by reducing reactive oxygen species[51]. Moreover, a triple transgenic mice model overexpressing APP and PS1 mutations together with human apoA-1 (APP/PS1/A1) showed that the presence of apoA-1 avoided learning and memory impairment associated with reduced neuroinflammation. In

particular, APP/PS1/A1 mice displayed increased HDL-associated PON activity and a decrease microglia astrocytes activation compared to APP/PS1 mice. In accordance, also cultured Aβ-induced hippocampal slide overexpressing apoA-1 showed a decreased production of MCP-1 and IL-6[45].

Recent evidence supports the role of HDL in preserving cognitive functions during aging. In facts, a centenarians Jews study revealed that plasmatic HDL levels are positively related with cognitive function determined by Mini-Mental State Examination (MMSE)[52]. In accordance, the Leiden 85 plus study, a population based study in patients that achieved 85 years of age, indicated that low HDL levels were significantly associated with a MMSE score of 25 points, independently of atherosclerotic disease[53]. In addition, low HDL levels were related to short memory defined by Short term verbal memory compared to high levels in middle-aged adults in the Whitehall II study and the decrease in HDL levels was associated with memory decline independently of confounding including education, employment, comorbidity and apoE genotype over 5 years follow-up [54].

A French cohort study in AD patients and control subjects evidenced lower plasmatic HDL and apoA-1 levels in AD patient compared to controls and that apoA-1 concentrations were correlated with the severity of the disease defined by the MMSE score[55]. Moreover, the Prospective cohort Manhattan study found that higher HDL levels (>55 mg/dL) were associated with a decreased risk of both probable and possible AD (hazard ratio, 0.4; 95% confidence interval, 0.2-0.9; P = 0.03) also after adjustment for age, sex, education, ethnic group and APOEε4 genotype[56].

Accordingly, another research in centenarians Ashkenazi Jews (mean of age 99 year), carriers of the polymorphism 1405V on the CETP gene, demonstrated that low CETP levels and increased levels of HDL-C and HDL particles of larger particles size were significantly correlated with peculiar longevity with a MMSE score > 25 points[57]. Finally, the phase II clinical trial ASSERT (A Stratified Sickle Event Randomized Trial), in which patients were treated for 12 weeks with RVX-208, a small molecule that stimulates apoA-1 gene expression, showed an increase in plasma Aβ levels compared to baseline. Thus, the plasma apoA-1 increased is associated with enhanced Aβ clearance from the brain[58] with peripheral “sink effect” that turned in decrease Aβ budern[59].

4. Lipid metabolism and transport in the Central Nervous System

4.1 Lipoproteins in the Central Nervous System

Lipoprotein metabolism and transport in the CNS have not yet been fully clarified, although there are some studies on lipoproteins and apolipoproteins in the brain focusing in the CSF. Lipoproteins metabolism in the CNS have generate interest in the few years especially for the relation between cholesterol and neurodegenerative disorders.

The first who described the lipoproteins present in the CSF were Swahn, Bronnestam and Dencker about 40 years ago[60], but it's only in the recent years that lipoproteins have generated interest due to the relationship with Alzheimer's disease pathogenesis. The CSF is a clear and colourless fluid present in the brain and spinal cord and it is secreted by ependymal cells in the choroid plexus of the lateral, third and fourth ventricles. CSF is used to investigate the cholesterol metabolism in the CNS due to its poor accessibility. CSF contains lipoproteins that have a size and density similar to plasmatic HDL and for this reason they have been identify as "HDL-like-particles". Lipoproteins in brain has not been fully studied yet, although some investigators have tried to characterize the lipoproteins composition in the CSF by different techniques i.e density gradient, sequential flotation ultracentrifugation, gel filtration or affinity chromatography[61]. In particular, lipoproteins fractioned by density gradient ultracentrifugation float at *d*=1.063 to 1.21 g/mL with the greater part at *d*=1.09 to 1.15 g/mL. These particles are mainly spherical and display a wider size range compared to plasma HDL, from 10nm to 22nm and different lipid and protein compositions with a prevalence of apoE and apoA-1. Other apolipoproteins present in the CSF are are apoJ, apoA-II, apoA-IV, apoD, and apoH, while apoB is lacking, due to its incapacity to cross the BBB[62].

Several groups reported the existence of specific subfraction of CSF lipoproteins detected, presenting some differences in terms of size, lipid and apolipoprotein composition[63–65]. For example, CSF lipoproteins fractioned by immunoaffinity columns displayed two different particles; one spherical containing apoE, with a size of about 15nm and another lower rate of particles containing apoA-1 that are smaller and spherical with a size of 12.9nm[66]. Another research group analyzed CSF lipoprotein by gel filtration. After the separation by affinity chromatography they isolated 4 lipoprotein classes that can be distinguished for

size, lipid and apolipoprotein composition: CSF-LpEA, CSF-LpE, CSF-LpA and sCSF-Lp[67] (**Table 1**). CSF-LpEA represents the principal class containing apoE and apoA-1 and others minor apolipoproteins i.e. apoA-IV, apoD, apoH and apoJ. They display a size of about 13-20nm and a lipid: protein ratio of 0.51:1. The second one is CSF-LpA mostly containing apoA-1 and apoA-II but also apoA-IV and apoD with a size between 13 and 18nm. The third class, CSF-LpE, described large particles with only apoE but any apoA-1 with a lipid protein ratio of 0.76:1. In the fourth class sCSF-Lp neither apoE nor apoA-1 was detected but only apoA-IV, apoD, apoJ; they have small particles size about 10-12nm [67].

About lipid composition, total amount of lipids in the CSF of healthy subjects is about 1.25mg/dl and cholesterol and phospholipid, the two main lipids, are about 0.4mg/dl[68 69,70]. CSF lipoproteins mainly contain cholesterol and phospholipid, mostly phosphatidylcholine (54%), sphingomyelin (20%) and phosphatidylethanolamine (13%). The ratio unesterified/esterified cholesterol differ from plasmatic HDL with a percentage of unesterified cholesterol of ≈35% compared to 25% of peripheral HDL[66].

	Plasma			**CNS**	
	Pre-beta HDL	**HDL3**	**HDL2**	**Glial-derived HDL**	**CSF HDL**
Size (nm)	<8.0	7.8-8.8	8.8-13.0	8.0-12.0	13.00
Density	1.125-1.25	1.125-1.21	1.063-1.125	1.00-1.12	1.063-1.21
Shape	Discoidal	Spherical	Spherical	Discoidal	Spherical
Apolipoproteins	ApoA-1	ApoA-1 ApoA-II	ApoA-1 ApoA-II	ApoE, apoJ	ApoE, ApoA-1
Major lipids	PL/UC	PL/UC	PL/UC	PL/UC	PL/UC

***Table 1**. Comparison between plasma and CSF lipoproteins[71]. PL: phospholipids, UC: unesterified cholesterol*

4.2 Lipoprotein synthesis, secretion and metabolism in the Central Nervous System

Lipoprotein synthesis, secretion and metabolism in the brain is very peculiar. First of all, apoB-containing particles including low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and intermediate-density lipoproteins (IDL) are not present in the CNS. Moreover, HDL-like particles, differently from plasmatic HDL, are mainly composed by apoE. ApoE is the major apolipoprotein present in the CNS and involved in lipid metabolism together with the apoA-1. ApoE is secreted in the brain and cannot cross the blood-brain barrier, as demonstrated in liver transplanted patients that presented in plasma the donor apoE phenotype but preserved their phenotype of birth in CSF lipoproteins[72].

ApoA-1, in contrast to apoE, is not secreted in the CNS but derives from the peripheral compartment by crossing the BBB through an endocytosis process[73].

The synthesis, secretion and formation of lipoproteins in the CNS have not been fully elucidated yet compared to plasma lipoproteins. Lipoproteins in the CNS are mainly synthetized by glial cells in particular astrocytes. Astrocytes secreted discoidal nascent lipoproteins of about 8-12nm mainly composed by phospholipids, unesterified cholesterol and apoE or apoJ[74]. These nascent discoidal particles are further enriched in cholesterol and phospholipid by several ATP-binding cassette (ABC) transporters, that are transmembrane proteins that used ATP hydrolysis to convey molecules through biological membranes and widely expressed in the brain[75]. ABCA1 transporter is express in the CNS and in astrocytes secretes nascent discoidal HDL- like particles[76]. After apoE secretion, ABCA1 generates low density HDL-containing apoE and further synthetized cholesterol is assembled to HDL through the interaction with ABCA1 in caveolin-enriched domain[77]. The transporter is implicated in the removal of excess of cholesterol from neurons, as demonstrated *in vitro* using reconstituted apoE containing particles[78].

The majority of the HDL-like particles found in the CSF are spherical and display different size compared to nascent discoidal lipoprotein secreted from astrocytes, supporting the idea that these particles undergo wide modification after secretion from astrocytes, similar to what occur during HDL plasmatic maturation[67]. The transporters ABCG1 and ABCG4 are well expressed in the brain cells including astrocytes, microglia, and neurons and promote further incorporation of cholesterol and phospholipids in the nascent HDL-like particles (**Figure 1**).

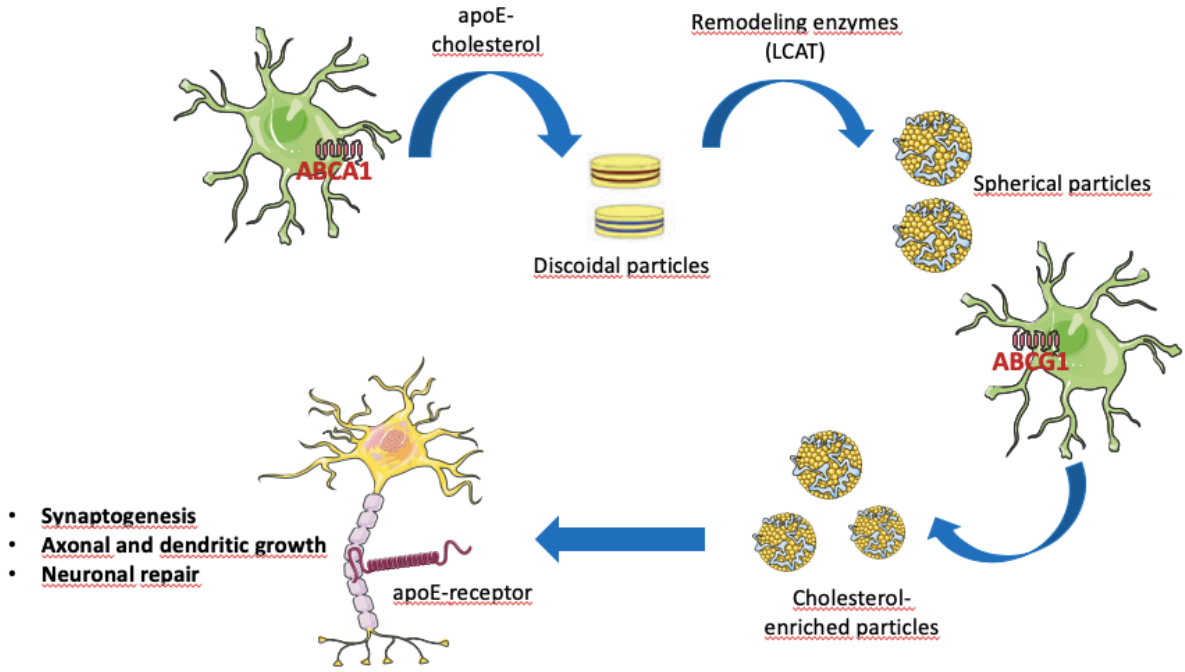

Figure 1| *Lipoprotein Synthesis and Metabolism in the CNS*

Little is known about brain lipoproteins maturation in contrast to plasmatic HDL, however several remodeling enzymes and lipid transfer proteins are also present in the CNS.

One of these enzyme is Lecithin cholesterol acyltransferase (LCAT), a soluble enzyme that converts free cholesterol and phosphatidylcholines to cholesteryl esters and lyso-phosphatidylcholines on circulating lipoproteins, mediating a key step in the maturation of plasmatic HDL[79]. LCAT is also synthetized within the brain, one of the three organs together with liver and testes, suggesting a crucial role in the maturation of brain lipoproteins. In human, CSF LCAT concentrations are approximately ≈5% of plasmatic LCAT[80]. The primary producer of active LCAT in the brain is astrocytes and astrocytes-derived LCAT is the only enzyme able to esterify free cholesterol on nascent discoidal apoE-containing particles[81]. It is known that apoA-1 is the first activator of plasmatic LCAT, however also apoE can enable LCAT, although less efficiently than apoA-1[81]. LCAT promotes the maturation of nascent discoidal apoE containing particles into mature spherical ones, considering that glial-derived apoE-containing particles are LCAT substrates.

Similarly, other two enzymes, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) are also involved in the remodeling of plasmatic HDL.

CETP has been also detected in the human CSF in a concentration of about ≈12% that of plasma. CETP is also present in conditioned medium from human neuroblastoma and neuroglioma cells indicating that this enzyme is synthetized and secreted in the brain where it contribute to exchange cholesteryl ester and triglycerides between lipoproteins[82]. A single-nucleotide polymorphism (SNP) in CETP gene is associated with a lower rate of memory decline and lower risk of incident dementia, including Alzheimer disease (AD)[83].

PLTP is an enzyme involved in biogenesis and remodeling of plasma HDL; its produced by neurons, astrocytes and microglia and its CSF activity represents about ≈15% of plasmatic one. It has been demonstrated that PLTP is highly expressed and secreted by brain capillary endothelial cells and actively involved in HDL genesis and remodeling at the BBB[84]. In particular, in an in vitro model of BBB, brain capillary endothelial cells secreted PLTP into the parenchymal side and the pretreatment with HDL3 and PLTP produced HDL particles of small and large size and increased the capacity of newly generated particles to take off cholesterol from brain capillary endothelial cells[84].

PLTP deficient mice presented reduce BBB permeability with decreased integrity of tight junction proteins and increased cerebrovascular oxidative stress[85]. In addition, PLTP levels were higher in brain tissues from AD patients compared to non-AD subjects[86].

Once reached the completed maturation, these newly generated brain HDL particles containing apoE can be internalized by neurons thought the interaction with specific lipoprotein receptors including the low-density lipoprotein receptor (LDLR), the very low-density lipoprotein receptor (VLDLR), the apoE receptor 2 (apoER2) and the low-density lipoprotein receptor-related protein 1 (LRP1) [87,88]. This process seems essential for the supply of cholesterol to neurons in order to maintain their physiological functions.

5. Apolipoproteins

5.1 ApoE: Sites of Synthesis, Secretion, and Regulation

ApoE is a multifunctional protein with a pivotal role in lipid metabolism, neurobiology and neurodegenerative diseases. It is a protein constituent of plasmatic lipoproteins firstly discovered in the early 1970s. ApoE is a glycoprotein with a molecular mass of 34KDa composed by 299 amino acids and it is the major apolipoprotein expressed in the CNS. It was firstly identified in plasma triglyceride-rich lipoproteins in combination with VLDL, IDL and unique subclasses of HDL[89,90]. The apoE concentration in plasma and CSF is about 40-70μg/ml and 3-5μg/ml respectively[66]. ApoE is encoded by a single gene on chromosome 19 [APOE]. APOE gene displays genetic polymorphism determined by three common alleles: APOE2, APOE3, APOE4 that are expressed codominantly and lead to different phenotypes E2E2, E2E3, E2E4, E3E3, E3E4 and E4E4. The three isoforms differ only by a single amino acids substitution: the most frequent isoform, considered the normal form, is apoE3 with a prevalence of 65-70% in the general population; ApoE4 differs from apoE3 for an Arg instead of Cys at residues 112 with a prevalence of 15-20%; conversely, apoE2 for a Cys instead of Arg at residue 158 with a prevalence of 8-10%[91,92]. These variations in apoE isoforms maybe result in an alteration of protein structure and consequent correlated biological functions. In particular apoE2 has a defective LDL receptor binding and is associated with a genetic disorder, the hyperlipoproteinemia type III[93], characterized by increased plasma chylomicron remnants and early atherosclerosis development.

On the other hand, apoE4 is one of the most strong risk factor for developing AD[94,95], while apoE2 in this context have shown a protective effect[96].

The principal apoE synthetizing organ is the liver followed by the brain expressing apoE in high concentration[72] but also spleen, kidney, adrenal gland, macrophages are sites of synthesis. In the brain apoE is synthetized by different cells type in particular astrocytes are the major source of apoE followed by microglia, oligodendrocytes, ependymal cells[97] while neurons generated a splice variant of RNA that is minimally translated[98]. Although in minor amount also hippocampal and cortical neurons in particular condition such as after injury may synthetize apoE[99]. apoE mRNA is translated into a protein of 317 amino acids with consequent cleavage of its signal peptide of 18 amino acids.

Before its secretion, apoE undergoes post translational modification including O-linked glycosylation and sialylation[100]; while, most of the plasmatic apoE about 80-85% is the asialo form due to post secretory de-sialylation[101]. On the contrary, apoE in the CNS is highly sialylated compared to the plasmatic form[102]. It's is not still clear whether post-translational modification could influence apoE metabolism and biological functions and also if it is dependent by the isoform or tissue. ApoE maturation occurs through the Golgi apparatus and secretion of apoE are inhibited when the endoplasmic reticulum membrane is enriched in cholesterol[103].

ApoE displays two structural domains linked to a hinge region; in particular the nuclear magnetic resonance (NMR) showed the entire apoE structure with a N-terminal domain (1-167 amino acids) and the C-terminal domain (206-299 amino acids) separated by the hinge region (168-205 amino acids)[104].

The N-terminal domain represents two thirds of apoE and, as shown by X-ray crystallography and NMR, it is composed by a four-helix bundle[105]. Helix 4 in the N-terminal domain contains the receptor binding region (136-150 amino acids) and mediates the interaction between apoE and the ligand binding domain of the LDL receptor[106]. Arginine at residue 158 regulates the conformation of the binding region and it is able to modulate indirectly the receptor binding capacity. For this reason, only apoE3 and ApoE4 that possess an Arg at residue 158, shown a physiological receptor binding capacity, instead of apoE2, which have a Cys and is lacking in LDL receptor binding capacity. On the other hand the C-terminal domain, that represent one third of apoE, contains the lipid-binding site (244-272 amino acids)[107]. In the C-terminal domain interface several hydrophilic amino acids are hidden; the unmasking of this residues destabilizes the domain and creates a great hydrophobic surface that mediates the binding of apoE to lipoproteins[104]. This is demonstrated by the observation that a truncated fragment apoE including amino acids from 1 to 244 displayed reduced capacity to bind lipoproteins compared to a truncated fragment containing amino acids from 1 to 272, that hold a full lipoprotein binding capacity[108]. However, the fondness of apoE3 and apoE2 for HDL whereas of apoE4 for VLDL and LDL could not be explained by the different amino acids in apoE isoforms (amino acid 112) since the residue is located in the N-terminal domain, while the lipid binding region is

in the C-terminal domain suggesting that these two domains interact to determine the protein conformation and consequent preference for the different lipoproteins[107].

ApoE also has two heparin-binding sites, each within the N-terminal and the C-terminal domains[109]. Analyzing crystallographic structure of apoE3 compared to apoE4 revealed that in apoE3 the presence of cysteine 112 is near to the glutamic acid in position 109 in helix 3 and to arginine 61 in Helix 2, while in apoE4 the presence of arginine in position 112, instead of a cysteine, caused a different adaptation of glutamic acid 109 and arginine 61. For this reason, arginine 112 is linked thought a salt bridge to glutamic acid 109 and the side chain of arginine 61 is redirect in the aqueous environment away from the helix, where arginine 112 can interact with glutamic acid in position 255 with consequent connection between the N-terminal and C-terminal domains leading to different protein conformation[93].

This domain connection might explain the preference of apoE4 for binding LDL and VLDL and the relevance of the interaction goes beyond the lipid binding capacity of apoE isoforms and could help to understand the role of apoE isoforms in AD. Indeed, it has been showed that apoE4 domain connection also happen in neuronal cells and could help to promote the deleterious effect of apoE4 in AD pathogenesis[110].

As already described in Section 4, the function of apoE is to convey lipids to different cells types and tissues; apoE alone is not stable but it requires the combination with lipids, indeed ABCA1 knockout mice, the transporter involved in apoE secretion and lipidation, displayed a reduced levels of apoE in cortex and CSF as well as small apoE containing particles indicating an anomalous lipidation of apoE[111].

ApoE supports the interaction between apoE containing particles and lipids complexes with various apoE receptors including the LDLR, LRP, the VLDL receptor, the apoER2 and gp330. Every apoE isoforms possess different capacity to bind the apoE receptors in particular the LDLR. The affinity of apoE3 and apoE4 for the LDLR is higher compared to apoE2.

Moreover, apoE also binds Heparan Sulfate Proteoglycans (HSPGs) on the cell surface with particular distinction based on the apoE isoform. HSPG is essential for the transfer of apoE containing particles to LRP and other receptors to mediate their internalization. The interaction between apoE and HSPGs in the initial step for the next internalization through HSPG-LRP pathway and its activity is not limited to hepatocytes but also in neurons[112]. HSPGs is widely express on the cells surface and mediated the uptake of different ligands

including tau, α-synuclein and soluble APP via electrostatic interactions[113,114]. In association with LRP1, HSPGs is also involved in the internalization of Aβ in neuronal cells that also hold an heparin-binding region[115]. For this reason, apoE thought HSPGs could influence Aβ aggregation and metabolism. In particular, apoE containing lipoproteins prevent Aβ binding and Aβ internalization thought HSPGs in neurons in a concentration but not in isoform dependent manner[116].

5.2 ApoE and AD pathogenesis

ApoE4 represents one of the most known genetic risk factors for late-onset Alzheimer's disease as demonstrated by several epidemiological studies[117]. AD patients with at least one apoE4 alleles have 3 or 4 times more risk to develop the disease and also a decrease age of the disease onset of about 8 years compared to the ones without the alleles. In contrast, the APOE ε2 allele appears to decrease AD risk. Several are the explanations available in literature that contribute to explain the association ApoE4-AD risk. For example, some evidence highlighted an effect of apoE4 on Aβ metabolism, with the consequence that apoE4 would enhance neuropathology and cognitive decline caused by Aβ. An *in vitro* study showed that both apoE4 and apoE3 bind to Aβ peptide forming stable complexes that do not detached by boiling with sodium dodecyl sulfate; however, Aβ form complexes with apoE4 more efficiently and promptly[118]. In addition, an *in viv*o study in an AD transgenic mice model expressing the different human apoE isoforms revealed that hippocampal amyloid deposition occurred in an isoform dependent manner with apoE4>E3>E2. They also observed, through an in vivo microdialysis, the Aβ levels in ISF (esplicita) as an index of Aβ clearance, founding a reduction in this parameter in AD transgenic mice carriers of apoE4, while Aβ synthesis was not affected by the different apoE isoforms[119].

Interestingly, hAPP young transgenic mice, in which Aβ accumulation is less pronounced and dependent of age, expressing one or two copies of human APOE3 or APOE4 gene did not modified Aβ accumulation. However, 12-month old mice expressing one or two alleles of apoE4 displayed significantly higher levels of Aβ accumulation and plaque burden than age-matched mice expressing one or two alleles of apoE3, indicating an isoform-dependent apoE effect on Aβ deposition[120].

Other evidence both *in vitro* and *in vivo* suggested an Aβ-independent role of apoE4 in developing AD. The first one points to the role of apoE4 in inducing neuronal and behavioral deficit as demonstrated by Buttini et al. that analyzed apoE KO transgenic mice in which human apoE3 or ApoE4 was specifically expressed in neurons at similar levels. They observed that apoE4 mice revealed an impairment in the ability to remember and to process spatial information; this behavior worsened with age and occurred in a more marked manner in the females. Moreover, the same mouse model showed that apoE3 expression, but not apoE4, protected against kainic acid-induced neurodegeneration[121,122]. Since this transgenic mouse model is not characterized by Aβ accumulation., this and other studies suggested an Aβ-independent role of apoE4 in causing neuronal and behavioral deficit *in vivo.*

Another possible mechanism underlying the pathogenic involvement of apoE4 in AD relates to the observation that apoE4 undergoes enhanced proteolysis compared to apoE3 trough the activity of a neuron specific chymotrypsin-like serine protease enzyme, generating fragments of about 12-29KDa, that have been shown to be neurotoxic[123]. This apoE4 increased susceptibility to proteolysis seems to be due to its peculiar conformation since the mutation in Arg in position 61 to Thr or Glu in position 255 to Ala, interfering with domain interaction, significantly inhibited the apoE4 tendency to undergo proteolysis[123].

A study in transgenic mice expressing carboxyterminal truncated apoE4 (Δ272-299) in the brain at 4 month of age showed cortical and neuronal abnormalities including phosphorylated tau and cytosolic straight filaments similar to nascent neurofibrillary tangles[124]. Mice expressing truncated apoE4 showed neurons degeneration and impairment in learning and memory suggesting an AD like neurodegeneration. These fragments include the receptor binding region and the lipid binding region and are the minimum apoE structure responsible for the neurotoxicity.

In humans, these apoE fragments are present in AD brain at higher concentrations compared to age and sex- matched non-AD controls, with an APOE gene-dose-dependent effect; moreover, carboxyterminal truncated fragments of apoE have been observed to be elevated in neurofibrillary tangles and amyloid plaques of AD patients[125;126]. Some authors hypothesized that as a consequence of injure or stress, neurons enhanced apoE expression;

however, the presence of apoE4 triggers proteolysis generating neurotoxic fragments leading to neurodegeneration.

ApoE4 fragments enter the cytosol and target cytoskeletal components or mitochondria. Among these components, apoE4 carboxyterminal fragments interacts with tau and neurofilament leading to neuronal inclusions similar to intracellular neurofibrillary tangles constituted of phosphorylated tau and neurofilaments with high molecular weight[126].

In addition, apoE4 fragments interacts also with mitochondria and for this interaction the lipid binding region, which contains the mitochondrial binding region, is critical [108]. Further analyses showed that mitochondrial dysfunction in AD depends on apoE genotype, and the consequence are more pronounced in apoE4 than in apoE3 carriers[127]. The disturbance in electropotential generation by apoE4 fragments could affects different neuronal mitochondrial functions such as synaptogenesis and the regulation of glucose metabolism as shown in middle-age patients carriers of the apoE4 isoform that presented impaired rates of glucose metabolism in several brain regions[128].

Differently from apoE4 fragments, the whole apoE4 is not able to bind tau, while apoE3 does it, and form stable complexes. Phosphorylation of tau prevented the binding of apoE3 with tau indicating that apoE3 only interact with non-phosphorylated tau[129] while apoE4 is associated with hyperphosphorylated tau[123]. Several studies showed an enhanced phosphorylation of tau in mice expressing human apoE4 in neurons but not in astrocytes, suggesting a cell-type specificity of apoE4 on tau phosphorylation[123]. The augmentation in tau phosphorylation observed in apoE4 transgenic mice are associated with the activation of Erk pathway[130].

Furthermore, astrocytes-derived apoE3 but not apoE4 induced hippocampal neurite outgrowth[131]. *In vitro* Neuro2A cells transfected with apoE3 and apoE4 incubated with β-VLDL, VLDL and CSF HDL-like particles displayed decreased neurite extension in the presence of apoE4 compared to apoE3[132]. This effect may be explained by a reduction in microtubules and a decreased ratio polymerized to monomeric tubulin in Neuro2A apoE4-treated then apoE3-treated cells[133].

Another mechanism possibly explaining the effect of apoE4 in neurodegeneration could be the effect of apoE4 on lipid transport in the CNS. It is known that apoE4 is degraded more

efficiently compared to apoE3 and apoE2[134] and that apoE4 showed decreased capacity and affinity to bind lipids[135].

Moreover, the capacity of apoE4 to promotes cholesterol efflux was decreased compared to apoE3, also when only the amino terminal portion have been evaluated[136]. For these reasons apoE4 could be associated with decreased lipid supply to neurons resulting in neuronal cholesterol depletion and consequent impaired neuronal functions. However other studies did not confirm this observation revealing that the different apoE isoforms did not influence the capacity to promote cholesterol efflux through ABCG1, ABCG4 and SR-BI[137]. The question on the involvement of apoE4 in brain cholesterol transport is thus still open and further studies are needed to come to a definitive conclusion on this issue.

5.3 Apolipoprotein J

Apolipoprotein J (apoJ), also known as Clusterin, is a human 80kDa glycoprotein firstly isolated in testis fluid and identified for its aggregating or "clustering" effect on Sertoli cells[138]. In the following years Clusterin has been found in different tissues under different names according to tissue localization and function including: testosterone repressed prostate messenger-2 (TRPM-2), serum protein-40,40 (SP-40,40)[139], complement cytolysis inhibitor (CLI), sulfated glycoprotein 2 (SGP-2) and apolipoprotein J (APOJ)[140]. Finally, it was concluded that all these proteins were produced from the same gene called CLU in 1992[141].

ApoJ is expressed in different tissues such as pancreas, lymphoid tissue, testis, prostate and brain[140], and present in biological fluids, and it is associated by a variety of functions including complement inhibition[139], chaperon function[142], lipid transport[143] and apoptosis[144].

In the CNS apoJ is mainly produced by astrocytes[135] and seems to contribute to the clearance of Aβ from the brain because apoJ containing lipoproteins can bind Aβ and are eliminated rapidly across the BBB via LRP-2[145].

Two Genomic Wide Association Studies (GWAS) have identified CLU, the gene encoding for apoJ as novel risk factor in AD[146,147], becoming the third most common lipidic genetic risk factor after APOE and BIN1; Subsequently, genetic studies have discovered several single nucleotide polymorphism (SNPs) as susceptible loci[148].

It has been shown that apoJ concentrations are increased in the hippocampus and cortex of the AD brain together with amyloid beta (Aβ) plaques[149] and this occurs also in the CSF[150]. In addition, increased plasma apoJ levels have been correlated with cortex atrophy and was also associated with greater burden of fibrillar Aβ in the brain[151] .

Moreover, endophenotype studies by analyzing apoJ CSF levels identified other loci that might be related to AD pathogenesis[152].

Considering the effect of apoJ on amyloid aggregation and consequent formation of amyloid fibrils and neuronal toxicity, a study revealed that apoJ is able to bind different oligomers of Aβ (from dimers to 50-mers) and to create long-term (from 50 to 200 hours), stable complexes. Consequently, apoJ can influence Aβ aggregation and prevented the further growth or dissociation of these oligomers to the formation of Aβ fibrils[153].

For example it has been demonstrated that the addition of a mix of physiologically chaperones including apoJ, preserved cell viability of neuroblastoma treated with CSF supplemented with Aβ and enhanced Aβ uptake by macrophage-like cells[154]. In the same way, a study on rat primary hippocampal cells displayed that pretreatment with apoJ prevented the increase of intracellular calcium, ROS generation and proapoptotic caspase-3 trigger by forming complexes with Aβ[155]. In another study, the treatment with apoJ before the injection of Aβ aggregates in rat brain hippocampus avoided Aβ-induced injury by improving learning and memory performance measured with the Morris Maze test and in immunohistochemical analyses decreasing inflammation and neuronal degeneration in rat brains[156].

On the other hand, considering the effect of apoJ on Aβ clearance, numerous mechanisms have been identified, among these a promotion of Aβ intracellular uptake and the transport across the BBB. In addition, in amyloid-treated human astrocytes an accumulation of clusterin have been observed together with the presence of vacuoles containing amyloid material[157]. On the opposite, the treatment with apoJ reduced Aβ oligomers uptake in human astrocytes incubated with Aβ, altering the uptake in vitro[158].

Finally, apoJ is also implicated in Aβ transport across the BBB, and Aβ clearance is enhanced when aggregate with apoJ in vivo through the activity of the receptor LRP2[145].

Recently, findings using an *in vitro* BBB model based on primary cerebral endothelial cells cultured on trans-wells to mimic the trafficking between the basolateral (brain) and apical

(blood) compartments have shown that the transport of labelled fluorescent Aβ from basolateral to apical compartment was increased when complexes with apoJ formed and blocking of LRP1/LRP2 pathways decrease this passage thought compartments[159]. All these finding demonstrated a relationship between apoJ and Aβ is relevant for amyloid clearance in vivo.

Additionally, animal models of amyloidosis investigate the correlation between apoJ and Aβ *in vivo* and conflicting results have been achieved. Unexpectedly, a study by *DeMattos et al* showed that PDAPP mice, a transgenic mouse model of AD, the genetic deletion of apoJ displayed significantly reduced amyloid deposition compared to apoJ expressing PDAPP mice. This phenomenon is accompanied by decreased neuritic dystrophy which points for a pro-amyloidogenic role of apoJ in this mouse model[160]. Subsequently, a second work from the same group demonstrated that the absence of apoJ and also ApoE significantly increased Aβ production and amyloid deposition[161].

Moreover, in another study the intracerebroventricular administration of an apoJ mimetic peptide [113–122] in AD transgenic mice for 2 weeks resulted in an improvement of cognitive function assessed by the Water Maze test. Immunohystochemistry analyses also revealed that amyloid plaque deposition is reduced in apoJ-treated group, together with a reduction of brain soluble $A\beta_{40}$ and $A\beta_{42}$ levels. Finally, western blot analyses of hippocampus and temporal cortex showed that the treatment increased expression of the receptor LRP-2 , one of the receptors involved in the $A\beta$ clearance from the brain[162].

In conclusion, several findings suggest an involvement of apoJ in AD, although is not clear whether apoJ is a causal factor or contributing factor to disease development.

6. ATP-binding cassette (ABC) transporters in the Central Nervous System

The human genome included 48 transcriptionally active ABC transporter genes that are divided into seven subfamilies (designated A–G) based on sequence homology. It is known that the subfamilies A and G are involved in the transport of lipids including phospholipids, sterols and bile acids[75].

ABC transporters are full-length transporters consisting of two hydrophobic transmembrane domains each containing six α-helices and two nucleotide binding domains (NBD-1 and NBD-2). Each NBD is characterized by a Walker A and Walker B motifs and an ABC signature sequence. ABC transporters use the energy derived from the hydrolysis of ATP, binding NBD domains, to transport substrates through the membrane against gradient concentration. Their role in the peripheral tissues in the transport of lipids through membrane is well-known; on the contrary little is known about their role in the brain. In facts, several ABC transporters are expressed in the brain and recent findings would establish either the specificity for the cell type and the brain region[75].

6.1 ABCA1 transporter

The subfamily A of the ABC transporters is composted by 12 members. The transporters ABCA1 compared to the other transporters, includes also two large extracellular loops with multiple sites for N-linked glycosylation.

ABCA1 is highly expressed in the adrenal system, uterus, lung, liver, kidney, gastrointestinal tract and testis. Peripheral ABCA1 is involved in HDL metabolism by being responsible for the HDL biogenesis at hepatic and intestinal level. In addition, ABCA1 is able to promote cholesterol efflux from the membrane of macrophages to the lipid free or lipid-poor apoA-I, the first step of the reverse cholesterol transport. Mutations in the ABCA1 gene cause Tangier disease, that is characterized by very low HDL levels associated with cholesterol accumulation and atherosclerosis[163].

First findings found ABCA1 in human and mouse brain; in particular, high ABCA1 expression has been found in brain regions including putamen, occipital lobe, amygdala, caudate nucleus, hippocampus and substantia nigra. Total brain expression is about 15 to 40% of the hepatic one as indicated by the qRT-PCR analysis in humans and mice[164]. ABCA1

expression have been evaluated also in different brain cells type, with detected ABCA1 expression in neurons, astrocytes, microglia and oligodendrocytes.

As already mentioned, ABCA1 is involved in cholesterol transport in the brain and since in the SNC membrane cholesterol is responsible for the APP processing and consequent Aβ production, ABCA1 have been investigated as a possible modulator of amyloid production and deposition.

The liver X receptors (LXRs) and retinoid X receptors (RXRs) regulate the expression of ABCA1 and are activated by specific ligands including 22(R)-hydroxycholesterol and 9-cis-retinoic acid which bind specific promoter regions to stimulate transcription. Therefore, ABCA1 mRNA and protein levels were increased following exposure to RXR/LXR agonists. This functionally translates in an increased in apoA-I and apoE cholesterol efflux from neurons and glia. Moreover, these ligands alone or in combination with apoA-I induced a reduction in APP stability with consequent decrease of amyloid production[78].

Considering that membrane cholesterol distribution, that is sensitive to the action of ABCA1[165] modulates APP processing, some studies focusing on the role of ABCA1 in the regulation of this process. Most of the findings supported a positive role of ABCA1 as shown in the Neuro2a neuroblastoma cell line in which LXR/RXR-induced ABCA1 expression have been demonstrated to decrease Aβ secretion[78]. Consistently, also CHO cells transfected with human APP695 displayed Aβ secretion of about 50%[166].

Studies *in vivo* in mice have confirmed the *in vitro* findings. ABCA1 KO mice displayed decreased apoE levels both in cortex and CSF. Moreover, the authors observed reduced cholesterol and apoE-containing particles in the CSF and in lipoproteins secreted from astrocytes obtained by ABCA1 KO mice suggesting impaired apoE lipidation[111].

Interestingly, a recent study with a small molecule LXR agonist, analogue of bexarotene, demonstrated a significant improvement of cognitive impairment, evaluated through the NOR and Morris Maze test in APP/PS1 transgenic mice treated for 15 days or three months. The treatment with the molecule decreased Aβ deposits in hippocampus and cortex of about 30% and Aβ soluble and insoluble levels and this effect seems to be mediated by an enhanced phagocytosis by microglia through apoE. Indeed, OAB-14 administration is associated with a significantly increase in ABCA1 and ABCG1 expression in association with

an the increase of lipidation of apoE[167]. Lipidated-apoE and Aβ formed a complex that can be phagocyted by microglia compared to apoE free with AB[168].

Also, the peptide CS-6253 that derived from the carboxyl terminal of apoE, activated ABCA1 in vitro and has been investigated for its role in transgenic mice in which endogenous apoE has been replaced with human apoE3 or apoE4. In apoE4 mice, the peptide treatment significantly decreased Aβ levels and tau hyperphosphorylation in hippocampal neurons leading to a phenotype similar to apoE3 mice. This effect is combined with increased apoE4 lipidation, suggesting that the detrimental effect of apoE4 in vivo are related to decreased ABCA1 activation and consequent insufficient apoE lipidation, that can be counterbalanced by the administration of an ABCA1 agonist[169].

With respect to ABCA1 and the modulation of amyloid production, AD mice models were treated with the LXR agonist T0901317 and after 6 days significant increase in ABCA1 expression and a decrease in APP amyloidogenic process with a reduction in sAPPβ in favor of sAPPα and a decrease in soluble $A\beta_{40}$ and $A\beta_{42}$ have been observed [170].

However, some doubt on the real correlation between ABCA1 expression and Aβ deposition and have been raised by an *in vitro* study that demonstrated that T0901317 can directly regulate APP processing by decrease γ-secretase activity[171], independently from ABCA1. Nevertheless, these last observations have been contradicted in a following study using APP/PS1 transgenic mice with loss in either LXRα and LXRβ resulted in surprisingly increased amyloid plaque deposition. In addition, the increase in Aβ deposition was related with a decrease in ABCA1 mRNA and protein levels[172]. Lastly, these and other in vivo studies showed that LXR modulate ABCA1 expression and in parallel Aβ deposition.

Several investigations have been conducted on genetic variations of ABCA1 and its association to AD in humans. Numerous studies confirmed an association between ABCA1 polymorphism and increased AD risk[173,174,175]. Accordingly, patients with a single nucleotide polymorphism in ABCA1 in the R219K allele are associated with later AD onset (mean 1.7 years) in healthy elderly patients[176]. In addition, a loss of function mutations in ABCA1 (N1800H), with a frequency of 1:500 in the general population, was associated with 4.13 higher risk of developing AD and cerebrovascular disease[177]. Also another rare variant in ABCA1 (rs137854495) has been found to be associated to AD in non-Hispanic white families suffering of LOAD AD[173]. However, a later meta-analysis of 13 studies failed to confirm this

association revealing that there was no significant correlation found between ABCA1 R219K, I883M and R1587K polymorphisms and risk for AD[178].

6.2 ABCG1 transporter

The subfamily G of the ABC transporters is composed by five transporters; all the transporters contain a single TMD and NBD. ABCG1 is the most representative transporters of this family. It is expressed in several tissues in mice and human and also in macrophages[75]. ABCG1 is also present in the brain as reported in several studies. In particular, ABCG1 expression has been found in some brain region including both the ventricular and mantle zones and in both gray and white matter of postnatal brains but also cortical layers, striatum and thalamus. At cellular level, ABCG1 is highly expressed in neurons, oligodendrocytes and astrocytes[166]. Like for ABCA1, LXL/RXR agonists enhance cerebral ABCG1 mRNA and protein expression. ABCG1, similar to ABCA1, has a pivotal role in maintaining cholesterol transport thought cellular membrane and the same function has been suggested for the CNS.

One of the characteristics that discriminate the two transporters for their capacity to promote cholesterol efflux is the kind of the extracellular cholesterol acceptor. In particular ABCA1, as mentioned before, interacts preferentially with apoA-I and pre-β HDL, while, ABCG1 mediates the efflux to mature HDL in the periphery and lipidated apoE-containing particles in the brain.

Based on the observation that ABCG1 is involved in the cholesterol transport in the brain the protein has been studied for its potential involvement in Aβ production and deposition. At this regard, CHO cells stably transfected with both human APP and ABCG1 have been investigated for the effect of ABCG1 on Aβ production. As in the case of ABCA1, a significant reduction in Aβ production but not in its clearance have been observed in ABCG1-overexpressing cells[166].

A later study in human APP-HEK cells did not confirm the previous observation: overexpression of ABCG1 showed an increased the Aβ production and also enhanced the secretion of sAPPα and sAPPβ[179]. The role of ABCG1 in APP processing has been further elucidated by *in vivo* studies as the one in AD transgenic mice overexpressing human ABCG1. Such study revealed that ABCG1 did not affect Aβ burden, with no difference in

$A\beta_{40}$ an $A\beta_{42}$ levels, neither APP in hippocampus and cortex and in cognitive performance[180]. Consistently, no effect on ABCG1 overexpression has been detected in mice in which behavioral tests have been assessed including locomotor activity and anxiety using the open field test and learning and memory performance evaluated by the Morris Maze test[181].

Finally, genetic studies founded a significant association between ABCG1 polymorphism and AD in individuals from Switzerland and Poland but not in Germany, Belgium, Sweden and Greece[182].

7. Lipoproteins receptors in the Central Nervous System

Specific receptors expressed on brain cells mediated the uptake of apoE containing lipoproteins. They represent an ancient family of structurally membrane proteins that bind apoE in different isoforms and lipidated status and other ligands. The receptors most involved in cholesterol trafficking in the brain belong to the low-density receptor (LDLR) family and include LDLR, LRP-1, VLDLR and apoER2[94] (**Figure 2**).

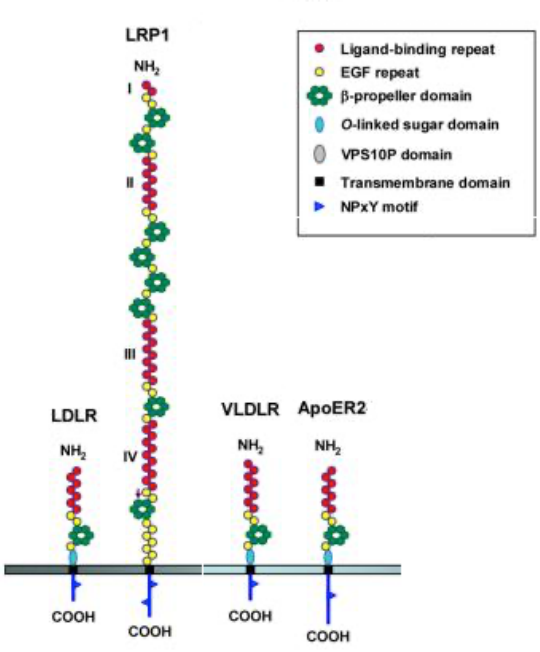

***Figure 2**| Structural organization of the LDLR family members. Modified from* [94]

7.1 LRP1

LRP1, also named as *L*DLR-*r*elated *p*rotein, is a large endocytic and signaling receptor that is widely expressed especially in the liver and brain. It binds several ligands including lipoproteins enriched in apoE, α2-macroglobulin (α2M), tissue-type plasminogen activator (tPA), blood coagulation factor VIII (fVIII) and APP[183]. LRP1 is produced in the endoplasmatic reticulum as a single transmembrane glycosylated protein of about 600kDa and then when reaches the *trans* Golgi compartment is cleaved by furin to produce two subunits, an extracellular one of 515 KDa and transmembrane one of 85 KDa, attached to the membrane through noncovalent association[184]. Successively LRP1 undergoes proteolytic processing and shedding resembling APP/Notch processing. Similar to the LDLR, ligands enter from the cell surface to intracellular compartment via receptor mediated endocytosis; LRP1 displays the highest levels of endocytosis rate compared to the other apoE receptors[185]. Disruption of the LRP1 gene in mice blocks early embryonic development[186].

Moreover, selectively *cre-loxP*- mediated LRP1 absence in differentiated neurons in mice results in abnormal functional neurotransmission with severe behavioral and motor abnormalities including hyperactivity, tremor, and dystonia even without histological visible alterations[187].

A conditional LRP1 forebrain knockout mice model showed significantly increase apoE levels while cholesterol levels decrease, suggesting an impairment in apoE lipoproteins catabolism[188]. The removal of LRP1 in mice also leads to dysregulation in cholesterol metabolism with decreased cholesterol brain levels. This deficiency is associated with age-dependent dendritic spine degeneration, synaptic loss and neuroinflammation. At 13-months of age mice slowly presented behavioral abnormalities including motor dysfunction and memory deficit and neurodegeneration[189].

With respect to the removal of Aβ from the brain, LRP1 seems to play a role in the receptor mediated cellular clearance pathway. Overexpression of a LRP1 minireceptor *in vitro* produced an elevated Aβ uptake and storage in neuronal lysosomes. On the other hand, LRP1 deletion significantly reduces neuronal Aβ uptake[190].

APP/PS1 mice with conditional forebrain neurons-specific knockdown of LRP1 showed increased brain Aβ levels and worsened amyloid plaque deposition in the cortex without compromising APP processing. *In vivo* microdialysis analysis revealed that Aβ clearance in

the brain interstitial fluid was decreased in LRP1 KO mice. These results indicate that deletion of LRP1 in neurons significantly suppresses the elimination of soluble Aβ from the ISF without affecting the principal Aβ degrading enzymes levels[191].

LRP1 is also involved in the clearance of Aβ thought the BBB as demonstrated by this study in which LRP1 has been specifically deleted in brain endothelial cells of C57BL/6 mice. The removal of LRP1 reduced brain efflux of radiolabeled Aβ. In addition, the deletion of brain endothelial LRP1 in 5xFAD mice decreased plasma Aβ levels and increased soluble brain Aβ with cognitive deficit and impaired spatial learning memory. [192]

In addition, LRP1 plays a pivotal role in the metabolism of apoE-Aβ complexes and apoE could compete with Aβ for the binding to LRP1, with derived impaired Aβ clearance[193].

In AD and with increasing age, LRP1 expression is reduced in total brain and capillaries[194]. Some genetic factors related to AD, such as APOE gene, could be associated with a decreased Aβ clearance through LRP1[145].

7.2 LDLR

The low-density lipoprotein receptor (LDLR) is a glycoprotein that plays an essential role in the removal of cholesterol-containing circulating lipoprotein particles from plasma. LDLR was firstly identified by Brown and Goldstein in 1973 during the investigation of the molecular basis of Familiar Hypercholesterolemia (FH), a genetic disorder caused by loss of function mutations of the LDLR encoding gene. It is located on the outer membrane surface of different cell types and it internalizes both apoB and apoE- containing particles of different densities thought electrostatic interaction between the acid amino acids present in the receptor and the basic ones present on apoB or ApoE. The LDLR is present on brain capillaries endothelial cells and astrocytes, where it is supposed to regulate Aβ clearance. Deletion of the LDLR in mice enhances of approximately 50% apoE levels in brain parenchyma and CSF, suggesting impaired apoE internalization by brain cells[195]. The comparison between LDLR and LRP1 showed important differences in the regulation of apoE and apoE lipoproteins metabolism in the CNS. First of all, LDLR is more expressed in glia rather than neurons while LRP1 Is predominantly present in neurons. Second, apoE-containing lipoproteins produced by astrocytes have more affinity for LDLR compared to LRP1[195], while recombinant apoE, apoE containing particles and HDL like particles isolated

from CSF bound LRP1[196]. This specificity for apoE receptor-binding is possibly dependent by the apoE conformation and lipidation status.

LDLR as well as other apoE receptor is associated with Aβ clearance. LDLR knockdown in primary astrocytes leaded a reduction of Aβ, however LDLR overexpression improved Aβ uptake and clearance independently of apoE presence[197]. Consistently, LDLR-deficient mice exhibited memory and learning deficiency. Interestingly, cholesterol-enriched diet in these mice exacerbate memory and spatial reference impairment followed by anti-oxidative imbalance and cortico-cerebral mitochondrial dysfunction[198,199].

Moreover, 5xFAD/LDLR KO mice have faster Aβ deposition and neurotoxicity that is not however associated to modification of APP expression[200]. On the other hand, brain overexpression of LRLR decreased Aβ aggregation and enhanced Aβ clearance, and furthermore ameliorated plaque-associated neuroinflammation[201]. LDLR overexpression also increased brain to blood transport of exogenously-administered Aβ and regulates endogenous Aβ clearance thought BBB efflux transport[202].

Concerning the LDLR, the results of genetic studies on association with AD are conflicting, despite the fact that a large study discovered a LDLR related SNP associated with AD in a sex- dependent manner[203].

7.3 VLDLR and ApoER2

The VLDLR and apoER2 are structurally very similar to the LDLR. In the peripheral body compartment, VLDLR and apoER2 internalizes apoE particles enriched in triglycerides like VLDL and IDL, however they cannot internalize LDL. In the brain, both receptors recognized apoE containing particles. These receptors interact with adaptor proteins to mediate cellular signaling; the best-known signaling pathways involving VLDLR and apoER2 is the reelin-mediated signaling, that plays a pivotal role in the control of neuronal migration and the formation of cellular layers during brain development and also involved in adult brain synaptic plasticity[204]. Accordingly, VLDLR/apoER2 double KO mice showed abnormalities in the lamination of cerebral cortex[205]. Lack of the VLDL receptor or the apoER2 exhibited contextual fear conditioning deficits, a behavioral test used to measure hippocampus-dependent memory induction and retention, and long-term potentiation deficiency[206].

In humans, mutation for the VLDLR leads to a rare genetic disorder that combines autosomal recessive cerebellar ataxia with mental retardation (disequilibrium syndrome) and cerebellar hypoplasia with quadrupedal locomotion[207]. This strongly support the involvement of the VLDLr in the brain physiology. Finally, meta-analyses on VLDLR polymorphism showed that VLDLR 5'-UTR genotype is associated with increased risk for AD [OR = 1.70] in the Asian population and is protective [OR = 0.48] in the non-Asian population[208]. This genetic study has not been confirmed by other suggesting that the association between VLDLR and AD should be further investigated[209].

8. Proprotein Convertase Subtilisin/Kexin Type 9 (PCSK9)

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a serine protease, representing the ninth member of the secretory subtilase family. It was firstly discovered in the brain as neural apoptosis-regulated convertase-1 (NARC-1) where it was supposed to play a role in neuronal development by increasing the recruitment of undifferentiated neural progenitor cells into the neuronal lineage[210]. It is produced as a soluble zymogen that is subjected to autocatalytic intramolecular conversion in the endoplasmic reticulum.

PCSK9 is a glycoprotein of 692 amino acids and the major domains, analogous to other family members, included a signal sequence, a pro-domain, a catalytic domain and a variable C-terminal domain, named V domain, with a fold never seen before in subtilisin family. PCSK9 possess a catalytic triad composed by Asp186, His226, and Ser386 overlapped with other subtilisin. PCSK9 is synthetized as a 72kDa precursor and the autocatalytic cleavage produced a heterodimer consisting in a 14kDa prodomain portion and a mature catalytic and C-terminal portion of 57KDa[211].

Almost all body PCSK9 is produced by the liver as indicated by tissue specific PCKS9 KO mice. The human plasmatic concentration of PCSK9 varies over a 100-fold range from 33 ng/ml to 2988 ng/ml[212]. The majority of circulating PCSK9 is associated with LDL but also with other lipoproteins like HDL and VLDL. In addition, in circulation PCSK9 can be found in two types: a mature form and a furin-cleaved form, that is not so active as the mature one, but provides again a residual activity. It has been seen that the majority of LDL binds PCSK9 in the intact form, while the furin-cleaved form is not bound to apoB-containing particles.

PCSK9 regulates cholesterol homeostasis by degrading the hepatic low-density lipoprotein receptor (LDLR). PCSK9 binds the LDLR in the extracellular surface while apoB100, the apolipoprotein constituent of LDL and the physiological ligand for the receptor, binds the receptor in a different site. After the internalization into the hepatocytes, the LDLR is shifted to the lysosomes where it can be recycled on the hepatic surface or degraded through hydrolytic activity. PCSK9 is capable to prevent the LDLR recycling, caused the receptor enzymatic degradation.

Since plasma concentration of LDL-C, one of the major proatherogenic factor in atherosclerosis, are defined by the percentage of LDL production and clearance, PCSK9 by regulating LDLR degradation represent a key player in peripheral cholesterol homeostasis.

Until 2003, just two gene mutations leading to hypercholesterolemia were known: one in the gene encoding for LDLR receptor causing familiar hypercholesterolemia (FH) and the other one in the gene encoding for apoB100, preventing the LDL binding to the receptor, causing familiar defective apoB100 (FDB). These autosomal dominant disorders by decreasing the LDL uptake in the liver and preventing their clearance, caused a marked increase in LDL-C levels resulting in accumulation of cholesterol in tissues and arteries[213].
In 2003, Abifadel et al. reported two mutations identified in French families, S127R and F216L, in the gene encoding for PCSK9 associated with hypercholesterolemia, so called "gain of function" mutations[214]. These PCSK9 mutations represent a small rate of autosomal dominant hypercholesteremia forms compared to LDLR and APOB. On the other hand, also "loss of function" mutations have been identified, i.e. Y142X and C679X in Afro-Americans, and R46L in Caucasians. They all associated with hypocholesterolemia and increasing the LDL clearance. These subjects presented decreased LDL-C, TC and TG levels[215] and an very low cardiovascular risk. As mentioned before PCSK9 is present mainly in the liver, but it is also expressed in other tissues and organs with specific functions, in particular in the vascular wall, in the kidneys, pancreas, intestine and brain (see the next chapter). Moreover, an association between PCSK9 and immunity has been suggested since sepsis and viral infections are influenced by the protein. The discussion on the role of PCSK9 in extrahepatic tissues are still in progress and in the next years we have to expected new and compelling studies that better clarify the biological roles of PCSK9.

8.1 Role of PCSK9 in neuronal development and apoptosis

A highly expression of PCSK9 have been observed in cells with increased proliferating activity, including embryonic telencephalon neurons; in addition, overexpression of PCSK9 enhances the recruitment of undifferentiated neural telencephalic progenitor cells toward the neuronal lineage[210]. The importance of PCSK9 in this phase has been shown also in undifferentiated P19 embryonal carcinoma cells: during neuroectodermal differentiation using retinoic acid, PCSK9 expression increased and achieved the maximum at day 2 indicating that initial PCSK9 increased expression may be necessary to regulate cell differentiation[216]. This is consistent with studies observed that PCSK9 is expressed during the gestational period in telencephalon and cerebellum but not in adulthood. In addition,

an initial studies showed that NARC-1/PCSK9 is transiently expressed during embryonic development in neurogenic centers such as those in the telencephalon and cerebellum, and that it is no longer expressed in mature CNS neurons of rodents (RE-OP)[210].

Mice brain co-expressed PCSK9 and LDLR during development and adulthood. Despite adult brain of PCSK9 KO mice showed similar LDLR and apoE protein levels in the RE-OP and olfactory bulb compared to WT mice suggesting that in adult mice PCSK9 does not enhance the degradation of LDLR. However, LDLR levels were 2.5-fold higher in telencephalon and cerebellum and are followed by a decrease in apoE levels, as a consequence of increased PCSK9 expression during development. In accordance, cell surface LDLR levels increased during brain development, indicating that protein LDLR levels upregulation increased apoE degradation[217]. Furthermore, no significant modification in cerebellum, cortex and hippocampus have been observed in PCSK9 KO mice[218] and this is consistent with the finding that human with LOF mutations for PCSK9 displayed no neurological deficit[219].

In zebrafish, embryonic knockdown of PCSK9 caused incomplete neurogenesis, disorganization of cerebellar neurons and loss of hindbrain-midbrain boundaries that led to embryonic death[216].

Neuronal apoptosis, a critical process occurring during brain development, that can also take place in adult brain of neurodegenerative disease, leads to neuronal loss network integrity. In cultured cerebellar granule neuros (CGN), PCSK9 has been described as one of the several genes that showed an upregulated expression during apoptosis followed by the removal of potassium and serum[220].

Consistently, rat cerebella granule neurons transfected with PCSK9 exhibited a proapoptotic effects that were partially reversible by a caspase inhibitor, consequently identifying both a caspase- dependent and independent mechanism of PCSK9 pro-apoptotic effect[221].

Recently, it has been reported that PCSK9 modulates apoptosis in human neuro glioma, a malignant tumor marked by aggressive proliferation and enlargement into adjacent brain tissues. The knockdown of PCSK9 in human neuroglioma cells enhancing apoptosis thought the activation of caspase 3 pathway and the decrease of anti-apoptotic proteins; on the contrary, the overexpression of PCSK9 decreased it[222]. Finally, it has been shown that RNA interference (RNAi)- mediated knockdown of PCSK9 inhibited activated caspase-3 and

reduced the levels of nuclear phosphorylated c-Jun in potassium withdrawal cerebellar granule neurons. PCSK9 RNAi resulted also in an increasing in apoER2 protein levels but not in VLDLR levels, with a consequent reduction in cell survival. This effect has been suggested to be a result of PCSK9 overexpression[223].

Although *in vitro* data demonstrated that PCSK9 can bind LDLR, VLDLR and apoER2, some *in vivo* studies observed that modifications in PCSK9 expression did not alter the expression of these receptor in adult mouse brain. While hepatic LDLR levels were modified in PCSK9 KO or PCSK9 overexpressing mice compared to WT, some contradictory data have been shown regarding LDLR levels in the brain of these animals[224]. These conflicting data may be justified by the fact that PCSK9 could have a cell and tissues specific functions or by the fact that PCSK9 it's not able to regulate receptors levels due to its lower brain concentration compared to plasma.

8.2 The role of PCSK9 and Alzheimer's disease pathogenesis

PCSK9, beyond regulating plasma cholesterol homeostasis, has shown to exert several extrahepatic effects that are object of intense investigation. Although, firstly identified in neurons, the involvement of PCSK9 in Alzheimer's disease (AD) is still unknown and the available results led to controversial conclusions[225]. Cerebral cholesterol is essential for neuronal development, neurite outgrowth, synaptogenesis and the repair of damaged membranes. As already said above, brain cholesterol, produced by astrocytes is transported by HDL-like particles and it's finally incorporated by neurons through the activity of specific receptors including LDLR, VLDLR, apoER2 and LRP1 that are targeted by PCSK9. As in peripheral tissues, brain PCSK9 may modulate lipid metabolism by degrading the receptors involved in brain cholesterol uptake. This hypothesis is supported by *in vivo* data displaying that LDLR expression in reduced by PCSK9 during brain development and following transient ischemic stroke[217]. For this reason, it is possible that the degrading activity of PCSK9 on lipoproteins receptors resulting in reduced cholesterol supplied to neurons with possible detrimental consequences[226] (**Figure 3**).

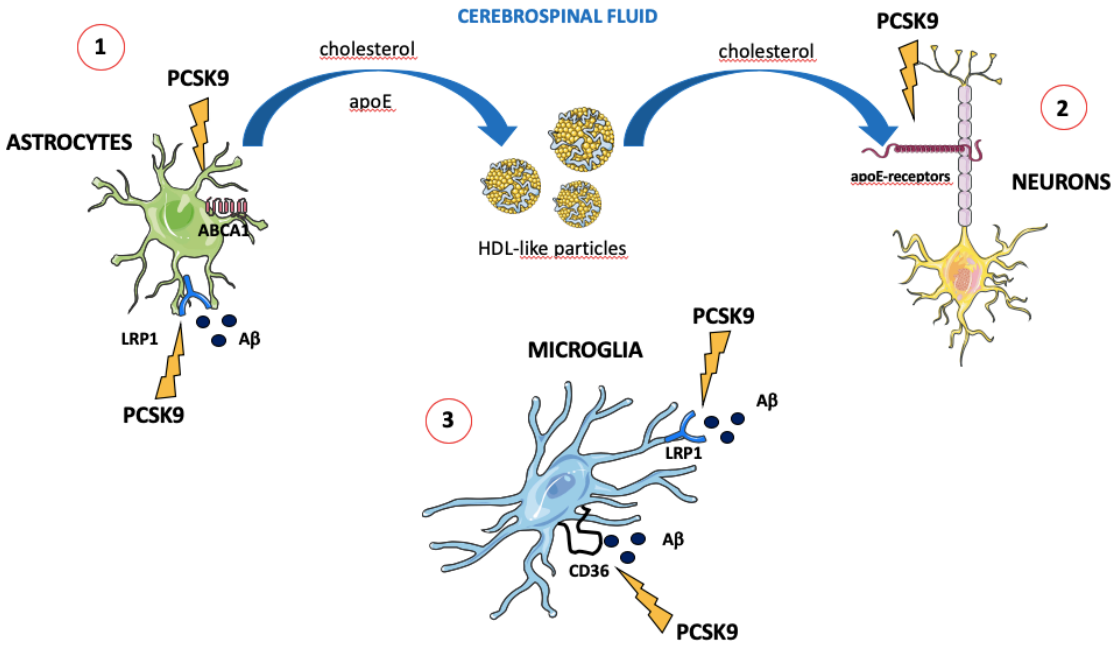

Figure 3 | *PCSK9 possible mechanism implicated in the pathogenesis of Alzheimer's disease*: **1**. In astrocytes, PCSK9 degraded LRP1, decreasing Aβ clearance and enhancing plaques deposition. PCSK9 reduces ABCA1 expression, preventing apoE lipidation and consequent generation of HDL-like particles; **2**. PCSK9 degrades neuronal apoE-receptors that internalize cholesterol through the interaction with apoE-containing particles impairing thus neuronal functions and leading to neurodegeneration. **3**. In microglia, PCSK9 degrades LRP1, decreasing Aβ clearance and enhancing its deposition within plaques. Moreover, PCSK9 modulates CD36 expression, resulting in Aβ-dependent neuroinflammation.

There are controversial data on PCSK9 and its involvement on AD pathophysiology. Regarding neuronal apoptosis, a preventive action of PCSK9 on neuronal apoptosis could take place thought the decrease of Aβ production[227] even though the mechanism of action of PCSK9 on Aβ production is still under investigation. In addition, PCSK9 deletion in mice resulted in increased levels of β-site amyloid precursor protein-cleaving enzyme 1 (BACE1), the enzyme involved in the generation of Aβ products, and consequent Aβ production in the brain. In addition, CHO cells overexpressing PCSK9 decreased the level of BACE1 while PCSK9 siRNA increased the level of BACE1 and secreted PCSK9 may stimulate the degradation of BACE1[228]. These data suggested that a protective role of PCSK9 by decreasing Aβ generation might inhibit neuronal apoptosis through the degradation of BACE1 in nerve cells.

On the contrary, in brain- damaged rats caused by ischemia-reperfusion injury, the pre-treatment with PCSK9 inhibitors reduced astrocytes and microglia activation and Aβ

expression. The pre-treatment ameliorated the loss of dendritic spine density caused by I/R injury and decreased neuroinflammation suggesting a negative role of PCSK9[229].

However, other authors indicated no evidence on PCSK9 and BACE1 levels and APP processing in mice brain[224]; so that further studies are needed to confirm whether or not PCSK9 modulate BACE1 activity.

The effect of PCSK9 on neurocognitive performance in pre-clinical models derives from the observation that the elimination of LRP1, which is sensitive to the degrading activity of PCSK9[230], conducts to a decrease in Aβ elimination and to worsened spatial learning and memory deficiency[192].

The scavenger receptor CD36 is implicated in fibrillar Aβ-mediated microglial activation and consequent innate immune response. PCSK9 increased the expression of CD36 in macrophages and microglia-like cells with possible consequent regulation of CD36 mediated Aβ clearance and innate response. Indeed, the recognition of Aβ by microglia depends also on the expression of CD36, that interacting with Aβ induces the release of proinflammatory cytokines. In addition, according to the hypothesis of Stewart and colleagues[231], Aβ binds to the CD36 receptors, triggering the assembly of a heterotrimeric complex with the Toll-like receptors (TLRs) 4 and 6, essential for host defence against pathogens[232].

Lastly, a possible modulation of cholesterol could be related also to the transporter ABCA1 which is involved in the lipidation of apoE- containing particles produced from astrocytes that transport cholesterol and lipid to neurons. PCSK9 has been shown to inhibit ABCA1-mediated cholesterol efflux in macrophages by downregulating protein and gene expression[233], that if confirmed also in the CNS, it would translate in impaired cholesterol supply to neurons. In this context, studies on the association between ABCA1 and AD are still controversial as well. On one side, carriers of the R219K SNP in ABCA1 gene displayed 33% lower cholesterol in the CSF compared to non-carriers and this variant was linked to delay in the onset of the disease by 1.7 years[176].

Also other studies observed an association between genetic mutations of ABCA1 gene and both decreased or increased AD risk[173,174].

8.3 PCSK9 and Alzheimer's disease in humans

In humans, several genetic studies have investigated the role of PCSK9 mutations on AD without reaching decisive conclusions. Firstly, Wollmer and colleagues analyzed PCSK9 among several genes with a role in cholesterol metabolism that has been matched with AD genes collected in the AlzGene database[234]. However, subsequent studies evidenced no association between PCSK9 polymorphisms and the risk of AD onset neither in a Japanese nor in a Swedish cohort study[235]. In accordance, in a recent Mendelian randomization study, loss-of-function mutations in PCSK9 gene were not related to an increase in the AD risk (Hazard Ratio (HR) = 0.50; p = 0.37)[236]. Moreover, the genetic analyses among African American REGARDS (Reasons for Geographic and Racial Differences in Stroke) participants with and without the PCSK9 loss-of-function variants C697X or Y142X, came to negative conclusions. The first endpoint was the neurocognitive performance and they concluded that participants with or without these variants displayed the same neurocognitive decline[237]. Consistently, in another study conducted in French Canadian participants, who carried PCSK9 LOF variants, in particular R46L and InsLEU, they found no differences on AD prevalence and on age of AD on set in patients carrier of PCSK9 LOF variants compared to non-carriers suggesting a neutral effect of PCSK9 on AD. Patients have been also stratified base on their apoE genotype and gender again without finding any difference[238].

A recent study identified two SNP, rs4927193 and rs499718 situated in PCSK9 intron 2 and 3 respectively, that is associated with AD risk in female[239]. Moreover, they also found increase PCSK9 protein and gene expression in frontal cortex of AD patients brain compared to controls[239].

In humans, PCSK9 has been detected in the CSF of healthy subjects even though a much lower concentration compared to plasma[240]. In addition, CSF PCSK9 concentrations seem to be unchanged during the day, not undergoing the typical diurnal pattern of plasmatic PCSK9 and suggesting a different regulation of PCSK9 in plasma and brain[240].

The recent use of PCSK9 inhibitors for the treatment of hypercholesterolemia raises the question about the potential side effects of these treatment in clinical studies. In particular, the FOURIER and ODYSSEY trials point out the potential correlation between the use of monoclonal antibody and neurocognitive adverse effects[241,242].

The EBBINGHAUS (Evaluating PCSK9 Binding Antibody Influence on Cognitive Health in High Cardiovascular Risk Subjects) trial, a prospective study aimed to investigate the effect of the treatment with Evolocumab in statin treated patients focusing on cognitive functions. This study enrolled a subgroup of 1974 patients from the FOURIER study of 40-80 years of age for 20 months, excluding patients with neurological disorders at the baseline. The results showed that no significantly differences have been observed in cognitive function test scores among Evolocumab and placebo groups[243]. One undelighted (??) limitation of the study is the short followed up of the study, that is the reason why a 5-year extension of the FOURIER study is ongoing to definitely answer to this question.

In these reported studies the lack of evidence on the effect of PCSK9 inhibitors on cognitive functions may be justify by two reasons. The first one could be explained by the presence of the BBB, indeed cholesterol levels in the plasma may not be translated into direct changing in brain lipids levels[244]. The second one is that the BBB limits the passage to monoclonal antibodies for their high molecular weight. Under healthy conditions, the BBB is intact and thanks to the presence of tight-junction capillaries that prevented antibody diffusion thought the transcellular route. In fact, antibody permeability from the circulation into brain has been reported to be approximately 0.1% in human patients and in animal models[245] with the exception of pathological conditions such as diabetes that might damage the BBB integrity resulting in increased permeability.

Aim

The overall objective of the present study was the characterization of lipoprotein-mediated brain cholesterol transport and its potential alterations in AD. In particular, the first specific aim of this project was to investigate whether brain cholesterol trafficking evaluated as CSF capacity to promote cholesterol efflux from cells is altered in AD and whether it correlates with the biomarkers of cognitive decline. The second objective was to evaluate whether PCSK9 levels were altered in AD and the possible mechanistic effect of the protein on brain cholesterol transport.

AD is a multifactorial neurodegenerative disease clinically characterized by a progressive cognitive impairment. Alterations of cholesterol homeostasis in the CNS have been associated to various neurodegenerative disorders, including AD, as shown by genomic-wide association studies that have identified several loci involved in lipid metabolism among AD susceptible genes. Among these, the apoE4 gene is the most important risk factor for AD onset[29]. Cholesterol synthesis in neurons is very high during embryogenesis, but it gets progressively lost in adult neurons, that rely on cholesterol produced from astrocytes for neuronal plasticity maintenance, synaptogenesis and regeneration after injury[246]. CNS cholesterol trafficking between astrocytes and neurons is mediated by HDL-like particles, similar to plasma HDL, identified in human in the CSF and containing mainly apoE[71]. HDL-like particles interact with membrane cholesterol transporters such as the transporters ABCA1 and ABCG1 and convey cholesterol from astrocytes to neurons. Only few observations suggest that in neurodegenerative diseases cholesterol transport between astrocytes and neurons mediated by HDL-like particles may be altered[247,248]. The lipid transport mediated by HDL, the unique lipoprotein particles identified in the CSF, has not been deeply investigated enough compared to what occurred for plasma HDL. In order to fill this gap of knowledge, the overall objective of the project was the characterization of brain HDL-mediated cholesterol transport and its potential alterations in AD. In particular, the specific aim of our project has been to investigate whether brain cholesterol trafficking, evaluated as CSF HDL-like particles capacity to promote cholesterol efflux from cells, is altered in AD. This goal has been pursued with a clinical observational study examining CSF of non-AD subjects, patients with AD and with other types of dementia not related to AD, to evaluate the ability of CSF HDL to promote cholesterol efflux specifically through the transporters ABCA1 and ABCG1.

With respect to the neuronal point of view, newly generated HDL-like particles can be finally uptaken by neurons through the binding of apoE to the LDLR family receptors (LRP1, LDLR, VLDLR and apoER2)[87,88]. These receptors are sensitive to the degrading activity of PCSK9, a serine protease firstly described to target hepatic LDLR but also identified in brain and detected in human cerebrospinal fluid[249,250]. PCSK9 modified activity might in principle be involved in the derangement of brain cholesterol trafficking, in lipoprotein homeostasis and in AD pathogenesis. The genetic studies and conducted so far in humans are not conclusive on the impact of PCSK9 mutations on AD.

At this regard, a further evaluation included also patients with other forms of dementia to establish what occur in non-AD neurodegenerative disease. With respect to the neuronal point of view, we also measured PCSK9 in CSF of AD patients to establish whether PCSK9 levels alterations occur in AD. In addition, we also integrated this part of the project by studying the influence of PCSK9 on cholesterol metabolism at mechanistic level using an *in vitro* approach. The objective of this part of the study was to investigate the molecular mechanism by which PCSK9 may exert a pathogenic role in AD and a deleterious effect on brain cells through the alteration of neuronal cholesterol homeostasis.

Material and Methods

1. Patients and samples

For the study on cholesterol efflux capacity of the CSF (CSF-CEC) we included a group of patients diagnosed with AD (n= 37), a group of patients with non-AD DEM (n=16), and a control group of patients with more than 20 heterogeneous conditions unrelated to DEM (controls; n=39). The CSF PCSK9 evaluation included a subgroup of these samples and in particular CSF from AD patients (n = 30) and age- and sex-matched non-AD controls (n = 30). All patients were recruited at Dementia Unit of the University of Parma and the Alzheimer Unit of the Department of Neurological Sciences of the University of Milano.

CSF samples were obtained by lumbar puncture for routine clinical diagnosis after the signature of an informed consent using a form approved by the local ethics committees Specimens were immediately stored at -80°C and slowly defrosted in ice only at the moment of utilization. None of the samples presented alterations at the macroscopic examination. The study was conducted in accordance with the ethical principles set forth in the Declaration of Helsinki. The control group included individuals with psychiatric disorders, hydrocephalus, tumors, peripheral neuropathy, and those who underwent lumbar puncture for suspected demyelinating disorders discharged with no neurological diseases. The AD diagnosis was performed according to NINCDS-ADRDA[251] and subsequent research criteria[252]. In the non-AD DEM group, patients were diagnosed as non-AD DEM according to established clinical criteria[253,254,255,256,257] and included behavioral variants of frontotemporal DEM, corticobasal syndrome, amyotrophic lateral sclerosis-frontotemporal spectrum disorder, semantic variant primary progressive aphasia, nonfluent primary progressive aphasia, progressive supranuclear palsy, and Parkinson's disease DEM.

1.1 Cell lines

J774 murine macrophages were grown in 10% FCS containing DMEM (both from Euroclone, Milano, Italy) in the presence of 1% penicillin-streptomycin (Thermo Fisher Scientific, Carlsbad, CA). J774 murine macrophages overexpressing or not PCSK9 were grown in the same medium as above but supplemented with 1% nonessential amino acids (Thermo Fisher Scientific, Carlsbad, CA). Chinese hamster ovary (CHO) cells were cultured in 10% FCS-containing Ham's F-12 (both from Lonza, Verviers, Belgium) in the presence of antibiotics (Zeocin and Geneticin from Waltham, MA, US). SH-SY5Y neuroblastoma cells

and SHSY5Y overexpressing PCSK9 were grown in 10% FCS containing DMEM (both from Euroclone, Milano, Italy) in the presence of 1% penicillin-streptomycin (Thermo Fisher Scientific, Carlsbad, CA). U87 glioblastoma-astrocytoma cells were grown in 10% FCS containing DMEM (both from Euroclone, Milano, Italy) in the presence of 1% penicillin-streptomycin and 4mM L-glutamine (both from Thermo Fisher Scientific, Carlsbad, CA). Primary human skin fibroblasts obtained from a control subject and from a patient with familiar hypercholesterolemia were isolated with informed consent from an underarm explant from a healthy donor and were kindly provided from Professor Sebastiano Calandra from the Department of Biomedical, Metabolic and Neural Sciences of the University of Modena and Reggio Emilia and Dr Claudio Rabacchi from the Department of Life Sciences, University of Modena and Reggio Emilia. These cells were grown in 10% FCS DMEM (both from Euroclone, Milano, Italy) supplemented with 1% penicillin-streptomycin, 1% nonessential amino acids, 1mM sodium pyruvate and 2mM L-glutamine (all from Thermo Fisher Scientific, Carlsbad, CA). Cells were maintained in sterile flasks and incubated at 37°C with 90-95% humidity and 5% CO2. When adherent cells reached about 90% confluency, they were washed with sterile PBS (Euroclone, Milano, Italy) and detached. Specifically, macrophages were detached mechanically with the use of a sterile scraper while all the other cells line were detached enzymatically with trypsin in order to be seeded in plates and utilized in the experiments.

1.2 Generation of human neuroblastoma SH-SY5Y overexpressing PSCK9

For transfection, ϕNX-A cells were seeded (density of 4x106) on a gelatin-coated 10cm^2 plate in 10% FCS DMEM. The following day, cells were transfected in complete medium with a transfection mix containing 6µg of DNA and 24µL of Turbofect reagent in 0,6ml of MEM without FCS. The transfection mix was prepared by adding DNA to the medium and vortexing and after a Turbofect reagent was added and incubated at room temperature for 15 minutes. The mix was added to ϕNX-A cells dropwise and after rocking, the plate was incubated overnight at 37°C.

SH-SY5Y cells were seeded on a 10cm^2 plate in complete medium, in parallel, ϕNX-A cells were replaced with complete medium. The next day, SH-SY5Y were transfected with the culture medium isolated and filtrated with 0.45 µm filter from ϕNX-A cells. The following

step was repeated for three consecutive days. SH-SY5Y were selected using complete medium added with Puromycin and incubated at 37°C for 48 hours. After 48h of selection, cells were harvest and seeded in complete medium for expansion and stocking. SH-SY5Y PCSK9 expression was evaluated using RT-qPCR analysis.

2. CSF Cholesterol Efflux Capacity (CEC) thought ABCA1 and ABCG1

We evaluated CSF-CEC through the cholesterol transporters ABCA1 and ABCG1 that are expressed in CNS and involved in the cholesterol cross-talk between astrocytes and neurons[71]. In addition, we also measured CEC through the passive diffusion process, a spontaneous desorption phenomenon that does not require the expression of membrane transporters and occurs following the cholesterol concentration gradient[258]. CSF was evaluated for its capacity to promote cholesterol efflux by slightly modifying a standard radioisotopic technique commonly used for the evaluation of serum HDL CEC and characterized by the utilization of specific cell models overexpressing the single cholesterol transporters[259]. In particular, we used J774 murine macrophages in basal conditions to measure CEC by passive diffusion; J774 cells treated with 0.3 mM cpt-cAMP (Sigma-Aldrich, St. Louis, MO), which upregulates the ABCA1 transporter[260], were used for total CEC; the specific ABCA1 CEC was then calculated as the difference in CEC between ABCA1-expressing J774 and J774 cells in basal conditions. ABCG1-mediated CEC was evaluated in hABCG1-expressing Chinese hamster ovary (CHO)-K1 cells and calculated as the difference in CEC between hABCG1-expressing and parent CHO-K1 control cells[261]. In all assays, cells were plated (density of 120,000 cells/well for J774 and 10,000 cells/well for CHO) and following 24 h were labeled with [1,2-3H]cholesterol (PerkinElmer, Milano, Italy) for 24 h in the presence of an inhibitor of the cholesterol esterifying enzyme acyl-CoA:cholesterol acyltransferase (Sandoz 58035; Sigma-Aldrich) to ensure that all cellular cholesterol would be in the free form. After labeling, cells underwent an equilibration time in medium containing 0.2% free fatty acid BSA (Sigma-Aldrich). During this time, J774 underwent ABCA1 upregulation with a cAMP analogue. Cells were then washed to remove any cell death and subsequently exposed to CSF from controls, AD, and non-AD DEM subjects for 4–24 h depending on the pathway evaluated. Prior to the utilization, we did not perform any sample fractionation to separate the HDL-like particles from the lipid-containing

nanoparticles that have also been identified in the CSF because of their negligible contribution to cholesterol efflux, as previously documented[248]. The concentration of CSF used as cholesterol acceptor was 30% (v/v) for both cell models. In the case of ABCA1 CEC, the choice was based on previous reports[248]. In the case of ABCG1 CEC, we assessed the optimal concentration of CSF to be used through preliminary dose-response experiments with a pool of CSF samples from control subjects. Like what was seen for ABCA1 CEC[248], incubation with increasing concentrations of CSF resulted in a significant, dose-dependent increase of ABCG1-mediated CEC (r^2 = 0.962, p=0.019; **Fig.1**). CSF-CEC was expressed as a percentage of the radioactivity released into the medium over the total radioactivity incorporated by cells. A parallel set of cells was incubated with medium alone to provide a background efflux that was subtracted from CEC values of CSF samples. To verify the cAMP-mediated induction of ABCA1 expression, reference normal lipid-free human apoA-I (Sigma-Aldrich) was used in each experiment. Similarly, as a control for ABCG1 CEC cell responsiveness, in each experiment we evaluated cholesterol efflux to native plasma HDL isolated from healthy donors by ultracentrifugation[262]. In addition, a pool of normal human sera, as a reference standard, was tested in each assay and its CEC value was used to normalize the patients' CSF-CEC values obtained in different experiments to correct for the interassay variability. The actual ABCA1 and ABCG1 expression in cells was demonstrated by the internal quality control obtained by cholesterol efflux induction with apoA-I for ABCA1-CEC and isolated normal human HDL for ABCG1-CEC and normal human serum for both pathways (mean ± SD effluxes are shown in **Table 1**).

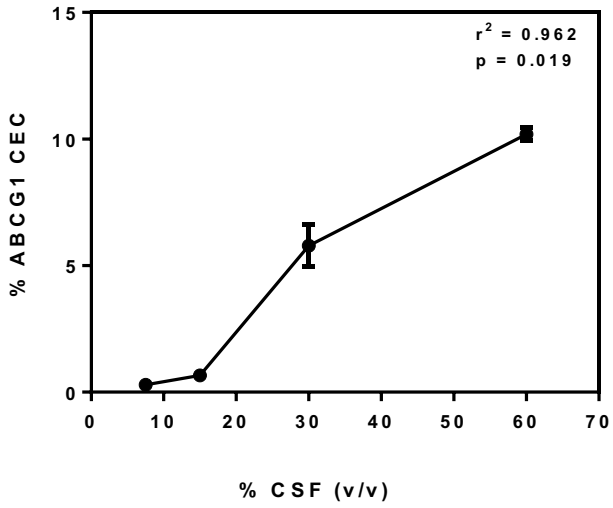

Figure 1. ABCG1-mediated cholesterol efflux capacity (CEC) to increasing concentrations of CSF (from 7.5% to 60%, v/v). The results of the regression analysis (r2 and p) are reported.

	J774 – cAMP	J774 + cAMP	p-value
Cholesterol efflux to apo A-I	0.51 ± 0.19	6.44 ± 0.64	<0.0001
Cholesterol efflux to normal human serum	7.69 ± 1.46	10.69 ± 1.84	<0.0001
	non-transfected CHO	**ABCG1-transfected CHO**	**p-value**
Cholesterol efflux to HDL	13.47 ± 3.00	19.54 ± 2.86	0.001
Cholesterol efflux to normal human serum	16.46 ± 3.88	22.30 ± 6.15	<0.0001

Table 1. Cholesterol efflux from unstimulated and cAMP-stimulated J774 macrophages to human apolipoprotein A-I and normal human serum and from non-transfected and ABCG1-transfected CHO cells to HDL and normal human serum. Data are presented as means ± standard deviations (SDs).

3. Biochemical analyses

The CSF neurobiomarker profile [amyloid β (Aβ) 1-42, total tau, and phosphorylated tau levels] was evaluated by ELISA (Fujirebio, Ghent, Belgium). Total apoE and apoE4 levels were measured in CSF by ELISA (MBL, Nagoya, Japan). The kit measures the amount of human total apoE and apoE4 specifically with high sensitivity using affinity-purified polyclonal antibody against total apoE and monoclonal antibody against apoE4. The minimum detectable concentration is 4 ng/ml and 8 ng/ml for total apoE and apoE4, respectively. Because apoE4 production is discrete and not continuous according to the null, heterozygous, or homozygous genotype, the apoE4/total apoE ratio could be used to identify the apoε4 genotype[263]. Based on this concept we stratified subjects in apoε4 carriers when the apoE4/apoE ratio was >0[264]. CSF apoA-I was measured by ELISA (Abcam, Cambridge, UK). The minimum detectable apoA-I concentration of the kit is 59 pg/ml. CSF PCSK9 levels were measured by ELISA (R&D Systems, Minneapolis, MN, USA).

4. Preparation of rHDL containing apoE (rHDL-apoE)

We prepared reconstituted HDL (rHDL) containing apoE, resembling the particles present in the human CSF and mediating the cholesterol transport between astrocytes and neurons in vitro. rHDL containing apoE were prepared using the cholate dialysis procedure according to previous methods[265; 266], by using a Tris-HCl buffer solution containing 10 mM Tris-HC1, 140 mM NaCl, and 1 mM EDTA, pH 7.4, was used. rHDLs containing ApoE⁄lecithin⁄cholesterol were prepared with a molar ratio of 1:100:2 and apoE concentration was 10μM. The lipid mixture of unlabeled cholesterol (Sigma-Aldrich) in ethanol, [1,2-3H] cholesterol (PerkinElmer, Milano, Italy) and lecithin (Sigma-Aldrich) in ethanol was evaporated to dryness under a stream of nitrogen. To these dried lipids, the proper amount of apoE [1mg/ml] in Tris-HCl buffer and made up to the mark of 0,7ml with buffer. An identical preparation without apoE was prepared in order was used to evaluate the non-apolipoprotein-mediated uptake of cholesterol. Then, a 725nM sodium cholate solution with a molar ratio lecithin: sodium cholate of 1:8 and mixed for 2 minutes at room temperature until obtaining a clear solution and incubated for other 30 minutes on a shaking bath at room temperature. The mixture was then extensively dialyzed for 24-48 hours at 4°C in order to remove the excess cholate. The dialysate was then adjusted to the

proper volume and filtered. The protein and cholesterol concentration were determined through the BCA and Amplex Red cholesterol assay according to the manufacturer's instruction (Thermo Fisher Scientific, Carlsbad, CA).

5. rHDL containing apoE internalization

The rHDL-apoE internalization into fibroblasts, macrophages and differentiated SH-SY5Y was carried out according to previous published methods [267;268]. In detail, cells were seeded in 10% FCS medium into 24-well plate (density of 200,000cells/ well for fibroblasts and 400,000 cells/well for J774). SH-SY5Y were plated at a density of 250,000 cells/well and differentiated for 6 days in the presence of all-trans retinoic acid (ATRA) [10μM] every other day to promote differentiation in neurons (ref). After medium removal, cells were incubated with DMEM containing 5% lipoprotein deficient serum (LPDS) for 24 hours. Then, cells were washed and DMEM containing labelled rHDL-apoE or rHDL without apoE were added for 3 hours. At the end of incubation, cells were washed with PBS and lysed with 0.1M NaOH on shacking plate at 4°C overnight. Aliquots of lysates were analyzed by scintillation counting, for evaluating the amount of labeled cholesterol incorporated by cells. Protein concentration in cell lysates was measured by BCA assay[269]. The amount of cholesterol internalized was calculated as c.p.m in lysates per mg cell protein.

6. Western blotting

SH-SY5Y were seeded in 6 well/plates in 10%FCS DMEM and differentiated for 6 days with ATRA as previously described. When reached the confluence, cells were washed twice with cold PBS and lysed on ice with RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich, Milano), using plastic cell scraper. The lysates were centrifugate at 8000 rpm for 10 minutes at 4°C and supernatant were transferred. Protein concentration was determined for each cell lysate using BCA assay. After protein quantification, an equal amount of proteins (40ug) were loaded on SDS-PAGE gel electrophoresis with molecular weight markers and run the gel to 100V for 1hour. After, proteins were transferred from the gel on a PVDF membrane (BioRad Laboratories, München, Germany) and then blocked for 1 hour in 5% non-fat dry milk in Tris-buffer containing 0,1% Tween-20. After, the membrane was incubated overnight with primary antibody solution against the protein

target at 4°C. PCSK9, LDLr, apoER2 and β-actin (all from Novus Biologicals, Biotechne, Milano) specific rabbit primary antibody were used. Then, the membrane was incubated with an anti-rabbit IgG horseradish peroxidase-conjugate (VWR), a secondary antibody solution for 1 hour at room temperature. A chemiluminescent substrate (Thermo Fisher Scientific, Carlsbad, CA) was applied to the blot according to the manufacturer's instructions and the chemiluminescent signals captured with a camera-based imager. An image analysis software (Fiji) was used to quantify the band intensity of the loading proteins and the control protein signals was used to normalize the target protein levels.

7. apoE-FITC preparation

Human recombinant apoE (PeproTech, Rocky Hill, NJ, USA) was coupled to fluorescein isothiocyanate (FITC, Sigma-Aldrich, Milano) and purified by chromatography on a Sephadex G25 column to isolate unbound FITC. After this passage, the apoE-FITC were extensively dialyzed against degassed PBS and finally against Dulbecco's modified Eagle's medium (DMEM) using a 12000-14000kD cut-off membrane (Spectra/por; Spectrum Medical Industries, Inc., Los Angeles, CA) to allow the complete elimination of free FITC. The protein obtained was quantified using a spectrometer and concentration was calculated as ratio between FITC/protein using the following formula: C [mg/ml] = Abs_{280}-(0.35 x Abs_{495})/1.4

8. Confocal laser scanning microscopy

The interaction between apoE-FITC and living differentiated SH-SY5Y was studied by confocal microscope LSM 510 META Zeiss (objective X63). SH-SY5Y were seeded on a coverslip fitting in a flow chamber kept a 37°C throughout the experiments. Normal culture medium was substituted by identical medium containing 1,5mg/ml purified apoE-FITC. After 40 minutes, cells were washed with fresh medium in order to remove all unbound apoE and Images were then acquired for other 50 minutes. During the observation period, the flow chamber was placed in a microincubator located on the microscope stage to provide stable, standard culture conditions.

9. Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA). Every experimental condition sample was run in triplicate and average values and SDs. The D'Agostino and Pearson omnibus normality test was used to verify whether parameters were normally distributed. Normally distributed parameters were presented as means ± SDs, and skewed continuous parameters were expressed as medians (interquartile ranges). Depending on variances analysis results, the two-tailed unpaired Student's t-test (for not statistically different variances) or two-sided nonparametric Mann-Whitney test (for statistically different variances) was applied to compare two groups. The comparison between more than two groups of subjects was performed by one- way ANOVA or the Kruskal-Wallis test for data normally and not normally distributed, respectively. In both cases, we corrected the results for multiple comparison by Dunn's post hoc test. Categorical variables were compared with the Chi-square test. The relationship between parameters was assessed by linear correlation analysis.

RESULTS PART I:
Brain HDL-mediated cholesterol transport: the astrocytic side

1. Cerebrospinal fluid cholesterol efflux capacity in Alzheimer's disease: an observational case-control study

1.1 Demographic and clinical parameters

Demographic and clinical data available for the cohort of the analyzed patients are reported in **Table 1**. AD patients were significantly older than controls (+15%), while the gender distribution was comparable among groups. The AD group showed the typical pattern of the neurobiomarkers utilized for diagnosis, represented by a significant reduction of $A\beta_{1-42}$ levels coupled with a marked increase of total and phosphorylated tau. On the other hand, both the controls and the non-AD DEM groups presented a physiological neurobiochemical profile. In accordance with apoE4 being the strongest AD risk factor[270], the apoE4 phenotype rate was significantly higher in the AD group.

	Controls (n = 39)	**AD (n = 37)**	**non-AD DEM (n = 16)**	**p-value**
Age (years)	60 ± 16	**69 ± 9***	63 ± 8	*p <0.01 vs controls
Male sex, n (%)	20 (51)	19 (49)	8 (50)	0.960
$A\beta_{1-42}$ (ng/L)	966 (782-1390)	**472 (381-562)***	742 (555-1414)	*p<0.0001 vs controls and non-AD DEM
t-Tau (ng/L)	120 (98-181)	**607 (297-978)*,#**	216 (103-549)	*p < 0.0001 vs controls. #p = 0.004 vs non-AD DEM
p-Tau (ng/L)	30 (26-38)	**84 (62-104)***	34 (16-50)	*p<0.0001 vs controls and non-AD DEM
apoE4 carriers, n (%)	7 (18)	**26 (70)***	5 (31)	*p<0.0001 vs controls and non-AD DEM

Table 1. Demographic data and diagnostic parameters of analyzed patients. Normally distributed parameters are presented as means ± SDs and skewed continuous parameters are expressed as medians (interquartile ranges). Statistically different values are reported in bold. CSF neurobiomarker ($A\beta_{1-42}$, total tau, and phosphorylated tau) values were available for 12/39 subjects in the control group and 13/16 subjects in the non-AD DEM group.

1.2 CSF- Cholesterol Efflux Capacity (CEC) thought the ABCA1 pathway

We first assessed the capacity of CSF to promote cell cholesterol efflux though the membrane transporter ABCA1, the first step of the brain HDL-mediated cholesterol transport. The effective ABCA1 expression in cells was proved by an internal quality control, the cholesterol efflux induction with apoA-1 and normal human serum, used as cholesterol acceptor in every assay (see Material and Methods Section 2). CSF from AD patients showed a reduced ABCA1-mediated CEC compared with controls (-73%; p = 0.001; **Figure 1**). On the other hand, CSF from non-AD DEM displayed an ABCA1-mediated CEC not different from that of the control group (p > 0.999). Considering the wide distribution of the efflux values of controls and to thus exclude the possibility that the difference between the control and AD groups was dragged by the highest nine CEC values in the controls, we ruled out them from the statistical analysis and verified the significant, although reduced, difference between the two groups (mean ± SD 0.39 ± 0.05% in controls in comparison with 0.21 ± 0.036% in AD; p = 0.048).

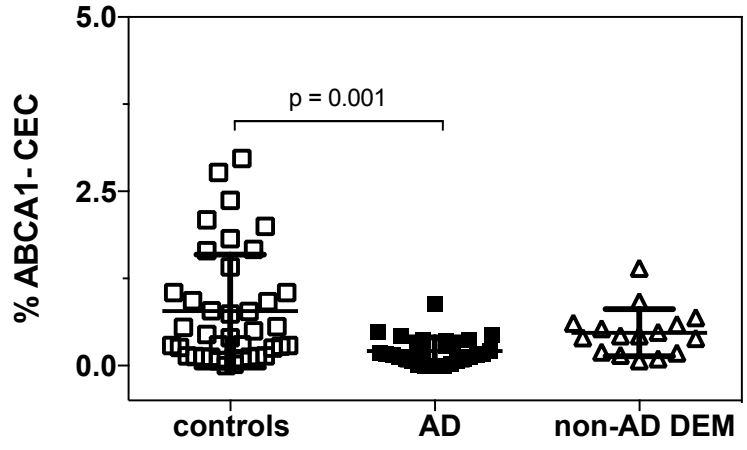

Figure 1. CSF ABCA1-mediated CEC in control subjects (empty square; n=39), AD patients (filled square; n=29), and non-AD DEM patients (empty triangles; n=16). CSF ABCA1- mediated CEC was measured as described in the Materials and Methods section. Each point of the scatter plot represents the mean of a triplicate analysis of each CSF sample. The plot reports the mean ± SD within each group.

1.3 CSF- Cholesterol Efflux Capacity (CEC) thought the ABCG1 pathway

In addition, we assessed the capacity of CSF to promote cell cholesterol efflux through the membrane transporter ABCG1. As for ABCA1, the effective ABCG1 expression in cells was proved by the internal quality control derived by cholesterol efflux induction with isolated normal human HDL and normal human serum (see Material and Methods section 2). In accordance with what has been detected for ABCA1 CEC, also the measurement of CSF ABCG1-mediated CEC displayed values significant reduced in AD patients compared to controls (-33%; p = 0.004; **Fig. 2**). Also in the case of ABCG1-efflux, the decrease seems to be specific for AD since CSF from patients with non-AD DEM showed CEC values similar to those of the control group (p > 0.999).

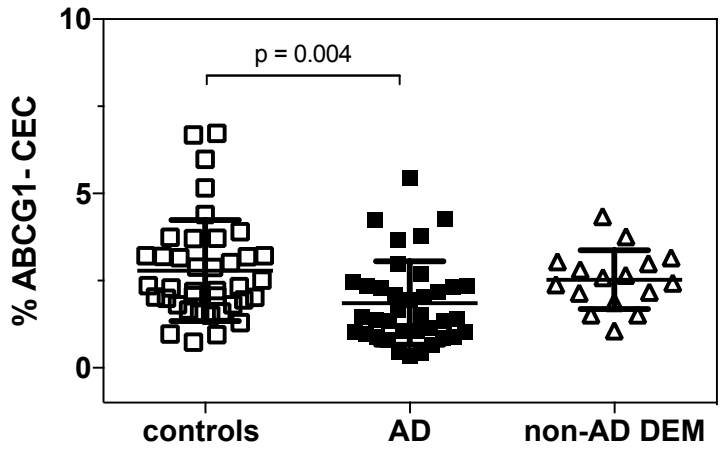

Figure 2. CSF ABCG1 CEC in control subjects (empty square; n=39), AD patients (filled square; n=37), and non-AD DEM patients (empty triangles; n=16). CSF ABCG1- mediated CEC was measured as described in the Materials and Methods section. Each point of the scatter plot represents the mean of a triplicate analysis of each CSF sample. The plot reports the mean ± SD within each group.

1.4 CSF- Cholesterol Efflux Capacity (CEC) thought passive diffusion

Lastly, the measurement of CFS-CEC thought the passive diffusion process displayed no differences between AD and control groups, while non-AD DEM patients showed significantly lower CSF-CEC values in comparison with control subjects (-40%; p = 0.002; **Fig. 3**).

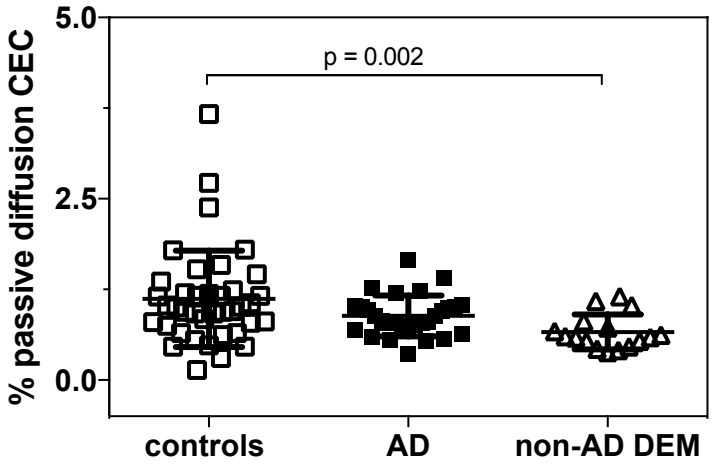

Figure 3. CSF passive diffusion CEC in control subjects (empty square; n=39), AD patients (filled square; n=29), and non-AD DEM patients (empty triangles; n=16). CSF passive diffusion CEC was measured as described in the Materials and Methods section. Each point of the scatter plot represents the mean of a triplicate analysis of each CSF sample. The plot reports the mean ± SD within each group.

1.5 Measurement of apoE and ApoA-1 levels in the CSF

The measurement of apoE levels, the principal apolipoprotein detected in the CNS[271], in the CSF of the three examined groups revealed no difference between AD and controls (**Fig. 4A**). However, non-AD DEM group showed significantly lower values of apoE levels compared to controls (p = 0.049). We also measured the CSF apoA-1 levels that were similar among the analyzed groups (**Fig.4B**).

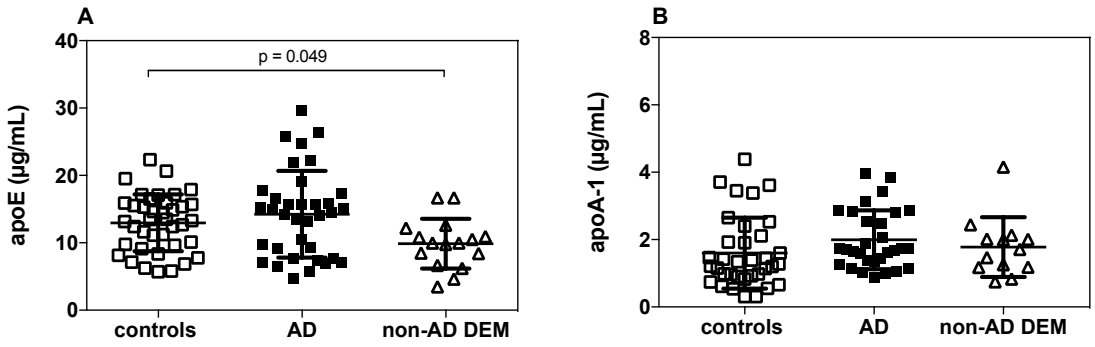

Figure 4. CSF levels (µg/ml) of (A) apoE and (B) apoA-1 control subjects (empty square), AD patients (filled square), and non-AD DEM patients (empty triangles). Each point of the scatter plot represents the mean of a duplicate analysis of each CSF sample. The plot reports the mean ± SD within each group. ApoE levels were evaluated in 35/39 CSF samples from AD patients. apoA-1 levels were evaluated in 30/39 CSF samples from control subjects, 35/37 samples from AD patients, and 13/16 samples from non-AD DEM patients.

1.6 CSF- Cholesterol Efflux Capacity based on apoE4 status

We have also stratified the CSF-CEC values according on the absence or presence of the apoE4 isoform founding no significantly differences in CSF ABCA1-, ABCG1- and passive diffusion CEC between carriers and non-carriers of the apoE4 phenotype.

	apoE4 carriers	apoE4 non-carriers	p-value
ABCG1 CEC (%)	2.27 ± 1.24	2.39 ± 1.41	0.969
ABCA1 CEC (%)	0.60 ± 0.70	0.43 ± 0.52	0.162
Passive diffusion CEC (%)	0.82 ± 0.36	1.03 ± 0.60	0.090

Table 2. CSF cholesterol efflux values stratification between carrier and non-carriers of the apoE4 isoform. Data are presented as mean ± SD considering carriers and non-carriers of the apoE4 isoform for each cholesterol efflux pathways considering ABCG1, ABCA1 and passive diffusion.

1.7 Correlations

Finally, we evaluated whether some correlations exist between the CSF-CEC mediated by each pathway analysed and other parameters considering age, apolipoproteins levels, and the CSF neurobiomarkers used for AD diagnosis. Results of linear-regression analyses considering all subjects together are reported in **Table 3**. None of the efflux pathways correlated with age. ABCG1 CEC was positively associated with CSF apoE levels. However, by analyzing the three study groups separately, a significant positive correlation was present only in the control group (r = 0.358, p = 0.027). A similar behavior was observed for the correlation between apoA-1 and passive diffusion, significant in the whole series of subjects but driven only by the control group (r = 0.413, p = 0.021). None of the CSF-CEC pathways correlated with apoE4 CSF levels. ABCG1 CSF-CEC correlated positively with Aβ 1-42, while ABCA1 CSF-CEC inversely correlated with both total and phosphorylated tau levels. All of these correlations were absent analyzing the three groups separately. The neurobiomarkers Aβ 1-42, total tau, and phosphorylated tau did not correlate with age, neither considering all subjects together nor the three groups separately.

	ABCG1 CEC (%)	**ABCA1 CEC (%)**	**Passive diffusion CEC (%)**
Age	r = -0.078	r = -0.102	r = -0.123
	p = 0.463	p = 0.358	p = 0.268
apoE	**r = 0.370**	r = 0.011	r = 0.170
	p < 0.001	p = 0.920	p = 0.132
apoE4	r = 0.046	r = -0.153	r = -0.174
	p = 0.675	p = 0.176	p= 0.121
apo A-1	r = 0.048	r = -0.061	**r = 0.354**
	p = 0.692	p = 0.626	**p = 0.003**
Aß $_{1-42}$	**r = 0.305**	r = 0.203	r = 0.068
	p = 0.025	p = 0.172	p = 0.647
t-Tau	r = -0.212	**r = -0.348**	r = 0.092
	p = 0.127	**p = 0.018**	p = 0.542
p-Tau	r = -0.225	**r = -0.294**	r = 0.269
	p = 0.106	**p = 0.048**	p = 0.070

Table 3. Relationship between parameters was performed by correlation analysis and the Spearman coefficients are indicated. Significant associations are shown in bold.

2. Cell cholesterol efflux characterization in astrocytic cell lines

2.1 Evaluation of cell cholesterol efflux to different cholesterol acceptor

In order to characterize the astrocytes for their ability to release cholesterol, we initially performed cholesterol efflux to different cholesterol acceptors including apoA-I, apoE, isolated plasma HDL and CSF from control patients as reported in **Figure 5**. An astrocytoma U87 cell line was treated with or without the LXR/RXR agonists, 22-OH cholesterol and 9-cis retinoic acid, that significantly upregulated the ABCA1 and ABCG1 transporters[233]. As expected, the treatment with LXR/RXR agonists (green bars) significantly increase the cholesterol efflux to all the acceptors apoA-I ($p<0.0001$), apoE ($p<0.0001$), isolated plasma HDL ($p<0.0001$) CSF at 15% but without reaching the statistical significance and at the highest percentage 30% ($p<0.001$).

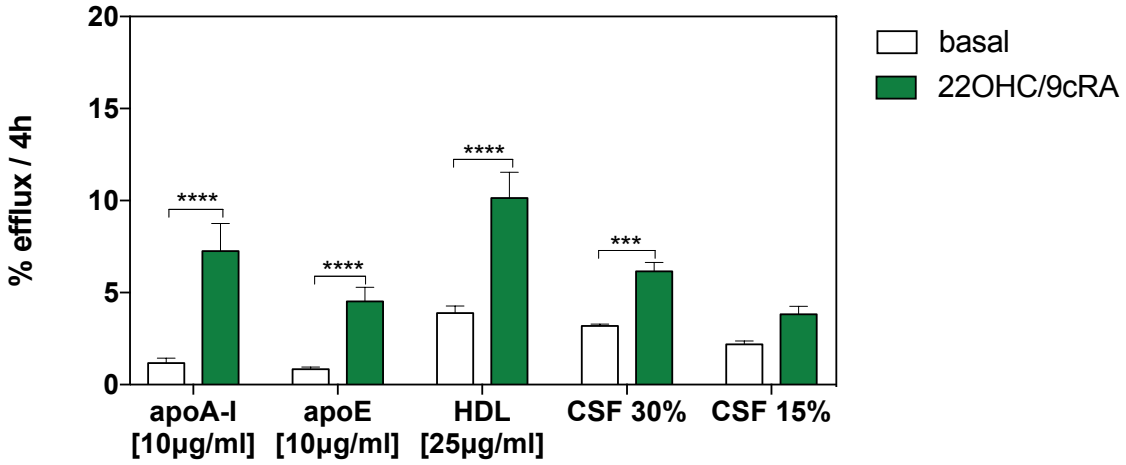

Figure 5. Characterization of cell cholesterol efflux from astrocytes. U87 cells were radiolabelled and subsequently incubated in the absence (white bars) or presence of LXR/RXR agonists (22OHC/9cRA) (green bars). Cholesterol efflux was promoted for 4 h to different cholesterol acceptor apoA-1 10µg/ml, apoE 10µg/ml, isolated human HDL 25µg/ml and 15% or 30% of CSF from a control subject. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the ordinary two-way ANOVA with a Sidak's multiple comparison test. A value of $p < 0.05$ was considered statistically significant. ***p<0.001, **** p<0.0001.

2.2 Cerebrospinal cholesterol efflux capacity in Alzheimer's disease

We also examined the ability of CSF to promote cholesterol efflux in a small subpopulation of the previously analyzed cohort of controls and AD subjects in an astrocytoma cell line. CSF from AD patients showed a trend towards decreased cholesterol efflux capacity values, although not reaching the statistical significance (**Figure 6**).

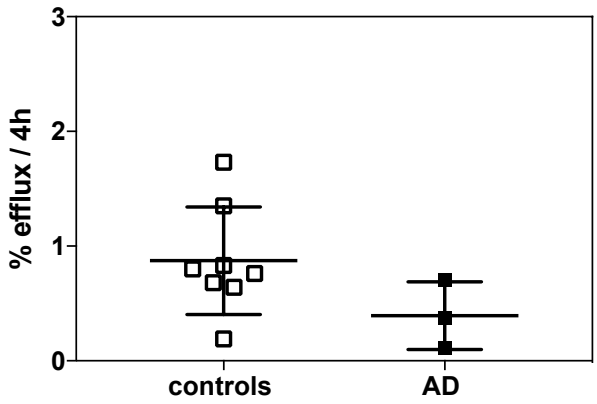

Figure 6. CSF-mediated CEC from control subjects (empty square; n=8) and AD patients (filled square; n=3) in astrocytes. U87 cells were radiolabelled and subsequently incubated in the absence or presence of LXR/RXR agonists (22OHC/9cRA), as described in the Methods section. Cholesterol efflux was promoted for 4 h to 30% of CSF. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the Kolmogorov- Smirnov test.

RESULTS PART II:
Brain HDL-mediated cholesterol transport: the neuronal side

3. Cerebrospinal fluid PCSK9 concentrations in Alzheimer's disease: an observational case-control study

3.1 Demographic and clinical parameters

For this observational case-control study, a subpopulation of the previous analyzed patients has been used for CSF PCSK9 evaluation. Demographic and clinical data available for the cohort of the analyzed patients are reported in **Table 4**. Age and gender distribution were similar among groups. AD patients displayed the typical CSF neurobiomarker pattern, characterized by a significant reduction of $A\beta_{1-42}$ levels, as an index of increased retention in the brain, associated with a marked augmentation of total and phosphorylated tau. Moreover, AD subjects presented a MMSE score below 23 points.

	controls (n=30)	**AD (n=30)**	**p-value**
Age (years)	60 ± 20	68 ± 8	ns
Male sex, n(%)	13 (43%)	12 (40%)	Ns
$A\beta_{1-42}$ (ng/L)	**1163 ± 414**	**537 ± 148**	0.0002
Tau (ng/L)	**138 ± 40**	**640 ± 461**	< 0.0001
Phospho-tau (ng/L)	**32 ± 7**	**78 ± 29**	< 0.0001
MMSE (points)	-	21.43 ± 4.14	na

Table 4. Normally distributed parameters are presented as means ± SDs, and skewed continuous parameters are expressed as medians (interquartile ranges). Statistically different values are reported in bold. Diagnostic parameters were available for 6 patients in the control group. Nonparametric two-sided Mann-Whitney test was applied to compare the two groups.

The clinical diagnosis for the control group are reported in **Table 5.** This cohort included patients that experienced neurological disorders not related to AD including psychiatric and neurological disorders, hydrocephalus, alcohol abuse etc.

Clinical diagnosis	Number of subjects (N=30)
Psychiatric disorders	9
Neurological disorders	7
Hydrocephalus	4
Not confirmed CNS disease	4
Alcohol abuse	1
Dural fistula	1
Hypoacusis	1
Other tumors disease	1
Graves-Basedow	1
Stroke	1

Table 5. Clinical diagnosis of control subjects

3.2 CSF apoE and apoE4 levels

We evaluated apoE levels in the CSF, as an indicator of CSF particles concentrations, that were similar between AD and control group (p = 0.6633; **Figure 7A**). Conversely, as expected apoE4 levels were higher in AD group compared to controls (p = 0.0068; **Figure 7B**).

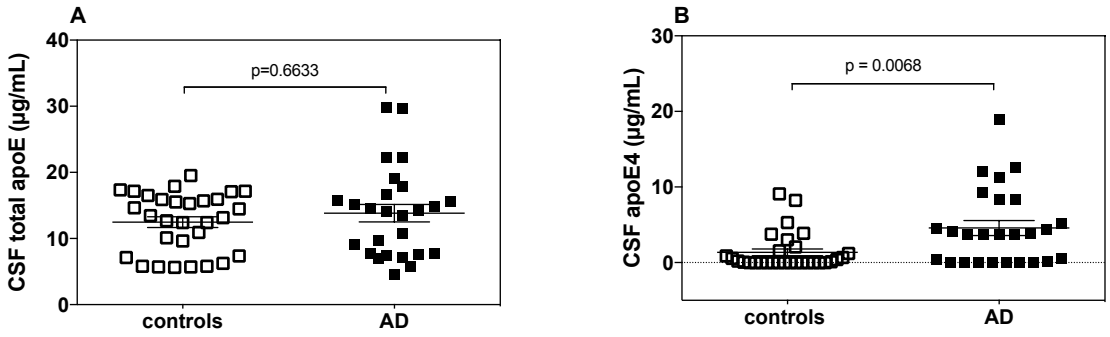

Figure 7. Total apoE (A) and apoE4 (B) levels in the CSF from control (empty squared; n=30) and AD patients (filled squared; n=30). Each sample was run in duplicate. Two-tailed unpaired t-test was applied to compare the two groups for apoE statistical analysis. Nonparametric two-sided Mann-Whitney test was applied to compare the two groups.

3.3 CSF PCSK9 levels

We also analyzed CSF PCSK9 concentrations that were significantly higher in AD patients compared to control subject (+1.45 fold; p= 0.0049; **Figure 8**).

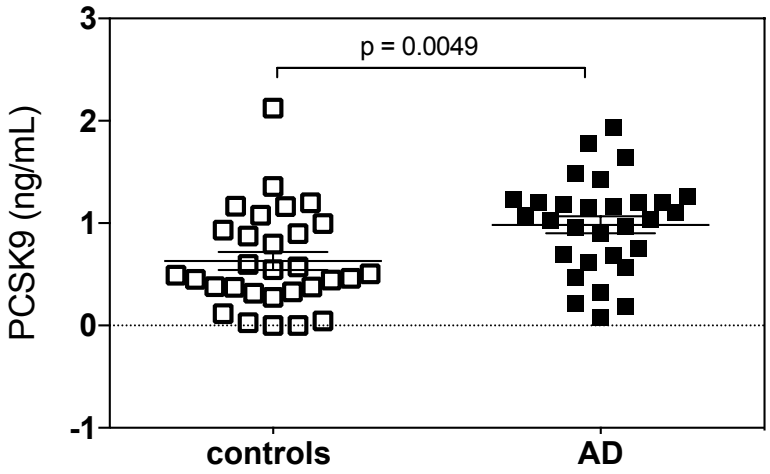

Figure 8. PCSK9 levels in CSF from AD (empty squares; n=30) and AD patients (filled squares; n=30). Each sample was run in duplicate. Two-tailed unpaired t-test was used to compare the two groups.

3.4 Relationship between CSF PCSK9 and apoE4 levels

Finally, considering all samples together we found a positive relationship between CSF PCSK9 and apoE4 levels (**Figure 9A**). Since apoE4 production is discrete and not continuous according to the null, heterozygous or homozygous genotype, the ratio apoE4/total apoE can be used to identify APOε4 genotype[264]. On the bases of this assumption, we identified as APOε4 carriers the subjects with apoE4/apoE that showed a ratio >0. Interestingly, we detected that CSF PCSK9 levels were slightly and almost significantly higher in apoE4 carriers among non-AD patients (+1.83; p= 0.0775; **Figure 9B**); this difference reached the statistical significance in the AD group (+1.43; p=0.0454; **Figure 9C**).

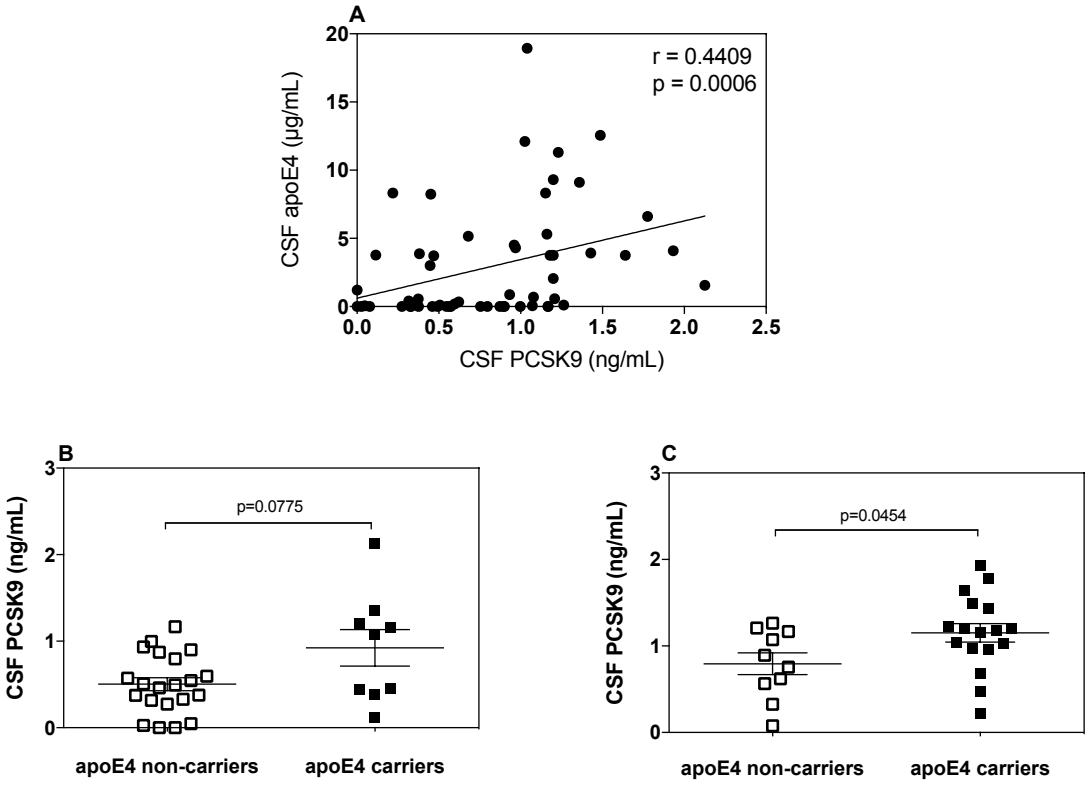

Figure 9. Relationship between PCSK9 and apoE4 levels in CSF from control (empty squares) and AD patients (filled squares). A. Correlation between CSF PCSK9 and apoE4 levels in pooled controls and AD. Statistical analysis was performed by nonparametric correlation and Spearman r is reported. B. PCSK9 levels in non-carriers (n=21) and carriers (n=9) of apoE4 among controls C. PCSK9 levels in non-carriers (n=10) and carriers of the apoE4 among AD group. Each sample was run in duplicate. Nonparametric two-sided Mann-Whitney test was applied in the case of control subjects and two-tailed unpaired Student's t-test was used for AD patients.

4. The pathophysiological role of PCSK9 in Alzheimer's disease: focusing on its influence on lipid metabolism though *in vitro* studies

4.1 Characterization of reconstituted HDL (rHDL) containing apoE: cholesterol efflux capacity

Firstly, we prepared rHDL-apoE resembling HDL-like particles present in the CSF to mimic the cholesterol transport between astrocytes and neurons. For this reason, based on previous published data[266] we anticipated that apoE-containing rHDL, obtained with an ApoE/lecithin/cholesterol in molar ratio of 1:100:2 as indicated in the Material and Methods section 4, would display a size of about 0.9nm resembling to mature HDL. According to previous observations[272], HDL with this size would be able to promote cholesterol efflux through the transporter ABCG1 and passive diffusion but not through ABCA1, that specifically promote efflux to immature, lipid poor discoidal particles[273]. By measuring their capacity to promote cholesterol efflux through ABCA1, ABCG1 and passive diffusion, we found that rHDL-apoE were effectively able to produce a significant cholesterol efflux through ABCG1 pathway and passive diffusion (**Figure 10A** and **10B**, p > 0, 0001 for both pathways). Considering passive diffusion even in a more efficient manner compared to plasma HDL. On the other hand, these rHDL-apoE were not able to promote cholesterol efflux through ABCA1 (**Figure 10C**).

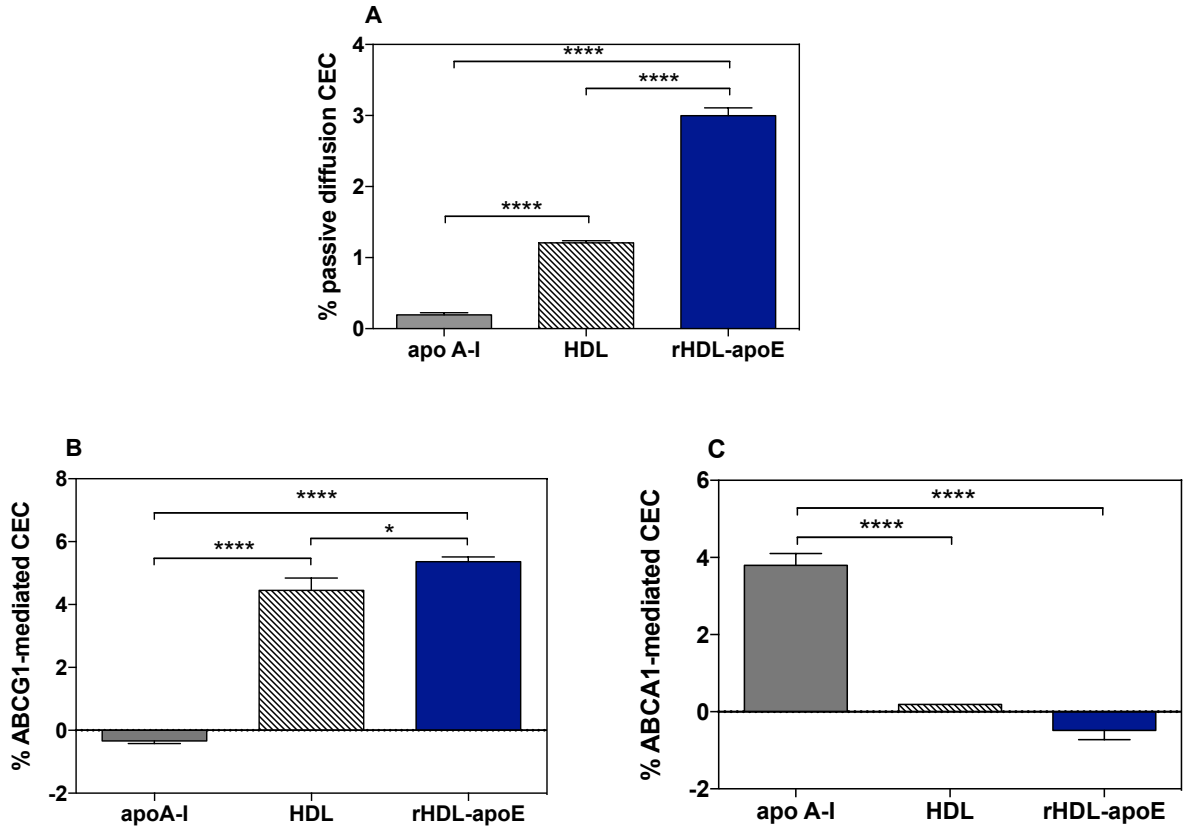

Figure 10. Characterization of rHDL-apoE for the capacity to promote cell cholesterol efflux. A. cholesterol efflux capacity though aqueous diffusion B cholesterol efflux capacity ABCG1-mediated C. cholesterol efflux capacity ABCA1 mediated. Cells were treated as described in Material and Methods section X. ApoA-1 and HDL are used as cholesterol acceptor in order to better characterized rHDL-apoE. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the ordinary two-way ANOVA with a Sidak's multiple comparison test. A value of $p < 0.05$ was considered statistically significant. *p<0.05,**** p<0.0001.

4.2 Characterization of reconstituted HDL (rHDL) containing apoE: cholesterol uptake

We then tested the capacity of rHDL-apoE to be internalized by fibroblast from control patients (CTRL) and from patients with homozygous familiar hypercholesterolemia (FH) that are characterized by the absence of the LRLR. Since the rHDL-apoE particles are internalized by neurons also through this receptor, that is sensitive to the degradation by PCSK9, we evaluated the uptake of these particles in the absence or presence of this receptor. We found that using rHDL-apoE, at both concentrations tested (30 and 60nM), as cholesterol donor, the uptake from fibroblast FH was markedly reduced compared to fibroblast from control subjects ($p<0,0001$ for both concentrations; **Figure 11A**) while rHDL prepared without apoE display a similar uptake between fibroblast ctrl and FH. In addition, we also evaluated the capacity of rHDL-apoE to be internalized in a macrophage cell line overexpressing PCSK9 or not (J774 control and J774 PCSK9) and we observed that the uptake of rHDL-apoE was significantly reduced in J774 overexpressing PCSK9 compared to the control ones, with an effect evident only at a concentration of rHDL-apoE of 60nM ($p=0.0017$; **Figure 11B**). Consistently also in this cellular system rHDL without apoE are not able to produce a reduction in cholesterol uptake in J774 PCSK9 compared to J774 control.

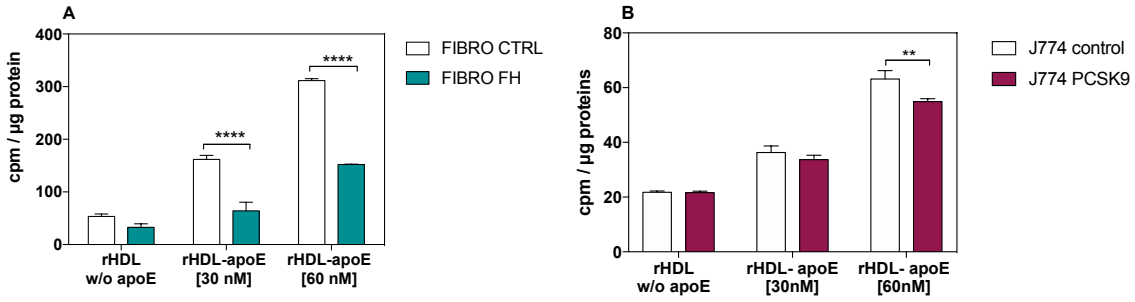

Figure 11. Characterization of rHDL-apoE for the capacity to be internalized. A. Cholesterol uptake in fibroblast from control patient (white bars; FIBRO CTRL) and with familiar hypercholesterolemia (green bars; FIBRO FH) of rHDL without apoE (rHDL w/o apoE) and rHDL-apoE in two different concentration [30-60nM]. B. Cholesterol uptake in control macrophages J774 (white bars; J774 control) and overexpressing PCSK9 (bordeaux bars; J774 PCSK9) of rHDL without apoE (rHDL w/o apoE) and rHDL-apoE in two different concentration [30-60nM]. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the ordinary two-way ANOVA with a Sidak's multiple comparison test. **p<0.01,**** p<0.0001.

4.3 Evaluation of the capacity of reconstituted HDL (rHDL) containing apoE to be internalized into neurons

We then explore the capacity of rHDL-apoE to be internalized using a neuroblastoma cell line (SHSY-5Y) differentiated into neurons after incubation with all-trans retinoic acid (ATRA) [10µM]. The uptake of rHDL-apoE was significant and occurred in a concentration-dependent manner (**Figure 12**).

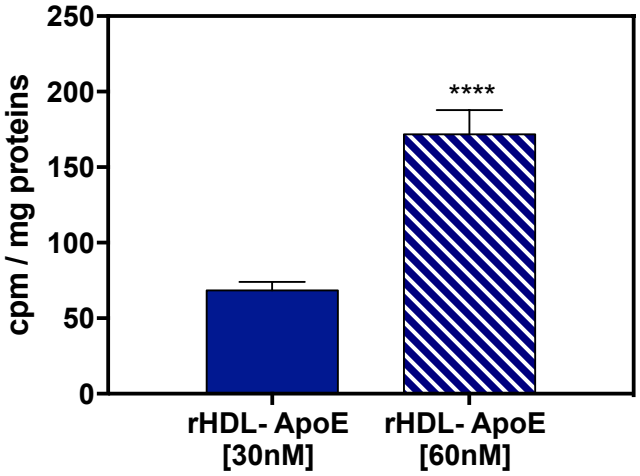

Figure 12 Characterization of rHDL-apoE for the capacity to be internalized in differentiated SH-SY5Y. A. Cholesterol uptake of rHDL-apoE in two different concentration [30-60nM]. Data were performed in triplicate and are expressed as mean ± SD. Unpaired t-test was applied to compare the different means. ****p>0.00001 compared to rHDL-apoE [30nM]

4.3 Gene and protein expression and secretion of PCSK9 in SH-SY5Y line

Subsequently, a line overexpressing PCSK9 was generated by our collaborator Prof. Nicola Ferri at the University of Padua. PCSK9 overexpression in SH-SY5Y differentiated with ATRA has been verified by gene and protein expression by Real-time PCR and Western Blot analyses, respectively (**Figure 13A**, **13C**, **13D**) while the PCSK9 protein secretion was quantified by ELISA assay (**Figure 13B**). The analyses revealed an increased gene and protein expression in SH-SY5Y overexpressing PCSK9 compared to control ones ($p>0.001$ and $p>0.01$ respectively). Also the protein secretion was significant in differentiated SH-SY5Y PCSK9 compared to control cells ($p > 0.0001$).

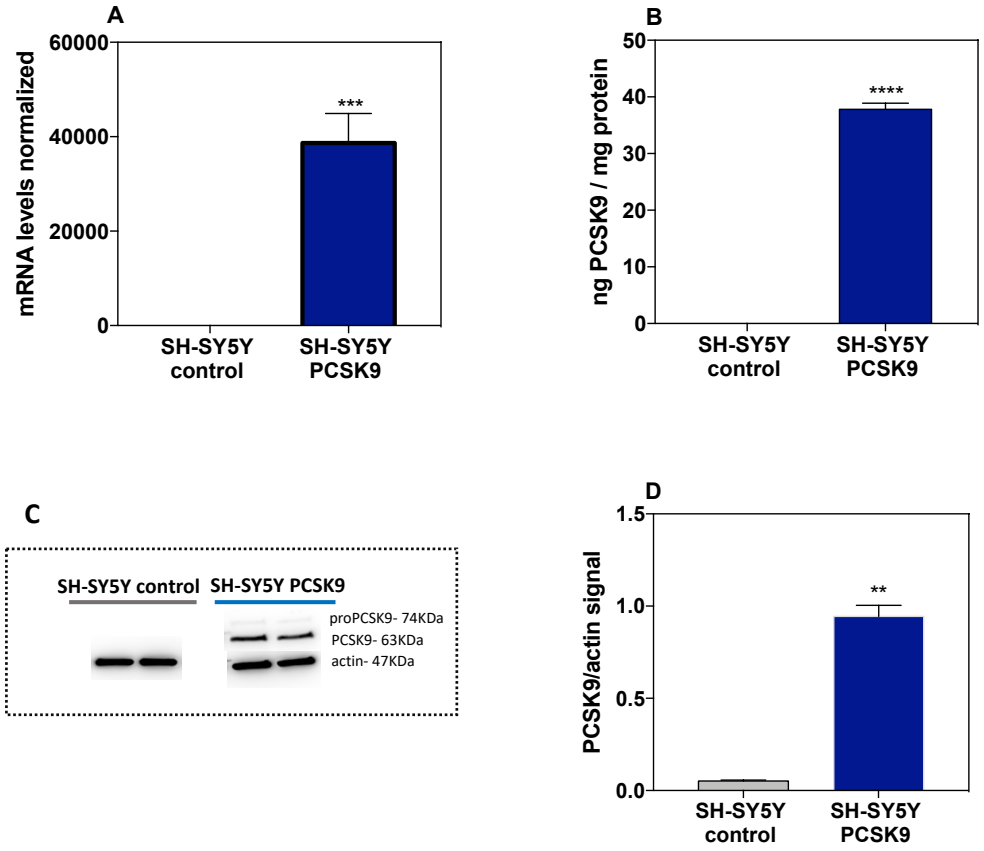

Figure 13. Gene and protein expression and secretion of PCSK9 in SH-SY5Y. **A.** PCSK9 mRNA levels have been analyzed by Real-time PCR after cell transfection. **B.** PCSK9 secretion was performed in conditioned medium from differentiated SH-SY5Y control and SH-SY5Y PCSK9 by ELISA kit. **C**. PCSK9 and proPCSK9 protein expression in differentiated SH-SY5Y control and SH-SY5Y PCSK9 evaluated by Western Blotting. **D.** Signal quantification measured with Fiji ImageJ software. Data are expressed as means ± SD. Unpaired t-test was applied to compare the different means. **p<0,01, ***p>0.001 and ****p>0.00001 compared to relative control cells.

4.4 Effect of PCSK9 on reconstituted-HDL (rHDL) containing apoE internalization into neuronal-like cells

As we did above (Figure 12) we successively evaluated the capacity of rHDL-apoE to be internalized in both neuronal cell overexpressing or not PCSK9. We observed that the uptake of rHDL-apoE in the presence of PCSK9 was significantly reduced in SHSY5Y overexpressing PCSK9 compared to control cells at both concentrations of rHDL tested [60nM and 120nM] ($p = 0.0232$ and $p = 0.005$ respectively; **Figure 14**).

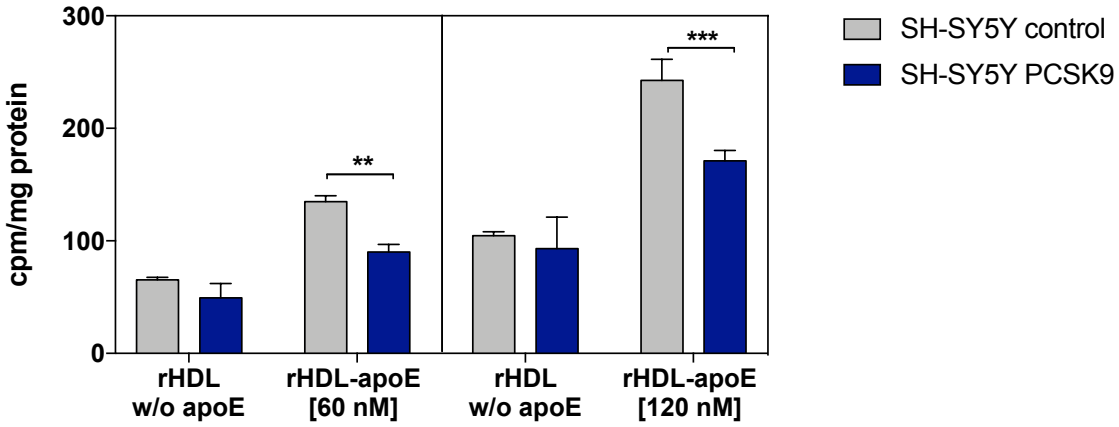

Figure 14 Characterization of rHDL-apoE for the capacity to be internalized in differentiated SH-SY5Y overexpressing or not PCSK9 (grey bars, SH-SY5Y control; blue bars, SHSY5Y PCSK9). Two different concentration [60-120nM] of rHDL-apoE have been tested. Data have been corrected for aspecific uptake mediated by rHDL without apoE. Data were performed in triplicate and are expressed as mean ± SD. Statistical analyses were performed using the ordinary two-way ANOVA with a Sidak's multiple comparison test. *p<0.05, **** p<0.0001.

4.5 Effect of PCSK9 overexpression on the interaction between fluorescinated- apoE and neuronal cells

We applied confocal laser scanning microscopy to investigate the interaction between fluorescinated-apoE (apoE-FITC) with SH-SY5Y overexpressing or not PCSK9. Before the incubation with apoE-FITC both cell models did not show any intrinsic fluorescence (**Figure 15A**). Interestingly, apoE-FITC were internalized with a process that become morphologically evident after 40 minutes of incubation. The first event observed in few minutes after the incubation with apoE-FITC was the appearance of granular pattern and widespread fluorescence outside the cells in both control and PCSK9 overexpressing SH-SY5Y. After 40 minutes a morphological evidence become valuable with fluorescent grains distributed along the cell membrane to outline the cells; this phenomenon was markedly more evident in the SH-SY5Y control compared to PCSK9 expressing cells (**Figure 15B, 15C, 15D**).

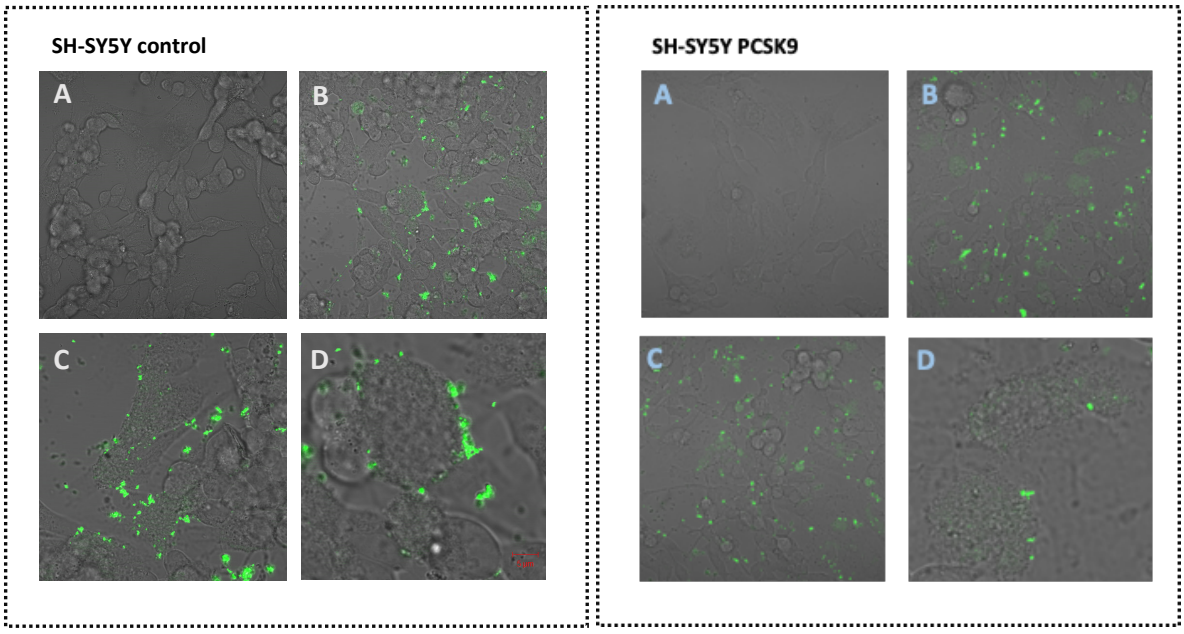

Figure 15. Confocal laser scanning microscopy findings. Internalization of apoE-FITC (green) in living differentiated SH-SY5Y. A. Picture taken before the incubation with apoE-FITC. B. Picture taken 40 minutes after free medium replacement C;D. High magnification detail of internalized apoE-FITC.

4.6 Effect of PCSK9 overexpression on LDLR and apoER2 protein expression

We also investigated whether the overexpression of PCSK9 on cholesterol uptake would lead to a modulation of LDLR-receptor family expression, that would be responsible for rHDL-apoE internalization. We evaluated the expression of LDLR and apoER2 by Western Blot in differentiated SH-SY5Y expressing or not PCSK9. The presence of PCSK9 significantly downregulated the expression of the apoE receptor, apoER2 ($p<0.5$, **Figure 16A** and **B**) and also LDLR ($p<0.05$, **Figure 16C** and **D**).

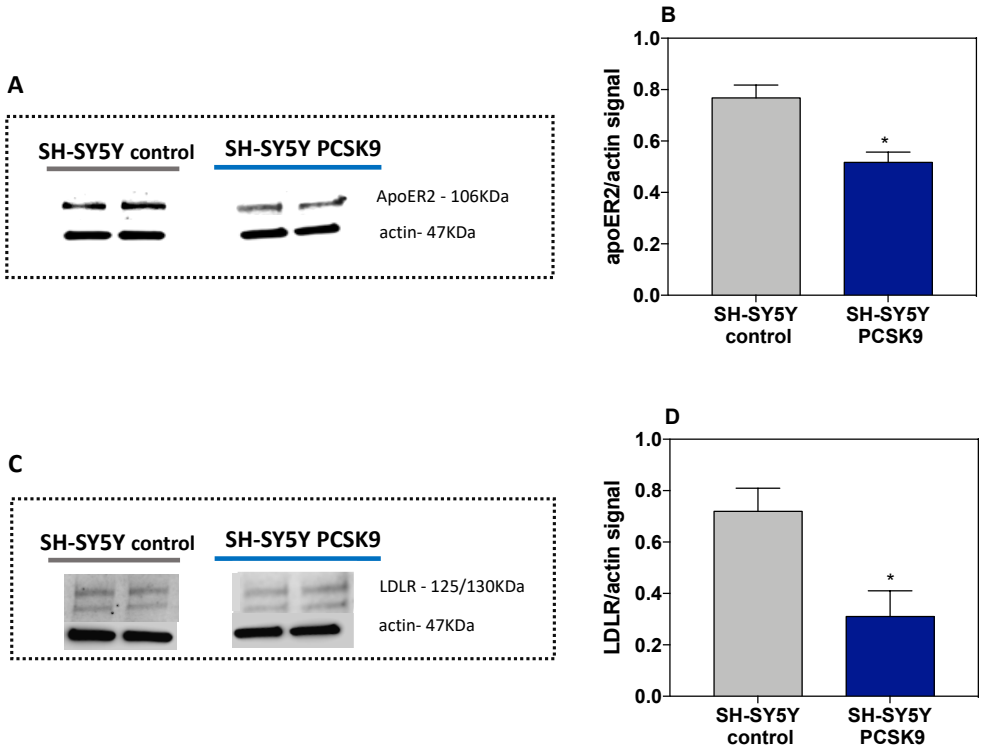

Figure 16. LDLR and apoER2 protein expression in differentiated SH-SY5Y control (green bar) and overexpressing PCSK9 (blue bar). A-C. apoER2 and LDLR protein expression analysis was performed by Western blotting (left panels). B-D. Signal quantification measured with Fiji ImageJ software (right panels). Data are expressed as means ± SD. Unpaired t-test was applied to compare the different means.

Discussion

Disruption in brain cholesterol homeostasis, including total amount, transport between cells and intracellular content are implicated in AD pathogenesis[274]. Considering brain cholesterol transport, both membrane expression of cholesterol transporters/receptors and appropriate functionality of CSF HDL-like particles are responsible of maintaining the physiological cholesterol flux between brain cells. We investigated brain cholesterol trafficking by analyzing the CSF HDL-like particles capacity to promote cholesterol efflux in control subjects, AD and non-AD DEM and examining its relationship with parameter used for AD diagnosis. Cholesterol efflux represents the first step of the lipoprotein-mediated brain cholesterol transport, allowing the generation of HDL-like particles able to carry cholesterol towards the neurons.

The observed reduction in CSF HDL-like particles capacity to promote cholesterol efflux through ABCA1 and ABCG1 could be relevant for cholesterol trafficking and possible leading to deleterious consequences for cholesterol supply to neurons. Indeed, neurons are not able to synthetize all cholesterol they require but depend also on cholesterol conveyed from CSF HDL like particles, that enriched in cholesterol by interacting with astrocytes membrane cholesterol transport ABCA1 and ABCG1[275]. For this reason, an impairment in CSF HDL particles functionality may translate into lower delivery of cholesterol to neurons resulting in loss of neuronal functions and consequent apoptosis and neurodegeneration. These results not only corroborate the prior findings by Yassine et al., that also described a reduction in CSF ABCA1-mediated CEC in AD patients[248] but also add a further element by reporting also an impairment in ABCG1-mediated CEC.

CSF-CEC primarily depends on HDL-like particles composition and size rather than particles concentration as reported for serum HDL[276]. To rule out the influence of HDL-like particles levels on their function we also evaluated apolipoproteins concentrations. CSF apoE is mainly produced from CNS[277] on the contrary apoA-1 derives from the peripheral circulation crossing the BBB through an endocytosis process[73]. The apoE and apoA-1 levels were comparable among our three analyzed group, reflecting similar HDL-like particles concentrations. This observation confirmed some previous studies[278,279,280] but not others[278,280,248] suggesting that further studies needed to clarify this aspect. Moreover, we did not find any correlation between CEC and CSF apolipoproteins levels in AD, while ABCG1-CEC and passive diffusion positive correlate with apoE and apoA-1, respectively.

Regarding our results, we may hypothesize that modification of CSF-CEC in AD seems to be related to alterations in HDL-like particles composition and quality rather than concentration. The reduction in CSF-CEC between control and AD patients may suggest an impaired phospholipids and triglycerides enrichment of HDL-like particles possibly due to a lower LCAT activity, that was previously found in AD patients[247] specifically affecting larger HDL particles, the major fraction found in the CSF[67], leading to reduced affinity for ABCG1 transporter[281]. Another hypothesis for the reduction in ABCG1 CEC in AD could be the different soluble Aβ distribution in CSF-HDL subclasses, with increased Aβ loading of the HDL-1 (large) subfraction[282]. This explanation could be in accordance with a study in which increased HDL content of serum amyloid A (SAA), another misfolded proteins implicated in the acute phase response, is associated with impaired serum HDL ABCG1-CEC[259]. Our results showing in the control group low values of ABCA1-CEC compared with those of ABCG1-CEC are consistent with the concept that in human CSF the most representative particles are large and spherical ones[67,71].

The impairment in CSF ABCA1-CEC has already been described by Yassine et al. not only in AD patients but also in subjects with Mild Cognitive Impairment, although less marked[248]. CSF-CEC through this pathway is also relevant since ABCA1 promotes the secretion of nascent apoE particles from astrocytes. Even if apoE concentration is similar among the three groups, ABCA1 is involved in apoE lipidation and ABCA1 deleted astrocytes have been shown to secrete particles of smaller size with decreased cholesterol suggesting a poorly lipidation with consequence in their functionality[111].

A loss-of-function mutation in ABCA1 are associated with high AD risk in the general population[177] and studies in APP23 mice lacking of ABCA1 showed an increase in amyloid deposition[283].

The specific alteration in CSF-CEC observed in AD and the differences with other type of DEM are further emphasized by our findings that only non-AD subjects presented a reduction in CSF-CEC thought passive diffusion. This result could be explained by the lower CSF apoE levels found in these patients since passive diffusion is an unmediated process conducted by concentration gradient and extracellular acceptor[284]. The lower apoE concentration found in non-AD DEM are in line with previous findings[285].

Our findings indicate also that the alteration in CSF-CEC is independent of the presence of apoE4 isoform, one of the major risk factors for AD. Our results are consistent with the ones of Yassine et al. that didn't find any correlation between ABCA1-CEC and apoE genotype[248]. However, bigger studies are needed to clarify this issue, since some previous published data *in vitro* performed using reconstituted apoE discs with different apoE isoforms suggested that ABCA1 and ABCG1 cholesterol efflux is not influenced by the apoE genotype[166,137] while others observed apoE isoform-dependent lipid efflux from neural cells in culture[136].

The relationship between CSF-CEC and neurochemical biomarkers used for AD diagnosis are indicative of a mechanistic association between HDL-like particle functionality and biochemical abnormalities implicated in Aβ peptide deposition and protein tau phosphorylation. This association appears to be independent of age because age did not correlate with both CSF-CEC and the AD neurobiomarkers. In particular, the CSF ABCG1-CEC directly correlate with soluble Aβ levels, indicating a reduced deposition of the insoluble form and in parallel, an inverse correlation between CSF ABCA1-CEC with phosphorylated tau levels, indexes of neurodegeneration. We did not speculate any mechanistic link, but it is interesting to consider that neuronal cholesterol membranes content is involved in Aβ processing. It has been in fact reported that decreased plasma cholesterol in neuronal membranes promoted the formation of Aβ peptide through the non-amyloidogenic pathway and lysosomal degradation of Aβ peptides in microglia. On the contrary increased cholesterol levels in neuronal membranes induce BACE-1 activity, thus enhancing amyloidogenic Aβ production[286]. Moreover, neuronal membrane lipid raft disturbances caused by cholesterol depletion has been shown to cause perturbations of membrane raft-associated cytoskeleton protein, including tau that is present in lipids rafts[287]. Therefore, altered cholesterol transport mediated by CSF HDL-like particle could be implicated in the intricate and misunderstood processing that is involved in the production of amyloid deposition and neurofibrillary tangles in AD.

These preliminary findings could represent a first step for better understand the CNS trafficking even if our study showed some limitations concerning the descriptive nature of the work, the relatively small sample size and the origin of cellular models not from cerebral source.

In the second part of my discussion, I will take into consideration the final part of cholesterol transport focusing on the processes regulating the uptake of the HDL-like particles in neurons thought the interaction with the LDLR family that are sensitive to the degrading activity of PCSK9. I will discuss the potential role of the protein PCSK9 in Alzheimer's disease. Our data showed an increase of PCSK9 levels in CSF of AD patients compared to control subjects. A potential implication of PCSK9 in neurodegenerative disease such as AD has been described later by Courtemanche et al, indicated a CSF PCSK9 levels increase not only uniquely in AD patients but also in subjects with other neurodegenerative conditions suggesting that PCSK9 may be not only associated with AD but more generally with neurodegenerative process[288]. Our data suggested that PCSK9 may be in principle implicated in AD and our findings are consistent with human brain section of AD autopsy-confirmed that showed an increase PCSK9 gene expression and protein levels in comparison with control patients[239].

A pro-apoptotic impact of PCSK9 in neurons has been reported[221]. It was also revealed an increased serum PCSK9 levels in either AD patients and MCI subjects[239]. On the contrary, other findings showed a protective role of PCSK9 since its absence increased the levels of BACE-1 in mice, the enzyme involved in the generation of Aβ peptide[228]. However, this last result has not been confirmed finding no evidence on the role of PCSK9 on BACE-1 levels in mice brain[224].

The PCSK9 genetic studies in humans are controversial. Some studies came to negative conclusions revealing any link between PCSK9 polymorphism and AD risk[235,289] . On the contrary, a recent genetic study identified two SNPs, rs4927193 and rs499718, located in PCSK9 intron 2 and 3 respectively, that were associated with late onset AD risk only in females[239] and are in accordance with our observation.

From our point of view, PCSK9 is a potential pathogenetic factor for its role as a regulator of neuronal apoE receptor expression including LDLR, VLDLr, LRP1 and apoER2[290]. Consistently, PCSK9 knockout mice showed enhanced brain expression of the LDLR[217].

To deeply elucidate the mechanism underling the implication of PCSK9 in AD pathophysiology we focused on its influence on brain cholesterol homeostasis. In particular, we made the hypothesis that PCSK9 may be a pathogenetic factor in AD though

the modulation of brain cholesterol metabolism and we explore this hypothesis through *in vitro* studies.

First of all, we have prepared rHDL-apoE resembling HDL-like particles found in the CSF to be used as cholesterol donor. We performed some *in vitro* studies to characterize these newly generated particles by testing their capacity to promote cholesterol efflux. In fact, based on the preparation methods, we expected a size of particles within the range of spherical and mature HDL, preferentially interacting with the transporter ABCG1[273].

Our data confirmed that rHDL-apoE are effectively able to promote cholesterol efflux thought ABCG1, but not thought ABCA1, that would promote efflux to more immature particles[272]. In order to better characterize their interaction with the LDLR, responsible for HDL-like particles uptake in neurons, we measured their capacity to be internalized in fibroblast from patients with familiar hypercholesterolemia, condition characterized by a defect of the LDLR expression[291]. We hypothesized that these particles would interact with the mentioned receptor and that the uptake was dependent on apoE since the rHDL without apoE did not appreciate the same behavior.

The observed reduction in rHDL-apoE uptake in differentiated SH-SY5Y overexpressing PCSK9 compared to control ones is in accordance with the possible role of the protein in interfering with cholesterol internalization by neurons. Whether the effect was mediated by the degradation of the receptor involved in the uptake was confirmed by the reduction of both LDLR and apoER2 implicated in HDL-like particles uptake that we observed in our experiments and that was also described by other authors[290]. According to our hypothesis, an *in vivo* study indicated that PCSK9 decreased LDLR expression during brain development and following ischemic stroke[217]. Subsequently, the degradation of lipoproteins receptor accomplished by PCSK9 may turn in less cholesterol supply to neurons with potential negative outcomes[226]. As already reported, PCSK9 is implicated neuronal apoptosis via activation of Bcl/Bax-caspase 9-caspase 3 pathway[292]. Interestingly, Asai and colleagues showed that a compound isolated from a Chinese herb, berberine, that has shown cholesterol lowering properties occurring also through a reduction of PCSK9 mRNA and protein levels[293], can decrease Aβ levels by modifying APP processing in human neuroglioma cells that stably express Swedish-type mutated human APP_{695} [294]. Moreover,

the administration of a small molecule inhibiting PCSK9 impeded dendritic spine loss thought the inhibition of Aβ aggregation and neuroinflammation[229].

The observation regarding the interaction between fluorescinated- apoE and neuronal cells confirmed that apoE displayed an amorphous morphology and tended to self-assemble. This behavior has already been reported with previous observations highlighting a tendency of apoE to aggregate through a monomer-dimer-tetramer association to further high molecular weight aggregates exhibiting an α-helical structure. This apoE self-assembling could be prevented by lipidation that stabilized apoE through the lipid binding and to verify this we are planning to repeat the confocal experiments after fluoresceination of rHDL[295].

In conclusion, we demonstrated an impairment in CSF capacity to promote cholesterol efflux through ABCA1 and ABCG1 in AD, possibly caused by quality alterations in CSF-HDL like particles. This defect relates with modifications of the neurobiomarkers of AD, Aβ, tau and p-tau, indicating the presence of a pathophysiological relationship between decrease neuronal cholesterol supply to neurons and neurodegeneration in AD. Our data, together with further findings clarifying the exact mechanism of CSF HDL-like particle dysfunction, may pave the way to developing new lipoprotein-based pharmacological treatments, like modulators of HDL-like particle maturation, to improve brain cholesterol transport in AD[296,297]. Regarding PCSK9 CSF levels, the findings of this study indicate a possible pathogenetic role of the protein in AD. In addition, our preliminary *in vitro* results suggest a possible role of PCSK9 in interfering with cholesterol supply to neurons. Further analyses are necessary to better understand other possible mechanisms involved in the pathogenetic role of PCSK9. At this regard, in order to confirm out hypothesis on the involvement of PCSK9 on AD pathophysiology, we planned to conduct in vivo study, In particular, in 5XFAD APP/PS1 transgenic mice, that recapitulate the major features of AD pathology, will be crossbred with mice in which PCSK9 will be knocked out. On these animals, the cognitive functions will be assessed, and analyses on brain and plasma from these mice will evaluate the influence of PCSK9 parameters of inflammation and cholesterol metabolism related to AD. In the case of these data will confirm the deleterious role of PCSK9 in AD, future pharmacological approaches using small molecules targeting PCSK9 may represent a potential innovative therapeutic approach to counteract AD.

Parts of this PhD thesis have been included in two publications: “ABCA1- and ABCG1-mediated cholesterol efflux capacity of cerebrospinal fluid is impaired in Alzheimer's disease” J Lipid Res. 2019 Aug;60(8):1449-1456. doi: 10.1194/jlr.P091033. Epub 2019 Jun 5 and “Increased PCSK9 Cerebrospinal Fluid Concentrations in Alzheimer's Disease” J Alzheimers Dis. 2017;55(1):315-320.

The PhD candidate contributed to *in vitro* experiment, statistical analyses and preparation of manuscripts Figures. The PhD candidate contributed also to the manuscript writing and revision.

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