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Wellinghton de Medeiros Barros

# Cholesterol metabolism and its relation to the brain's acetylcholinesterase homeostasis: a preclinical study

Florianópolis
2022

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Thesis presented to the Neurosciences Graduate Program from the Federal University of Santa Catarina as a partial and mandatory requirement for earning the title of Ph.D. in Neurosciences.
Supervisor: Prof. Eduardo Luiz Gasnhar Moreira, Ph.D.

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Wellinghton de Medeiros Barros

**Cholesterol metabolism and its relation to the brain's acetylcholinesterase homeostasis: a preclinical study**

O presente trabalho em nível de doutorado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Certificamos que esta é a **versão original e final** do trabalho de conclusão que foi julgado adequado para obtenção do título de
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Prof. Eduardo Luiz Gasnhar Moreira, Dr.
Orientador

Florianópolis, 2022

*Dedico este trabalho à minha família*
*de onde sempre encontrei apoio,*
*força e amor incondicionais.*

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*"A experiência é o nome que*
*damos aos nossos erros."*

*Oscar Wilde*

*"Somos o que fazemos, mas somos*
*principalmente o que fazemos*
*para mudar o que somos."*

*Eduardo Galeano*

## RESUMO

**Introdução:** Alterações no metabolismo do colesterol no encéfalo têm um papel significativo na fisiopatologia da doença de Alzheimer (DA). Estudos anteriores demonstraram que camundongos suíços alimentados com uma dieta rica em colesterol apresentaram comprometimento da memória espacial de curto prazo, e foi interessantemente correlacionado com um aumento na atividade catalítica da acetilcolinesterase (AChE) no córtex pré-frontal e hipocampo. Embora o colesterol não consiga atravessar a barreira hematoencefálica (BHE), seu metabólito oxidado, o 27-hidroxicolesterol (27-OH) - o oxisterol mais abundante no plasma - pode atravessar livremente a BHE e tem sido observado que suas altas concentrações medeiam a disfunção diversos sistemas encefálicos, promovendo, entre outras coisas, declínio cognitivo e neurodegeneração. No entanto, a relação entre altos níveis de 27-OH e AChE permanece desconhecida. **Objetivos e métodos:** Portanto, os procedimentos experimentais neste estudo visaram elucidar o envolvimento da 27-OH na homeostase da AChE, particularmente sua expressão gênica, densidade proteica e atividade catalítica em neurônios cultivados, bem como em células neurais de camundongos que superexpressam o gene CYP27A1 e, finalmente, testar a hipótese de que, se o colesterol plasmático aumenta a atividade da AChE nas células do hipocampo de camundongos alimentados com dieta hiperlipídica (HFD), então a associação de um medicamento hipolipemiante (ou seja, sinvastatina) com um medicamento inibidor da atividade da AChE (ou seja, donepezil) resultaria em uma melhor resposta em vários parâmetros. **Resultados:** Observamos que o tratamento com 27-OH aumenta a densidade proteica da AChE em neurônios hipocampais, bem como sua atividade catalítica em neurônios córtico-hipocampais; Camundongos que superexpressam o gene CYP27A1 apresentaram alterações constitutivas na expressão gênica e densidade proteica de AChE no cérebro. Ainda, camundongos alimentados com HFD apresentaram aumento do peso corporal, proteínas totais e níveis elevados de colesterol plasmático. Também observamos que o tratamento concomitante de sinvastatina e donepezil resultou em uma diminuição da atividade catalítica da AChE no hipocampo de animais alimentados com uma dieta hiperlipídica que acabou culminando em um melhor desempenho em uma avaliação de memória espacial de curto prazo.

**Conclusões**: No geral, os presentes achados sugerem que a 27-OH é um modulador da homeostase da AChE no encéfalo, e propomos que o tratamento com a associação de sinvastatina e donepezil pode ser uma abordagem interessante no manejo do comprometimento cognitivo na DA.

**PALAVRAS-CHAVE:** Oxisterois, Acetilcolinesterase, Sistema Colinérgico, Déficits de Memória, Demência, Doença de Alzheimer

# RESUMO EXPANDIDO

## Introdução

A manutenção da homeostasia do colesterol é essencial para o funcionamento neuronal normal e evidências científicas nas últimas décadas sugerem que o metabolismo do colesterol parece estar envolvido na patogenia da doença de Alzheimer (DA). Essa é a doença neurodegenerativa mais prevalente entre indivíduos acima de 60 anos de idade e resulta em uma progressiva disfunção cognitiva associada com um gradual declínio de memória e outras funções cognitivas e executivas e a um progressivo desenvolvimento de desordens afetivas e comportamentais. Evidências científicas apontam que o aumento do colesterol de membrana estimula o aumento da atividade catalítica de β- e γ-secretases e reduz a atividade da α-secretase, promovendo, assim, a formação do peptídeo beta-amilóide (Aβ). Além disso, já foi demonstrado que o colesterol medeia o metabolismo do Aβ, tais como em seus processos de fibrilação, transporte, degradação e *clearance*. Ademais, estudos experimentais com roedores hipercolesterolêmicos induzidos por dieta mostraram acúmulo de tau e fosfo-tau, disfunção da barreira hemato-encefálica (BHE) e neuroinflamação, diminuição do número de neurônios colinérgicos no núcleo basal de Meynert e diminuição dos níveis corticais de acetilcolina, bem como deficits cognitivos, as quais são características bem conhecidas da DA. A neurotransmissão mediada por acetilcolina é fundamental para o correto funcionamento do sistema nervoso e a próxima relação entre disfunções do sistema colinérgico e demências já são bem estabelecidas. A acetilcolinesterase (AChE) é a principal enzima responsável por finalizar a transmissão colinérgica por hidrolisar a acetilcolina e a sua inibição por drogas inibidoras da acetilcolinesterase (AChEIs) (e.g., donepezil) representa a estratégia terapêutica dominante no manejo do tratamento sintomático da DA.

Em um estudo prévio, nós demonstramos que camundongos Swiss alimentados com uma dieta hipercolesterolêmica por dois meses apresentaram déficit de memória espacial de curto-prazo (um evento que foi correlacionado com um aumento da atividade catalítica da AChE no córtex pré-frontal e hipocampo). Também foi observado um aumento concentração-dependente da atividade da AChE em células SH-SY5Y tratadas com colesterol LDL durante 24h. Esses achados ganham particular relevância em vista de evidencias cientificas que demonstraram que altos níveis de colesterol no sérum afetam negativamente a eficácia de longo-prazo do tratamento com AChEIs na progressão das funções cognitivas em pacientes com DA. Assim, uma relação causal entre esses eventos

pode ser sugerida, ou seja, o aumento da atividade catalítica da AChE no córtex pré-frontal e hipocampo causados pela hipercolesterolemia e a baixa eficácia de longo-prazo de AChEIs em pacientes hipercolesterolêmicos com DA. Entretanto, é incerto como o colesterol plasmático poderia afetar as funções encefálicas uma vez que o mesmo não consegue transpassar a BHE. Por outro lado, os oxisterois - metabólitos oxidados do colesterol – conseguem transpassar a BHE livremente para ambos os lados. Interessantemente, já foi observado que o 27-hidroxicolesterol (27-OH), o qual é o oxisterol mais abundante presente no plasma, consegue transpassar a BHE e que há um constante fluxo desse oxisterol da circulação para o sistema nervoso central (SNC). Além disso, devido à relação direta entre colesterol e 27-OH na corrente sanguínea, o aumento nos níveis de colesterol no plasma provavelmente resulta em um aumento na captação de 27-OH no encéfalo. No entanto, em condições não patológicas, o 27-OH permanece em níveis rasos no encéfalo devido a um metabolismo muito eficiente. A função deste oxisterol no encéfalo permanece desconhecida. No entanto, tem sido postulado que ele é um modulador da homeostase cerebral do colesterol e, curiosamente, vários estudos observaram uma associação entre altos níveis de 27-OH e déficits de memória, DA e outros processos neurodegenerativos. A produção de 27-OH é dada pela atividade catalítica da enzima CYP27A1, e foi observado que camundongos *knockout* para a CYP27A1 apresentam déficits de memória amenizados quando alimentados com uma dieta rica em lipídios. Esses achados sugerem que o 27-OH pode contribuir para os déficits de memória causados pelo colesterol dietético.

**Objetivos**

Diante dessas evidências, o presente estudo teve como objetivo elucidar o envolvimento da 27-OH na expressão gênica, densidade proteica e atividade catalítica da enzima AChE em neurônios hipocampais e córtico-hipocampais em cultura, bem como em células corticais e hipocampais de camundongos com superexpressão de CYP27A1. Além disso, dada a evidência anterior de que a hipercolesterolemia aumenta a atividade da AChE no hipocampo de camundongos, também testamos a possibilidade de que a associação de um medicamento hipolipemiante (sinvastatina) com um medicamento AChEI (donepezil) resultaria em uma melhor resposta em diversos parâmetros em camundongos alimentados com dieta hiperlipídica.

**Metodologia**

Esse estudo foi dividido em três *sets* experimentais. 1° *set*) Utilizamos cultura primária de neurônios hipocampais e cortico-hipocampais e tratamos com duas concentrações de 27-OH

por 6h e observamos a expressão gênica, densidade proteica e atividade catalítica da AChE; 2° *set*) observamos a homeostasia da AChE em camundongos geneticamente modificados que superexpressavam a CYP27A1; e, finalmente, o 3° *set*) buscamos observar se o tratamento concomitante de sinvastatina + donepezil resultaria em uma melhor resposta em diferentes parâmetros em camundongos alimentados com uma dieta hiperlipídica. Especificamente nesse último conjunto de experimentos trabalhamos com camundongos Swiss fêmeas de 3 meses de idade que foram divididos aleatoriamente em grupos alimentados com uma dieta padrão para roedores ou com uma dieta hiperlipídica. Após 41 dias de dieta, os camundongos foram submetidos a testes comportamentais, como: campo aberto (41º, 42º e 43º dias), e tarefa de reconhecimento de objetos (44º dia). No 45º dia, os camundongos foram novamente subdivididos aleatoriamente em quatro grupos diários de tratamento orogástrico: veículo, Sinvastatina (1 mg/kg), Donepezil (3 mg/kg) e a associação de Sinvastatina e Donepezil (1 + 3 mg/kg, respectivamente) por quinze dias. Após isso, os camundongos foram submetidos a testes comportamentais como campo aberto (61º dia) e tarefa de realocação de objetos (62º dia). Ao final do protocolo (63º dia) os camundongos foram privados de alimentos por seis horas, anestesiados e tiveram seu sangue coletado, e cérebro e fígado dissecados para posterior análise bioquímica.

**Resultados**

Observamos que o tratamento com 27-OH aumenta a densidade proteica da AChE em neurônios hipocampais, bem como sua atividade catalítica em neurônios córtico-hipocampais; Camundongos que superexpressam o gene CYP27A1 apresentaram alterações constitutivas na expressão gênica e densidade proteica de AChE no cérebro. Ainda, camundongos alimentados com HFD apresentaram aumento do peso corporal, proteínas totais e níveis elevados de colesterol plasmático. Nós também observamos que o tratamento concomitante de sinvastatina e donepezil resultou em uma diminuição da atividade catalítica da AChE em células hipocampais de animais alimentados com uma dieta hiperlipídica que acabou culminando em um melhor desempenho em uma avaliação de memória espacial de curto prazo.

**Considerações finais**

No geral, os presentes achados sugerem que a 27-OH modula a homeostasia da AChE no encéfalo. Propomos que o tratamento com a associação de sinvastatina e donepezil pode ser uma abordagem interessante para o manejo do comprometimento cognitivo na DA, especialmente em pacientes dislipidêmicos.

**PALAVRAS-CHAVE:** Oxisterois, Acetilcolinesterase, Sistema Colinérgico, Déficits de Memória, Demência, Doença de Alzheimer

## ABSTRACT

**Introduction:** Alterations in cholesterol metabolism within the brain have a significant role in Alzheimer's disease (AD) pathophysiology. Previous studies demonstrated that Swiss mice fed a high cholesterol diet displayed short-term spatial memory impairment, and it was interestingly correlated with an increase in the acetylcholinesterase (AChE) catalytic activity in the prefrontal cortex and hippocampus brain areas. Even though cholesterol cannot transpass the blood-brain barrier (BBB), its oxidized metabolite, 27-hydroxycholesterol (27-OH), the most abundant oxysterol in plasma, can freely cross the BBB and it has been observed that its high concentrations mediate the disruption of several systems within the brain, promoting, among other things, cognitive decline and neurodegeneration. However, the relation between high levels of 27-OH and AChE remains unknown. **Aims and methods:** Therefore, the experimental procedures in this study aimed at elucidating the involvement of 27-OH on the homeostasis of AChE, particularly its gene expression, protein density, and catalytic activity in cultured neurons as well as in neural cells of CYP27A1 overexpressing mice and, finally, to test the hypothesis that if plasma cholesterol increases the AChE activity in the hippocampal cells of high-fat diet (HFD) fed mice, then the association of a lipid-lowering drug (i.e., simvastatin) with an AChEI drug (i.e., donepezil) would result in a better outcome in several factors. **Results:** We observed that the treatment with 27-OH increases AChE's protein density in hippocampal neurons as well as its catalytic activity in cortico-hippocampal neurons; CYP27A1 overexpressing mice showed constitutive alterations in gene expression and protein density of AChE in the brain. Moreover, mice fed a HFD presented body weight gain, as well as an increase in plasma total protein levels and plasma cholesterol concentrations. Furthermore, we observed that the concomitant treatment of simvastatin and donepezil resulted in a decrease of AChE's catalytic activity in the hippocampal cells of the HFD-fed mice which ended up resulting in a better performance in a short-term spacial memory task. **Conclusions:** Overall, the present findings suggest that 27-OH is a modulator of the AChE homeostasis within the brain, and we propose that the treatment with the association of simvastatin and donepezil might be an interesting approach in the treatment management of cognitive impairment in AD.

**Keywords:** Oxysterols, Acetylcholinesterase, Cholinergic System, Memory deficits, Dementia, Alzheimer's Disease

## LIST OF ACRONYMS AND ABBREVIATIONS

| | |
|---|---|
| **24-OH** | 24-hydroxycholesterol |
| **27-OH** | 27-hydroxycholesterol |
| **ABC** | ATP-binding Cassette transporters |
| **ACAT** | Acylcholesterol Acyltransferase |
| **Acetyl-CoA** | Acetyl Coenzyme A |
| **AChE** | Acetylcholinesterase |
| **AChE$_E$** | Erythrocytic Acetylcholinesterase |
| **AChEI** | Acetylcholinesterase Inhibitory drug |
| **AChE$_R$** | Readthrough Acetylcholinesterase |
| **AChE$_S$** | Synaptic Acetylcholinesterase |
| **AD** | Alzheimer's Disease |
| **AICD** | APP Intracellular Domain |
| **ANOVA** | Analysis of Variance |
| **APH-1** | Anterior Pharynx defective 1 |
| **apoA** | apolipoprotein A |
| **apoB100** | apolipoprotein B100 |
| **apoC** | apolipoprotein C |
| **APP** | Amyloid Precursor Protein |
| **Arc** | Activity Regulated Cytoskeleton associated protein |
| **AT** | Adipose Tissue |
| **ATP** | Adenosine Triphosphate |
| **Aβ** | Amyloid-β Peptide |
| **BACE1** | Beta-Secretase |
| **BBB** | Blood-Brain Barrier |
| **BCSFB** | Blood–cerebrospinal fluid barrier |
| **BSA** | Bovine Serum Albumin |
| **BuChE** | Buthyrylcholinesterase |
| **CAT** | Catalytic acyl pocket |

| | |
|---|---|
| **CEUA** | Ethics Committee on the Use of Animals |
| **CFS** | Cerebrospinal Fluid |
| **ChAT** | Choline Acetyltransferase |
| **CHT** | Choline Transporter |
| **CM** | Chylomicron |
| **CSF** | cerebrospinal fluid |
| **CTFα** | α-C terminal fragmente |
| **CTFβ** | C-terminal fragments β |
| **CYP27A1** | Enzyme sterol 27-hydroxylase |
| **Cyp27Tg** | CYP27A1 Transgenic mice |
| **DMSO** | Dimethyl Sulfoxide |
| **DPZ** | Donepezil |
| **DTNB** | 5,5'-dithio-bis-(2-nitrobenzoic acid) |
| **EDTA** | Ethylenediaminetetraacetic Acid |
| **ELISA** | Enzyme-linked Immunosorbent Assay |
| **ER** | Endoplasmic Reticulum |
| **FDA** | Food and Drug Administration |
| **FPPS** | Farnesyl-Pyrophosphate Synthase; |
| **GAPDH** | Glyceraldehyde-3-phosphate Dehydrogenase |
| **GC–MS** | Gas Chromatography–mass spectrometry |
| **GFP** | Green Fluorescence Protein |
| **GGPPS** | Geranylgeranyl-diphosphate synthase |
| **HDL** | High-density lipoprotein |
| **HFD** | High fat Diet |
| **HMG-CoA** | 3-hydroxy-3-methylglutaryl-CoA |
| **HMGCR** | HMG-CoA reductase |
| **IL-1β** | Interleukin 1β |
| **ISF** | Interstitial Fluid |
| **LCAT** | Lecithin-Cholesterol Acyltranspherase |
| **LC–MS/MS** | Liquid Chromatography tandem–mass spectrometry |
| **LC-PUFAs** | Long-chain Polyunsaturated Fatty Acids |

| | |
|---|---|
| **LDL** | Low-Density Lipoprotein |
| **LDLr** | Low-Density Lipoprotein receptor |
| **LDM** | Lanosterol 14-demethylase |
| **LPL** | Lipoprotein Lipase |
| **mAChRs** | Muscarinic Acetylcholine Receptors |
| **MCI** | Mild Cognitive Impairment |
| **MK** | Mevalonate Kinase |
| **MMP** | Metalloproteinases |
| **MN** | Minneapolis |
| **MVD** | Diphosphomevalonate Decarboxylase |
| **MWM** | Morris Water Maze |
| **nAChRs** | Nicotinic Acetylcholine Receptors |
| **NaCl** | Sodium Chloride |
| **NFTs** | Neurofibrillary Tangles |
| **NO** | Nitric Oxide |
| **NOS** | Nitric Oxide Synthase |
| **OBJ** | Object |
| **ORT** | Object Recognition Test |
| **P-tau** | Hyperphosphorylated tau |
| **PAS** | Peripheral Anionic Site |
| **PBS** | Phosphate Buffered Saline |
| **PEN-2** | Presenilin Enhancer 2 |
| **PMK** | Phosphomevalonate Kinase |
| **PRiMA** | Proline-rich Membrane Anchor |
| **PS** | Presenilins |
| **PS1** | Presenilin 1 |
| **PS2** | Presenilin 2 |
| **RAGE** | Advanced glycosylation end product-specific receptor |
| **ROS** | Reactive Oxygen Species |
| **RT-PCR** | Real-Time Polymerase Chain Reaction |

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|---|---|
| **SBC** | Sociedade Brasileira de Cardiologia |
| **SC5D** | Sterol C5-desaturase |
| **SD** | Standard Diet |
| **SEM** | Standard Error of the Mean |
| **SERM** | Selective Estrogen Receptor Modulator |
| **SIMV** | Simvastatin |
| **SQS** | Squalene Synthase |
| **TC** | Total Cholesterol |
| **TG** | Triglycerides |
| **TNF-α** | Tumor Necrosis Factor alpha |
| **UFSC** | Universidade Federal de Santa Catarina |
| **VAchT** | Vesicular ACh Transporter |
| **VEH** | Vehicle |
| **VLDL** | Very Low-Density Lipoprotein |
| **WT** | Wild Type |
| **α2M** | α2-macroglobulin |

## LIST OF SYMBOLS

Å - ångström

α - alpha

β - beta

γ - gama

Δ - delta

μ - micro

## FIGURE LIST

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**SUMMARY**

## 1. Introduction

Since its isolation from gallstones at the time of the French Revolution, cholesterol has been extensively studied (Goedeke & Fernández-Hernando, 2012). However, its chemical structure only became fully known in 1927, through the work of Nobel Prize-winning chemist Heinrich Wieland. It is noteworthy that the extensive research on cholesterol metabolism resulted in no less than thirteen Nobel Prizes, being the molecule whose study laureate more researchers throughout history (Machado, 2016). Cholesterol is the most abundant member of a family of compounds known as sterols, and is a principal constituent of animal plasma membranes (Yeagle, 1985; Song & Kenworthy, 2014). Structurally, it is an amphipathic molecule containing three main domains: a hydrophobic region at C17 (nonpolar side chain), a hydrophilic region with a β-hydroxyl group at C3 (polar head), and a central four ring structure which gives the molecule extreme rigidity (Fig. 1) (Grouleff *et al*., 2015; Schade *et al.,* 2020). Because of this structure, it participates in many functions that are critical to normal cellular function, for instance: cholesterol determines the cell's permeability to ions and gases - the more cholesterol in the membrane, the less the permeability (Schade *et al.,* 2020). Therefore, cholesterol concentration in membranes varies depending upon the function of the membrane, from the highly permeable mitochondria (low cholesterol concentration) to the external cell membrane with limited permeability (high cholesterol concentration) (Schade *et al.,* 2020). Furthermore, cholesterol is also a biosynthetic precursor of steroid hormones (i.e. adrenal and gonadal hormones) (see Fig. 1), vitamin D and bile acids, as well as playing a critical role in synaptic transmission and plasticity (Goldstein & Brown, 1990; Yeagle, 1991; Simons & Ikonen, 2000; Pfrieger, 2003; Maxfield & Meer, 2010; Goedeke & Fernández-Hernando, 2012).

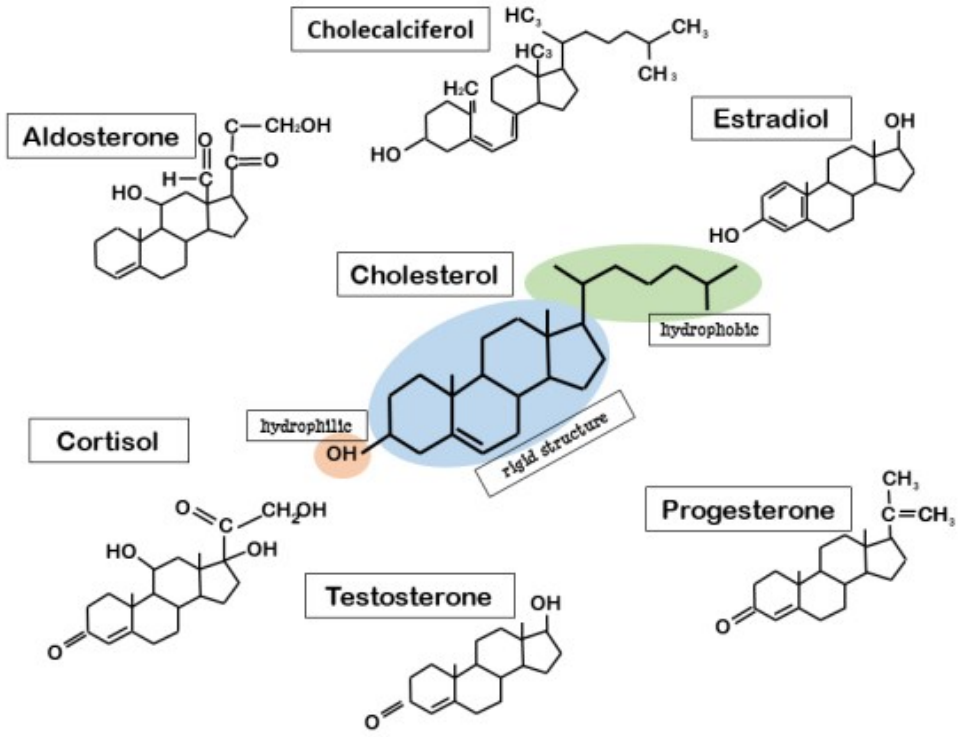



**Figure 1. Cholesterol chemical structure and some of its biological derivatives** (obtained from Schade *et al*., 2020 with authors' permission).

Nonetheless, despite its undeniable importance, abnormal cholesterol levels can cause severe cellular consequences and may lead to the pathogenesis of several diseases (Maxfield & Tabas, 2005; Goedeke & Fernández-Hernando, 2012). Because of this, the human body has evolved and developed complex and highly specialized mechanisms to regulate and maintain cholesterol biosynthesis, transport, metabolism, and excretion processes, which will be the focus of the next chapter.

## 1.1. Cholesterol biosynthesis, transport, metabolism and excretion

Due to the numerous fundamental functions, cells must have a sufficient and continuous supply of cholesterol. However, while some cells produce cholesterol in excess in order to supply other cells, others need exogenous cholesterol due to their limited biosynthetic capacity (Moreira, 2013). Under physiological conditions,

approximately 80 % of systemic cholesterol is endogenously synthesized (via *de novo*[1] biosynthesis - around 800 mg/day) while the remainder comes from the diet (Harvey & Ferrier, 2012). Because of this, mammals do not need dietary cholesterol intake (Lecerf & Lorgeril, 2011).

The mevalonate pathway, which is the name of the endogenous cholesterol biosynthetic pathway, involves a highly complex series of - at least - thirty enzymatic reactions that take place at the cytoplasm and endoplasmic reticulum (ER) (Fig. 2). This tetracyclic compound containing twenty-seven carbon atoms is synthesized from a single precursor, acetyl coenzyme A (acetyl-CoA), as the building block (Bloch, 1965; Bloch 1987).

Cholesterol biosynthesis begins with the production of acetoacetyl-CoA by the action of acetyl-CoA acyltransferase 2 (also known as cytosolic acetoacetyl-CoA thiolase). Acetoacetyl-CoA is then converted by the cytosolic 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase to produce HMG-CoA (Chang *et al*., 2017). In the next step, HMG-CoA is reduced to mevalonic acid by the action of the enzyme HMG-CoA reductase (HMGCR) - an integral protein of the endoplasmic reticulum membrane - in the reaction considered to be the rate-limiting step of sterol synthesis and the main regulation point of this pathway. The activity of this enzyme is regulated by negative feedback mechanisms, both at protein and transcriptional levels (Goedeke & Fernández-Hernando, 2012). Most of the mevalonic acid is converted through a series of biochemical reactions to lanosterol, the first sterol precursor with 30 carbons (Chang *et al*., 2017). Through several additional enzymatic reactions that occur mainly at the ER membrane, lanosterol can then be diverted into either the Bloch pathway producing cholesterol via desmosterol, or the Kandutsch-Russell pathway, via 7-dehydrocholesterol (Sharpe & Brown, 2013) (all enzymatic reactions of the mevalonate pathway can be seen at Fig. 2).

[1] - The term "*de novo"* means "newly made from simple components" as opposed to "acquired from the diet" or "recycled from preexisting complex components" (Brody, 1999)

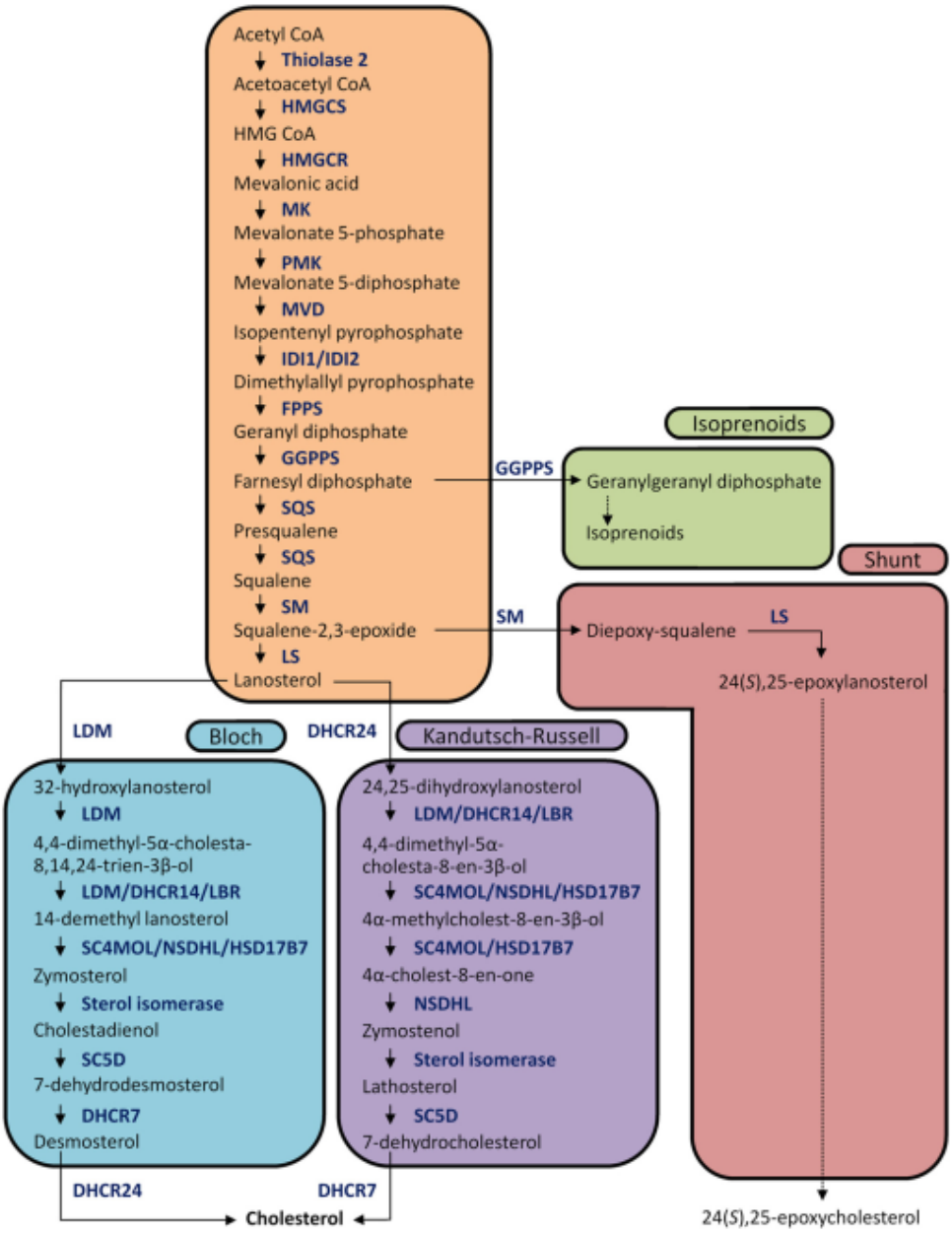



**Figura 2. Cholesterol biosynthesis pathway.** The mevalonate pathway leads to lanosterol production, which can then be diverted into either the Bloch pathway, producing cholesterol via desmosterol, or the Kandutsch-Russell pathway, via 7-dehydrocholesterol. Two other branches also diverge from the mevalonate pathway. Isoprenoids are produced by geranylgeranyl-diphosphate synthase (GGPPS) acting twice to convert farnesyl diphosphate to geranylgeranyl diphosphate, and flux through the shunt pathway occurs when SM acts twice to convert squalene 2,3-epoxide into diepoxysqualene, eventually leading to the production of 24(S),25-epoxycholesterol.

MK (mevalonate kinase); PMK (phosphomevalonate kinase); MVD (diphosphomevalonate decarboxylase); FPPS (farnesyl-pyrophosphate synthase); SQS (squalene synthase); LDM (lanosterol 14-demethylase); SC5D (sterol C5-desaturase) (obtained from Sharpe & Brown, 2013 with authors' permission).

Given the insolubility of lipids (such as cholesterol, cholesteryl esters, triglycerides (TGs), phospholipids, and fat-soluble vitamins), it requires particular types of biochemical machinery to facilitate their assimilation and distribution within the body (Brody, 1999). The biochemical apparatus includes bile salts, apolipoproteins[2], serum albumin, and vitamin-binding proteins (Brody, 1999). The lipropoteins are spheroidal macromolecules with a hydrophobic core containing triacylglycerol, vitamins, and esterified cholesterol, and a hydrophilic coat containing free cholesterol, phospholipid and apolipoprotein molecules (Hegele, 2009; Marques *et al*., 2018). The main TG-carrying lipoproteins are chylomicron (CM) and very low-density lipoprotein (VLDL), whereas the main cholesterol-carrying lipoproteins are low-density lipoprotein (LDL) and high-density lipoprotein (HDL) (Hegele, 2009).

Cholesterol and other dietary lipids are absorbed in the intestinal lumen and transported through CM, the largest and least dense lipoproteins, containing a high proportion of TG (Kane *et al*., 1980). In the liver, cholesterol has three major fates: conversion to bile acids, secretion into the bloodstream (packaged in lipoproteins for transport to extrahepatic tissues), and insertion into the plasma membrane (Brody, 1999).

VLDL can be remodeled, mainly in muscle and adipose tissue (AT), by the action of lipoprotein lipase (LPL) that removes TG from this lipoprotein, transferring apolipoprotein A (apoA) and apolipoprotein C (apoC) from VLDL to HDL and obtaining cholesterol esters (Moreira, 2013). The product of this remodeling is LDL, which supplies peripheral tissues with cholesterol. Cellular uptake of LDL is regulated by the LDL receptor (LDLr) and by the apolipoprotein B (apoB100) (Vance & Vance, 2008; Goldstein & Brown, 2009; Nelson & Cox, 2011).

Excess cholesterol influx inhibits the activity of HMGR enzyme, which will result in cholesterol biosynthesis inhibition, and stimulates cholesterol esterification

[2] - The term *apolipoprotein* is used when referring only to the protein, whereas the term *lipoprotein* refers to the complex of apolipoprotein and lipid (Brody, 1999).

through increased acyl-CoA: cholesterol acyltransferase (ACAT) enzyme activity. ACAT covalently attaches a fatty acid to the free hydroxyl group on the cholesterol molecule, producing a cholesteryl ester that can be stored in the cell (Harvey & Ferrier, 2012).

The last of the main types of lipoproteins, the high-density lipoprotein (HDL), is synthesized mainly by the liver but also in the small intestine, as a protein-rich particle that contains relatively little cholesterol and whose main function is the reverse[3] cholesterol transport (de Oliveira, 2015). HDLs are involved in transferring cholesterol from cells that contain excessive amounts to the liver or to tissues that are growing and need cholesterol (Huston, 1997). HDL particles contain apoA1 (the defining protein of the HDLs) and acquire cholesterol directly from the plasma membrane. This transfer is mediated by members of the adenosine triphosphate (ATP)-binding cassette (ABC) transporters family. Around 1g of cholesterol is eliminated from the body each day, and approximately half is excreted in the feces after conversion to bile acids (the remainder is excreted as unmetabolized – unchanged – cholesterol) (Moreira, 2013). The bile acids formed play an important role in the solubilization and absorption of fats, cholesterol, vitamins and drugs. Approximately 95% of bile acids are reabsorbed from the intestine and reach the liver via the hepatic portal vein (Degoma & Rader, 2011).

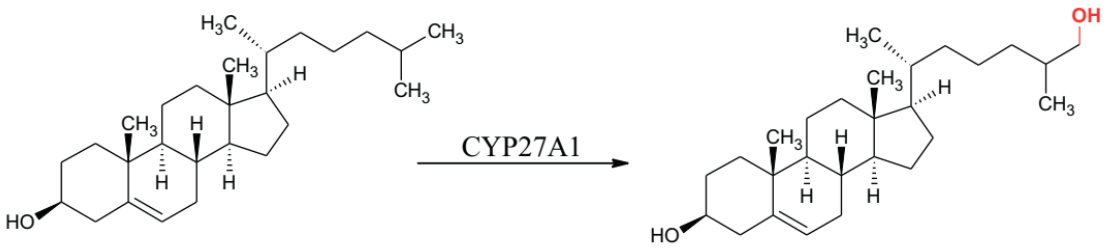
### 1.2 Oxysterols: Focus on 27-hydroxycholesterol

The cholesterol molecule is relatively easily oxidized, and it may be transformed into numerous oxidation products, including oxysterols (Zmysłowski & Szterk, 2019). Hence, oxysterols can be defined as oxygenated forms of cholesterol possessing one or more additional oxygen-containing functional groups (Brown *et al*., 2021). The oxygen-containing functional groups generally comprise hydroxyl, keto (oxo), hydroperoxyl or epoxide groups (Brown *et al*., 2021). Chemically, adding one or more oxygenated functional groups to a 27-carbon cholesterol molecule changes its behavior (Dias *et al*., 2019) and tends to render oxysterols more water soluble than

[3] - Reverse cholesterol transport is a mechanism by which HDL captures excess cholesterol from extrahepatic tissues and delivers them to the liver, where it will be redistributed to other tissues or removed from the body by the gallbladder (Marques *et al.,* 2018).

cholesterol, conferring distinct physicochemical properties (Brown *et al*., 2021). Primary oxysterols can be classified into side-chain (modified at C24, C25 or C27) or ring-modified (C7) oxysterols, while secondary oxysterols have more than one modification (Brown *et al*., 2021). These compounds are produced via auto-oxidation or formed enzymatically by a variety of enzymes (DeLuca, 2004). Autoxidation is mediated by free radical species (e.g., superoxide, hydroxyl radicals, lipid, and hydrogen peroxide) or occurs stoichiometrically by non-radical reactive oxygen species (ROS) (e.g., singlet oxygen, hypochlorite, and ozone) (Payne & Hales, 2004; Li & Chiang, 2009; Zerbinati & Iuliano, 2017).

Nevertheless, the most abundant oxysterols in human plasma are generated from the enzymatic reaction of mitochondrial or endoplasmic reticulum cholesterol hydroxylases, which are part of the cytochrome P450 family (Russell, 2000), with 27-hydroxycholesterol (27-OH) (Fig. 3) being the most abundant amongst all (Brown & Jessup, 1999). 27-OH is generated by the action of cholesterol 27-hydroxylase (CYP27A1). The activity of this enzyme is regulated by the rate of supply of cholesterol substrate to the inner mitochondrial membrane where this enzyme resides, and it is believed that this is a significant mechanism by which cells can eliminate excess cholesterol (Brown *et al*., 2021).




**Cholesterol** **27-hydroxycholesterol**

**Figure 3. Under the action of cholesterol 27-hydroxylase (CYP27A1), cholesterol is converted into 27-hydroxycholesterol (Source: the author).**

How oxysterols are compartmentalized and transported within the cell is yet poorly defined (Brown *et al*., 2021). However, as it happens with cholesterol, oxysterols

can be exported from cells by transporters like ABCA1 and ABCG1 (Terasaka *et al*., 2007), and they are transported in the circulation complexed to albumin or bound to lipoproteins (Lin & Morel, 1996). They can be present on the surface of lipoproteins in the free form, or within the core as oxysteryl esters, owing to esterification by lecithin-cholesterol acyltranspherase (LCAT) in the plasma (Dzeletovic *et al*., 1995). Particularly, 27-OH is mainly transported in the bloodstream in association with HDL and LDL particles (Babiker & Diczfalusy, 1998; Burkard *et al*., 2007) being taken up by cells either by LDLr or plasma membrane surface exchange (Luu *et al*., 2016).

Regarding its function in the periphery, it has been seen that 27-OH is a selective estrogen receptor modulator (SERM) and induces the growth of estrogen receptor-positive high-grade tumors (Nelson *et al*., 2011; Wu *et al*., 2013). Additionally, 27-OH could stimulate metastasis by its action on liver X receptor (LXR) signaling (Umetani, & Shaul, 2011). In addition to breast cancer, 27-OH was found to promote the growth of several other cancer types through estrogen receptors, including endometrial cancer, thyroid cancer, melanoma, lung, colon, and prostate cancer (Kim *et al*., 2022). 27-OH enhances the bone reabsorption by inhibiting bone formation and decreasing bone mineral density in bones due to its activity through estrogen receptors (DuSell *et al*., 2010). However, interesting results have been reported regarding the 27-OH-mediated regulation of estrogen receptor activity. Umetani and colleagues (2007) suggested that 27-OH is a competitive antagonist of estrogen receptor action in vascular cells. Because estrogen increases vasodilation responses by modulating nitric oxide synthase (NOS) expression, 27-OH may increase cardiovascular disease risk (Kim *et al*., 2022). Thus, 27-OH has both agonist and antagonist functions in the estrogen receptor depending on the tissue and cellular context (Kim *et al*., 2022). Moreover, 27-OH is the most common oxysterol found in atherosclerotic plaques, and its accumulation correlates with the severity of the lesion and affluence of macrophages (Upston *et al*., 2002; Lorea-Valencia *et al*., 2019a). A recent study has shown that 27-OH can induce instability of atherosclerotic plaques by mediating an inflammatory cascade (Gargiulo *et al*., 2015).

Oxysterols are inactivated by esterification or conversion into bile acids (in some instances, the essential need for bile acids can be met solely by the metabolism of oxysterols) (Schwarz *et al*., 1996). Oxysterols are usually measured by gas chromatography–mass spectrometry (GC–MS) methods incorporating selected-ion

monitoring (Dzeletovic *et al*., 1995; Schött & Lütjohann, 2015) or liquid chromatography-tandem–mass spectrometry (LC–MS/MS) methods exploiting multiple reaction monitoring (Stiles *et al*., 2014; Griffiths & Wang, 2019).

## 1.3 Cholesterol & the Central Nervous System

Regarding the cholesterol in the central nervous system (CNS), it is known that cholesterol is a major constituent of the human brain, and the brain is the most cholesterol-rich organ (Dietschy & Turley, 2004). Despite representing only 2% of total body mass, the brain contains about 25% of total body cholesterol (Björkhem *et al*., 1998; Björkhem & Meaney, 2004). The average brain cholesterol content is around 15–30 mg/g tissue, whereas the average in other tissues is 2–3 mg/g tissue (Dietschy, 2009). Brain cholesterol is found in two major stores: the smaller one is subject to relatively fast turnover rates (half-life of 5–10 months, 8 mg/g) and is made up of the plasma membranes of neurons (10%) and glial cells (20%); whereas the larger pool (containing up to 70% of the CNS cholesterol) consists of the myelin sheaths of oligodendrocytes (40 mg/g) with very slow turnover (half-life of approximately 5 years) (Russell *et al*., 2009; Petrov *et al*., 2016). In contrast, the half-life of plasma cholesterol is only a few days (Dietschy & Turley, 2004). In neuronal membranes, cholesterol is concentrated in microdomains called lipid rafts. These lipid rafts define the functional properties of ionic channels, neurotrophic factors and neurotransmitter receptors, as well as their locations in specific sites on membranes, thus creating a platform for initiating, propagating and maintaining signal transduction events (Paratcha & Ibáñez, 2002).

The rate of cholesterol synthesis is highest during the period of active myelination by oligodendrocytes (the first few weeks/months after birth) (Petrov *et al*., 2016). Following myelination the production of cholesterol in the mature brain drops by 90% (Dietschy, 2009; Petrov *et al*., 2016). In the CNS, cholesterol is a required component for synapse and dendrite formation (Goritz *et al*., 2005; Fester *et al*., 2009), axonal guidance (De Chaves *et al*., 1997; Koudinov & Koudinova, 2005), neurite growth, neurotransmitter release, signal transduction, membrane trafficking (Dietschy & Turley, 2004). Its depletion leads to synaptic and dendritic spine degeneration, failed neurotransmission, and decreased synaptic plasticity (Koudinov & Koudinova, 2005).

Cholesterol biosynthesis is crucial in the brain because the brain is separated from the peripheral pool of cholesterol by the blood-brain barrier (BBB), which - under physiological conditions - is impermeable to plasma lipoproteins (Zmysłowski & Szterk, 2019). Thus, brain cholesterol originates almost exclusively from *de novo* biosynthesis through the mevalonate pathway. The steady concentration of this sterol remains essentially constant under normal physical conditions, however, a fraction of the pool is constantly replaced (Zhang & Liu, 2015). Mechanisms must be in place to constantly excrete or degrade cholesterol and to constantly supply an equivalent amount of new sterol to the cell plasma membranes (Zhang & Liu, 2015). These two processes also must be so tightly regulated that the steady-state concentration of cholesterol in the brain remains essentially constant (Zhang & Liu, 2015). In this sense, a series of complex mechanisms are involved in regulating cholesterol by influencing its biosynthesis, transport, storage, and elimination from the brain, which will be briefly explained in the next sections.

### 1.3.1 Biosynthesis of cholesterol in the brain

Cholesterol biosynthesis within the brain is a very active, complex and resource-intense process in order to meet brain demands. Essentially all the synthesized cholesterol in the brain comes from the astrocytes, even though it is known that neurons can synthesize cholesterol at a very low rate. In the adult brain, astrocytes not only synthesize, but also internalize and recycle cholesterol released from degenerating nerve terminals to deliver it back to neurons (Jurevics & Morell, 1995; Pfrieger & Ungerer, 2011). This could allow neurons to spend energy on other processes, such as generating action and electrical potentials, since cholesterol biosynthesis involves several different reactions and intermediates (Pfrieger & Ungerer, 2011). *De novo* cholesterol synthesis takes place primarily in the ER of the astrocytes. Under cholesterol depletion or increased cholesterol demands, sterol regulatory element-binding protein cleavage-activating protein (SCAP) binds to sterol regulatory element-binding proteins (SREBPs) and transports it from ER to Golgi apparatus, where SREBPs will be cleaved by site-1 and site-2 proteases (S1P and S2P) forming a mature SREBPs (mSREBPs). The N terminal domains of mSREBPs translocate to the nucleus to bind sterol regulatory

elements (SRE) in the promoter regions of all (over 30) target genes encoding enzymes of cholesterol biosynthesis and uptake, including the HMGCR (Brown & Goldstein, 1997; Horton *et al*., 2002; Anchisi *et al*., 2013; Martin *et al*., 2014; Leoni & Caccia, 2015). Acetyl-CoA molecule enters in the mevalonate pathway and end up producing cholesterol. On the other hand, during high cholesterol concentrations, the SREBP-2/SCAP complex is retained in the membranes of the ER by the retention proteins INSIG-1 and -2 (insulin-induced protein 1 and 2) (Petrov *et al*., 2016) preventing the transportation of the complex to the Golgi apparatus. Newly synthesized cholesterol leaves the ER by vesicular and non-vesicular mechanisms (by means of carrier proteins) and is targeted to the plasma membrane, thus maintaining a low ER cholesterol content (Petrov *et al*., 2016). Moreover, Besides the biosynthesis of cholesterol, astrocytes could internalize and recycle the cholesterol that originates from degenerating nerve terminals (Jurevics & Morell, 1995; Sun *et al*., 2015).

### 1.3.2 Transport and storage of cholesterol within the brain

To transport the cholesterol, the astrocytes generate apolipoproteins, mainly apoE, to form a complex with the cholesterol which can be secreted out of the astrocytes by the activity of one or several ABC transporters, such as plasma membrane transporters ABCA1, ABCG1, and ABCG4 (Bu, 2009; Hayashi, 2011; Yu *et al*., 2014; Lane-Donovan *et al*., 2014). These complexes will be taken up by neurons by receptors belonging to the family of LDL: LDLr and LDL-receptor-like proteins (LRP, LRP1B, LRP2/megalin, LRP4, LRP5/6, LRP8/APOER2, LRP11/SORL1) (Vance *et al*., 2005; Petrov *et al*., 2016). Among them, the LDLr and LRP1 are the main receptors for the uptake of apoE-containing lipoprotein particles in the brain (Zhang & Liu, 2015). Interestingly, the interaction between ApoE-particles and their receptors triggers multiple signaling networks essential to neuron survival and function (Hayashi, 2011; Lane-Donovan *et al*., 2014). These receptors undergo endocytosis and will fuse with lysosomes that contain the acid lipase to hydrolyze the complexes, and release the free cholesterol in the cells (Sun *et al*., 2015). The liberated cholesterol exits late endosomes/lysosomes via NPC1-and NCP2-mediated pathways to reach the plasma membrane or the membrane of the ER, whereby the cholesterol content regulates the

genes involved in cholesterol homeostasis via negative feedback (the SREBP-2/SCAP/INSIG-1 pathway) (Peake & Vance, 2012; Petrov *et al*., 2016). This cholesterol can be used by the neurons to perform different physiological functions within the cell. The spare cholesterol could be esterified by acyl-coenzyme-A cholesterol acyltransferase (ACAT) in the ER (Sun *et al*., 2015). They will serve as the cholesterol storage in the cytoplasm lipid droplets and meet the neurons' demands (Brown & Jessup, 2009).

### 1.3.3. Elimination of cholesterol from the brain

The brain lacks pathways for cholesterol degradation, thus the degradation of this sterol in the brain depends on the liver (Sun *et al*., 2015). Nonetheless, given the presence of a preserved BBB, cholesterol is unable to cross and reach circulation. Despite a little cholesterol (1–2 mg) reaching the circulation each day via the CSF by an APOE-dependent mechanism (Pitas *et al*., 1987), the great majority of the brain cholesterol has other ways to leave the CNS. The main cholesterol elimination pathway within the brain is converting cholesterol into 24OH, which can then transpass the BBB and reach the systemic circulation. The enzyme cholesterol 24-hydroxylase, encoded by the CYP46A1 gene, is responsible for forming this oxysterol (Lund *et al*., 1999). This gene is highly expressed in neurons (Heverin *et al*., 2004). As the main form of the spare cholesterol is excreted from the brain to the circulation, the 24OH is metabolized in the liver (Saucier *et al*., 1993). In humans the flux of 24-OH across the BBB is about 6–7 mg/24 h (Lütjohann *et al*., 1996; Björkhem *et al*., 1998). In rats (Björkhem *et al*., 1997) and mice (Xie *et al*., 2003) the production of 24-OH is equivalent to about two-thirds of the cholesterol synthesis in the brain (Björkhem *et al*., 2009). Following its secretion from the brain, 24-OH travels through the circulation, is taken up by the liver, and is metabolized. In man, about half of the 24-OH reaching the liver is metabolized into the primary bile acids cholic acid and chenodeoxycholic acid while the remainder is converted into conjugates of either 24-OH itself or a 24S,27-dihydroxylated metabolite (Björkhem *et al*., 2001; Björkhem *et al*., 2009). Besides the cholesterol elimination mediated by 24OH, the reverse cholesterol transport pathway, which is mediated by the ATP transporters such as ABCA1 and ABCG1 (Martin *et al*., 2010), may also mediate

the cholesterol efflux in the brain (Sun *et al*., 2015). In the brain, ABCA1 is generally expressed in neurons, astrocytes, and the BBB (Panzenboeck *et al*., 2002; Koldamova *et al*., 2003). Experiments show that ABCA1 on BBB could interact with APOA1 and thus facilitate the assembly of HDL particles in circulation (Koldamova *et al*., 2010).

The figure 4 summarizes the main steps of cholesterol metabolism in the brain.

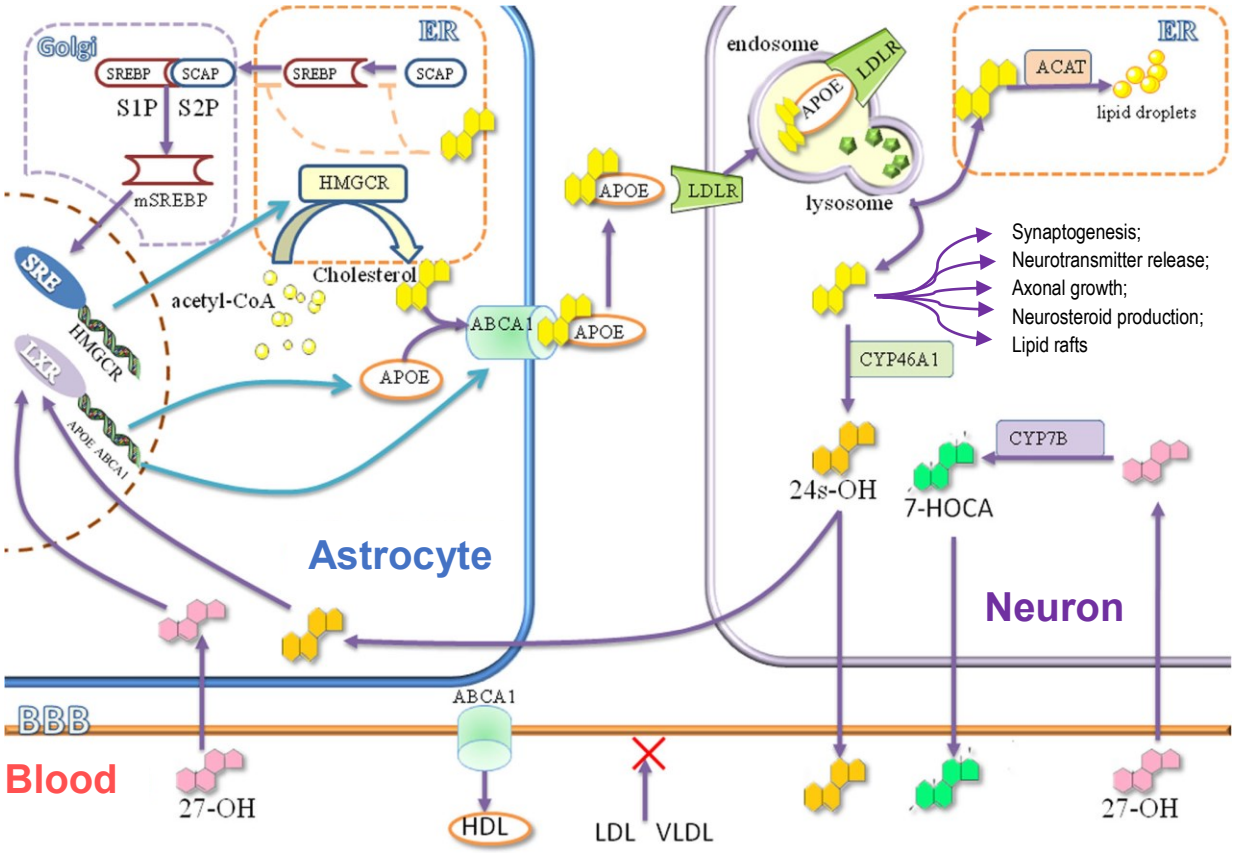



**Figure 4. The main steps of cholesterol metabolism in the brain: neuron-glial interplay.** The major input of cholesterol into the brain comes from in situ synthesis in the astrocytes. Under cholesterol depletion or given increased cholesterol demands, sterol regulatory element-binding protein cleavage-activating protein (SCAP) binds to sterol regulatory element-binding proteins (SREBPs) and transports it from ER to Golgi apparatus, where SREBPs are going to be cleaved by site-1 and site-2 proteases (S1P and S2P) forming a mature SREBPs (mSREBPs). This transcription factor translocates itself to the nucleus to regulate gene transcription. In the nucleus, mSREBP binds to sterol regulatory elements (SRE) and it end up upregulating the gene expression of

several enzymes in the mevalonate pathway, including the HMGCR, which is the rate-limiting step in the cholesterol biosynthesis. On the other hand, when cholesterol accumulates in the ER it inhibits the function of SCAP/SREBP pathway and provide the negative feedback to HMGCR. The synthesized cholesterol binds to APOE and forms a complex to be secreted out of astrocytes by ABCA1 transporter. This complex then binds to LDLr to be taken up by the neurons and be internalized to the endosomes by endocytosis, which will then fuse with lysosomes, to release the free cholesterol in neurons. This cholesterol, can then be used by the neuron to different functions synaptogenesis, neurotransmitter release, axonal growth, neurosteroid production, as well as it can go to the lipid rafts in the cell membrane. The extra cholesterol can be esterified by acylcholesterol acyl transferase (ACAT) to lipid droplets as the storage in ER or it can be metabolized into 24-OH through the action of CYP46A1 enzyme which is the main pathway where the brain excretes the spare cholesterol into the circulation through the BBB that prevents direct transport of cholesterol. 27-OH from the circulation can also be metabolized to 7-HOCA in neurons by the action of CYP7B. Both 24-OH and 27-OH are agonists of liver X receptors (LXR) which, when activated, increase the expression of APOE and ABCA1, thus facilitating cholesterol efflux. There is also evidence suggesting that ABCA1 on BBB could interact with APOA1 and thus facilitate the assembly of HDL particles to the circulation. In the brain, cholesterol is transported as a component of high density lipoprotein (HDL)-like particles which are similar to peripheral HDLs in their ability to both acquire and discharge cholesterol and phospholipids. The major apolipoprotein component of these particles in the CNS is apolipoprotein E (apoE), which mainly mediates the transport of lipids between astrocytes and neurons (modified from Sun *et al.,* 2015).

### 1.4. Hypercholesterolemia

Hypercholesterolemia can be characterized by a plasma cholesterol concentration equal to or greater than 200 mg/dL. It is one of the main causes of cardiovascular diseases. According to the latest data collected and published by the Brazilian Society of Cardiology (SBC, *in Portuguese: Sociedade Brasileira de Cardiologia*), about 77 million Brazilians have high cholesterol levels (SBC, 2017). In

this way, and due to the high rate of deaths due to heart attacks and strokes (300 thousand per year), the Brazilian government created the National Cholesterol Control Day (celebrated on August 8th) to shed light on the importance of keeping check on circulating cholesterol levels. Hypercholesterolemia can be resulted by two different etiological factors: behavioral or genetic. Regarding the behavioral component (which represents the great majority of the cases), it usually results from unhealthy nutritional factors such as obesity and a diet high in saturated fats (Bhatnagar *et al*., 2008). On the other hand, a common example of the genetic factor is monogenic familial hypercholesterolemia, an autosomal dominant disorder in which the plasmatic cholesterol is raised from birth usually due to defects in the LDLR gene, which encodes the LDL receptor (Goldstein *et al*., 2001). In the present thesis, however, we are not going to explore hypercholesterolemia's genetic (familial) factor. Notwithstanding, a review about this specific content can be seen by Defesche and colleagues (2017).

Until the 1980s, treatment strategies for hypercholesterolemia had limited effectiveness. At that time, clinical options were limited to dietary changes, bile acid sequestrants drugs[4], fibrates[5] and probucol[6]. However, the current main treatment of hypercholesterolemia is done by a class of drugs that inhibits the HMGCR (which is the rate limiting step in the cholesterol biosynthesis pathway), called statins. These drugs are markedly more efficacious in lowering LDL levels than previously available methods and the beneficial effects of lowering cholesterol levels with statins were confirmed in studies evaluating primary and secondary prevention of cardiovascular diseases (Pinal-Fernandez *et al*., 2018). In fact, a prospective meta-analysis of data from 90,056 participants in 14 randomized clinical trials on the cardiovascular effects of statins found similar proportional reductions in the risk of developing new major

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[4] These agents sequester bile acids in the intestine. As a result, there is a reduction in the absorption of exogenous cholesterol and an increase in the metabolism of endogenous cholesterol to bile acids. (Rang *et al*., 2011).

[5] Fibrates or fibric acid derivatives increase LPL activity, increasing the degradation of chylomicrons and VLDL in muscle and adipose tissue, and enhance the clearance of LDL by the liver (Rang *et al*., 2011).

[6] Powerful antioxidant. However, it decreases both LDL and HDL levels. Its mechanism of action is not fully elucidated and is associated with several side effects (Rang *et al*., 2011).

vascular or coronary events in patients regardless of their age, sex, cholesterol levels, presence of diabetes, hypertension, previous myocardial infarction or other coronary heart diseases (Baigent *et al*., 2005). Remarkably, it is estimated that each mmol/L of LDL reduction decreases by 22% the rate of major vascular events, 14% vascular mortality and, 10% the all-cause mortality per year (Pinal-Fernandez *et al*., 2018).

On the other hand, advances in the treatment management of high-mortality associated diseases, such as cardiovascular diseases, have also resulted in an increase in the life expectancy of the world population and consequent increase in the prevalence of aging-associated pathologies, such as neurodegenerative diseases. Interestingly, several studies have observed that many cardiovascular risk factors, such as obesity, hypertension and hypercholesterolemia are also considered risk factors for neurodegenerative diseases, especially Alzheimer's disease (AD). (Casserly & Topol, 2004; Kivipelto *et al*., 2005; Beach *et al*., 2007; Solomon *et al*., 2009). Concomitantly, the impact of the use of statins in cognition and the incidence of neurodegenerative diseases has been a matter of debate over the past decades. Recently, two systematic reviews and meta-analysis of observational studies have suggested that the use of statins are significantly associated with a decreased risk of dementia[7] and AD (Poly *et al*., 2020; Olmastroni *et al*., 2022).

### 1.5. Alzheimer's Disease

AD is a neurodegenerative disorder that represents the main cause of dementia and is quickly becoming one of this century's most expensive, lethal, and burdening diseases (Scheltens *et al*., 2021). Approximately 36 million people worldwide have AD, and due to the increase in life expectancy, the global prevalence is expected to increase to 115 million people by 2050 (Querfurth & LaFerla, 2010; Wisniewski & Goñi, 2014). In 1907, the

[7] Dementia is an umbrella term for loss of cognitive functioning — thinking, remembering, and reasoning — to such an extent that it interferes with a person's daily life and activities. Some people with dementia cannot control their emotions, and their personalities may change. Dementia ranges in severity from the mildest stage, when it is just beginning to affect a person's functioning, to the most severe stage, when the person must depend completely on others for basic activities of living (Extracted from the official website of the U.S.A. National Institute of Aging. Link: https://www.nia.nih.gov/health/what-is-dementia#:~:text=Dementia%20is%20the%20loss%20of,and%20their%20personalities%20may%20change).

German physician Alois Azheimer described two pathological alterations in the brain of a female patient suffering from dementia (Alzheimer, 1907). These two lesions represent the main pathological hallmarks of the disease (Fig. 5), and their observation during postmortem examination is still required for the definitive diagnosis of AD (Laferla *et al*., 2007). These two strongly-stained pathological features found within the AD brains are the amyloid plaques and neurofibrillary tangles (NFTs). It was not until the mid-1980s that it was discovered that the plaques consist of aggregates of a small peptide called amyloid-β (Aβ) (Glenner & Wong, 1984; Masters *et al*., 1985), whereas the NFTs are composed of intraneuronally aggregates of the tau protein, which becomes abnormally hyperphosphorylated (Grundke-Iqbal *et al*., 1986; Ihara *et al*., 1986; Kosik *et al*., 1986; Goedert *et al*., 1988). While both plaques and NFTs are individually seen in other forms of neurodegeneration (Suzuki *et al*., 1995; Gotz & Ittner, 2008; Malek-Ahmadi *et al*., 2019; DeTure & Dickson, 2019) their occurrence together is largely unique to AD (Rudge, 2022).

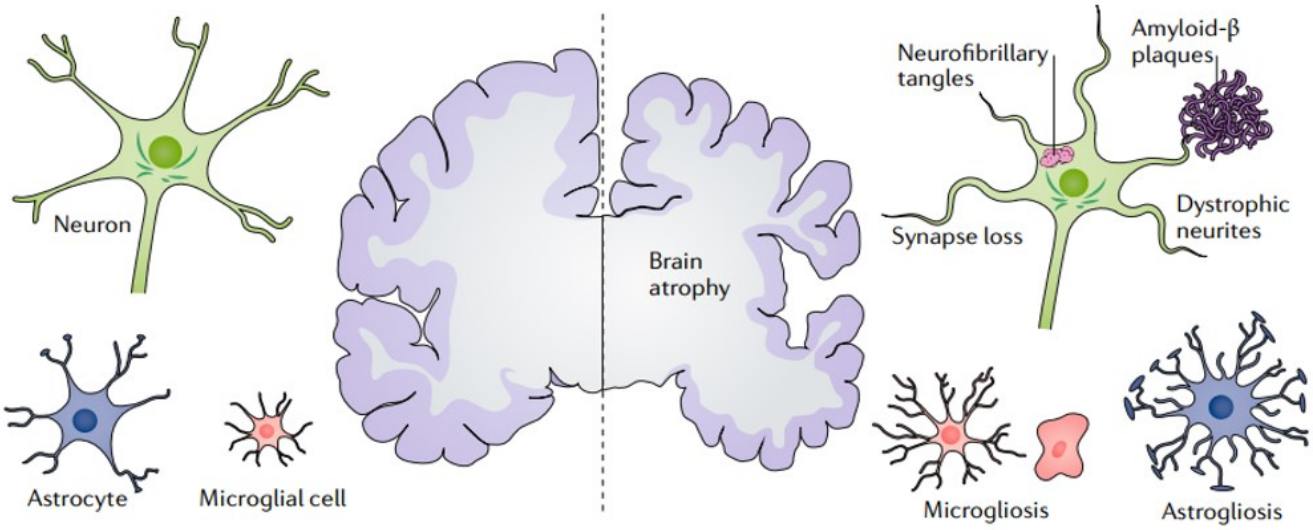


**Figure 5. The defining pathological hallmarks of Alzheimer's Disease.** At the gross anatomical level, Alzheimer's Disease is characterized by brain atrophy associated with loss of synapses and neurons. At the microscopic level, deposition of extracellular amyloid-β plaques and intraneuronal neurofibrillary tangles is observed, in association with dystrophic neurites and loss of synapses, as well as microgliosis and astrogliosis (Obtained from Congdon & Sigurdsson, 2018 with authors' permission).

Regarding Aβ, it is a peptide of 36 to 43 amino acids. It is formed from the sequential proteolytic cleavage of a transmembrane protein called amyloid precursor protein (APP) by a set of enzymes called secretases (Haass & Strooper, 1999; Haass & Selkoe, 2007; Herring *et al*., 2011), as shown in figure 6. The processing of this transmembrane protein can occur by the non-amyloidogenic pathway[8], which is the most common pathway, or by the amyloidogenic pathway (Fig. 6). The production of Aβ requires the initial action of β-secretase (BACE-1) on APP, forming a C-terminal fragment, which is an immediate substrate for a γ-secretase complex of enzymes[9]. γ-secretase mainly acts at positions 40 and 42, generating $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, respectively (LaFerla *et al*., 2007). Amyloid plaques mainly consist of $A\beta_{1\text{-}42}$, while the solubility properties of $A\beta_{1\text{-}40}$ allows its deposition in cerebral vessels (Weller *et al*., 1998).

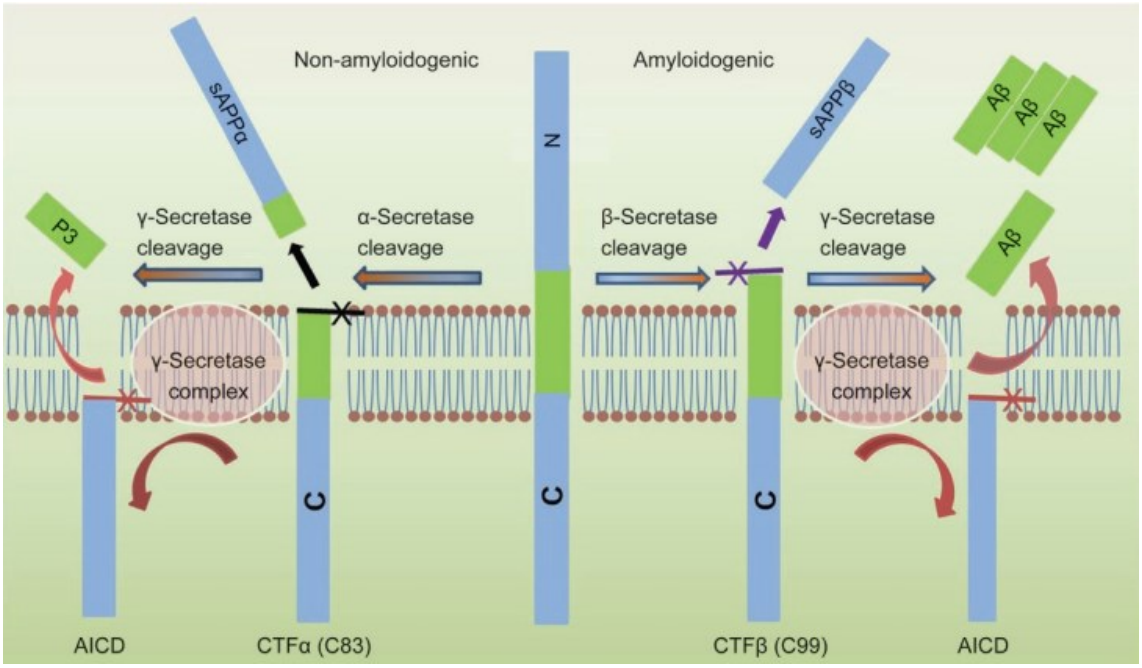


**Figure 6. Human APP proteolytic pathways**. APP proteolysis in the non-amyloidogenic pathway and amyloidogenic pathway. Non-amyloidogenic processing of APP refers to the

[8] In the non-amyloidogenic pathway, APP cleavage occurs within the Aβ sequence by α-secretase followed by γ-secretase, which results in the production of a fragment called p3 and prevents the production of Aβ (Haass & Selkoe, 2007).

[9] The γ-secretase is high molecular weight complex of enzymes that consists of four essential subunits: presenilins (PS, including PS1 and PS2), nicastrin, anterior pharynx defective 1 (APH-1), and presenilin enhancer 2 (PEN-2) (Strooper, 2003; Kimberly *et al*., 2003; Iwatsubo, 2004).

sequential proteolytic cleavage of APP by membrane bound α-secretases, which cleave within the Aβ domain to generate the membrane-tethered α-C terminal fragment CTFα (C83) and the N-terminal fragment sAPPα. CTFα is then cleaved by γ-secretases to generate extracellular P3 and the APP intracellular domain (AICD). Amyloidogenic processing of APP is carried out by the sequential action of membrane bound β- and γ-secretases. β-Secretase cleaves APP into the membrane-tethered C-terminal fragments β (CTFβ or C99) and N-terminal sAPPβ. CTFβ is subsequently cleaved by γ-secretases into the extracellular Aβ and AICD (Chen *et al*., 2017).

An imbalance between the production and clearance of Aβ in the brain and their aggregation, cause Aβ to accumulate with the subsequent formation of Aβ senile plaques. Soluble Aβ can be removed from the brain by various clearance systems, including enzymatic degradation and cellular uptake, transport across the BBB and blood–cerebrospinal fluid barrier (BCSFB), interstitial fluid (ISF) bulk flow, and cerebrospinal fluid (CSF) absorption into the circulatory and lymphatic systems (Tarasoff-Conway *et al*., 2015) (to see the clearance systems of Aβ and tau see Table 1). This peptide tends to aggregate to form dimmers, and oligomers and eventually it will form amyloid plaques. These oligomers and amyloid plaques are associated with several neurotoxic events that end up causing cholinergic degeneration and, in this way, being responsible for the signs and symptoms of AD.

**Table 1. The clearance systems of Aβ and Tau from the brain.**

| **Clearance System** | **Amyloid-β** | **Tau** |
|---|---|---|
| **Blood–brain barrier clearance** | Majority of Aβ clearance<br><br>LRP1 efflux;<br>ABCB1 efflux;<br>ApoE-mediated efflux;<br>*α*2M–mediated efflux;<br>LRP2-mediated efflux;<br>RAGE influx. | Unknown |
| | Ubiquitin–proteasome pathway;<br>Autophagy–lysosome | Ubiquitin–proteasome pathway;<br>Autophagy–lysosome |

| | | |
|---|---|---|
| **Degradation clearance**<br><br>*Intracellular* | pathway;<br>Endosome–lysosome pathway;<br>Proteases. | pathway;<br>Endosome–lysosome pathway;<br>Proteases. |
| *Extracellular* | Proteases<br>Glial phagocytosis | Unknown |
| **ISF bulk flow clearance**<br><br>*CSF sink* | Contributes to Aβ clearance | Unknown |
| *Perivascular drainage* | Contribution % to Aβ clearance is unknown | Unknown |
| *Perivascular glymphatic* | Contributes to Aβ clearance (55–65%)<br>Likely to facilitate blood–brain barrier clearance | Might contribute to clearance of non-endocytosed tau |
| **CSF absorption clearance**<br><br>*Circulatory* | Arachnoid villi;<br>Blood–CSF barrier transporters<br>(e.g. LRP1 efflux) | Arachnoid villi |
| *Lymphatic* | CSF lymphatic absorption | Unknown |

Zlokovic *et al*., 2000; Glabe, 2001; Weller *et al*., 2008; Iliff *et al*., 2012; Yoon & Jo, 2012; Chesser *et al*., 2013; Iliff *et al*., 2014; Tarasoff-Conway *et al*., 2015

This neurodegenerative disease is characterized by a progressive and irreversible decline in the number of cholinergic neurons that project mainly from the basal forebrain to cortical areas and hippocampal formation (Gallagher & Colombo, 1995; Mufson *et al*., 2003; Schliebs & Arendt, 2011). Clinically, this neuropathology is characterized by a severe cognitive impairment, memory loss, alterations of emotions, mood disorders, and personality changes (such as, e.g., a mixture of aggressive behavior and hallucinations)

(McKhann *et al*., 1984; Waldemar *et al*., 2007; Van Beek & Claassen, 2011; Mendez, 2021). These cognitive, emotional, and behavioral outcomes are strongly associated with the widespread neuronal death that gradually affects different brain areas. Severely affected regions include the entorhinal cortex, hippocampus, amygdala, neocortex, and some subcortical regions, which include cholinergic neurons of the basal forebrain, serotonergic neurons of the dorsal raphe nucleus, and noradrenergic neurons of the locus coeruleus (Braak & Braak, 1994; Geula & Mesulam, 1994; Dekosky *et al*. al., 1996; Kar *et al*., 2004). Over time, a person afflicted with this devastating disease progressively loses their ability to live and function independently, and, ultimately, end up dying from it.

Even though more than a century has passed since its discovery, the etiology of AD is still a matter of debate. Mutations in genes related to APP processing characterize AD of genetic origin (familial early-onset AD, patients younger than 65 years) (de Oliveira, 2015). The main genes involved are the genes that encode APP, presenilin 1 (PS1) and presenilin 2 (PS2) (Levy *et al*., 1990; Goate, 1994; Bertram & Tanzi, 2008). Thus, in the familial form of AD, the accumulation and deposition of Aβ are related to the increased production of this peptide (de Oliveira, 2015). However, mutations in these genes represent only a small proportion, with AD of a sporadic origin (late onset) accounting for 95% of all AD cases. The etiology of Aβ deposits in sporadic AD is, in most cases, unknown (Rocchi *et al*., 2003; Rocchi *et al*., 2009). Biochemical investigations from biopsy and autopsy indicate that several neurotransmitters and modulators, including acetylcholine, serotonin, noradrenaline and somatostatin, are differently altered in the brain of individuals with AD (Geula & Mesulam, 1994). However, the primary, and most consistently reproduced, event is a profound reduction in the activity of the acetylcholine synthesis enzyme, choline acetyltransferase (ChAT) in the neocortex, an event that positively correlates with the severity of dementia (Davies & Maloney, 1976; Perry *et al*., 1977; Auld *et al*., 2002). In fact, reduced choline uptake, acetylcholine release, and loss of cholinergic neurons in the basal forebrain indicate a deficit of selective presynaptic cholinergic in the hippocampus and neocortex of individuals with AD (Kar *et al*., 2004).

In this sense, drugs that potentiate central cholinergic function, such as acetylcholinesterase inhibitory drugs (the main enzyme responsible for terminating the cholinergic neurotransmission by rapidly hydrolyzing acetylcholine), have an important effect on the symptomatic treatment of AD (Trinh *et al*., 2003; Marucci *et al.,* 2021). Since

this thesis comprises one of the components of the cholinergic system, for instance, acetylcholinesterase, the next topic will explore the entire system a bit further.

### 1.6. Cholinergic System

The cholinergic system plays a critical role in the neural modulation of the mammalian brain (Li *et al*., 2018). It regulates several aspects of brain function, including sensory processing (Minces *et al*., 2017), attention (Sarter *et al*., 2005), sleep (Xu *et al*., 2015), and arousal (Szymusiak, 1995), as well as learning and memory processes (Everitt & Robbins, 1997; Sarter & Parikh, 2005; Bertrand & Wallace 2020).

Acetylcholine (ACh) was first identified by Dale (1914) for its actions on heart tissue. It was later recognized as a neurotransmitter by (Loewi, 1921), who initially named it "Vagusstoff" because it was released from the vagus nerve (Maurer & Willian 2017). Since then, the intricate workings of ACh synthesis and synaptic communication have been identified (Maurer & Willian 2017).

Cholinergic neurotransmission within the brain is now well-defined. It all starts with the synthesis of ACh in the terminal axon of the presynaptic neuron. Firstly, an acetyl-CoA is combined with a choline molecule by the action of the choline acetyltransferase (ChAT) enzyme, hence producing ACh. ACh then penetrates the vesicular ACh Transporter (VAchT). On arrival of an action potential, the voltage-dependent calcium channels open and the calcium enters into the pre-synaptic neuron and releases the VAchT to the synaptic cleft by exocytosis. Then, ACh can bind to two different membrane-bound receptor classes: (1) G protein-coupled muscarinic acetylcholine receptors (mAChRs), and/or (2) ligand-gated nicotinic acetylcholine receptors (nAChRs). After finishing its interactions with its receptors, ACh is hydrolyzed by acetylcholinesterase (AChE), the main enzyme responsible for terminating the cholinergic transmission within the CNS and that is localized in the post-synaptic membrane of the post-synaptic neuron, releasing acetate and choline. This choline is then recycled back into the pre-synaptic neurons by a choline transporter (CHT), and it is then combined with an Acetyl-CoA to resynthesize ACh. The whole steps in the cholinergic neurotransmission can be seen in figure 7.

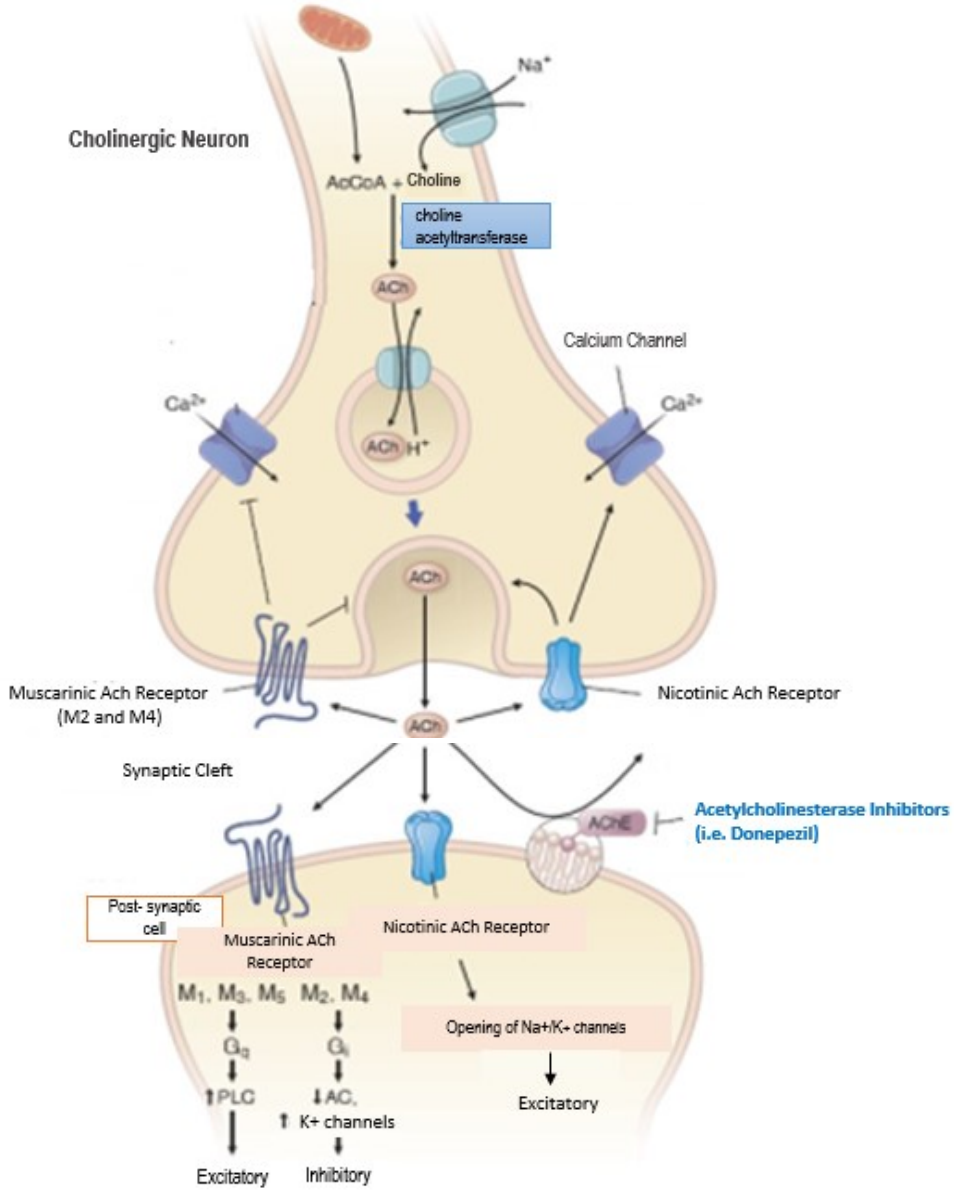


**Figure 7. Cholinergic neurotransmission within the brain.** Cholinergic neurons are responsible for synthetize the neurotransmitter Acetylcholine (ACh). In the pre-synaptic neuron a molecule of acetil-Coa is combined with choline by the action of choline acetyltransferase (ChAT) enzyme producing the neurotransmitter ACh. This newly neurotransmitter penetrates the vesicular ACh transporter (VAChT) and, on an arrival of an action potential, the voltage-dependent calcium channels open and allow calcium to

enter the pre-synaptic neuron. This action makes the VAchT to fuse with the presynaptic membrane and end up releasing its content by exocytosis. In the synaptic cleft ACh can then bind to either muscarinic and/or nicotinic ACh. After finishing its interactions with its receptors, ACh is then hydrolyzed into choline and acetate by the action of Acetylcholinesterase (AChE), which is localized in the post-synaptic membrane of the post-synaptic neuron, thus, releasing acetate and choline. This choline is then transported back into the pre-synaptic neuron by a choline transporter (CHT), to be recycled and to resynthetize ACh (Modified from Golan *et al*., 2014).

Two major cholinergic projection pathways occur in the brain (Fig. 8): 1) The magnocellular basal forebrain cholinergic system, which includes the nucleus basalis of Meynert, the medial septal nucleus, and the vertical and horizontal limbs of the diagonal band of Broca (Bertrand & Wallace, 2020). The basal forebrain cholinergic system has extensive projections to neocortical regions, as well as to the basolateral amygdala and olfactory bulb, hippocampus, and entorhinal cortices (Bertrand & Wallace, 2020). 2) The brainstem cholinergic system which includes the pedunculopontine nucleus and the laterodorsal pontine tegmental nucleus, projects primarily to thalamic structures and to basal forebrain regions (Bertrand & Wallace, 2020).

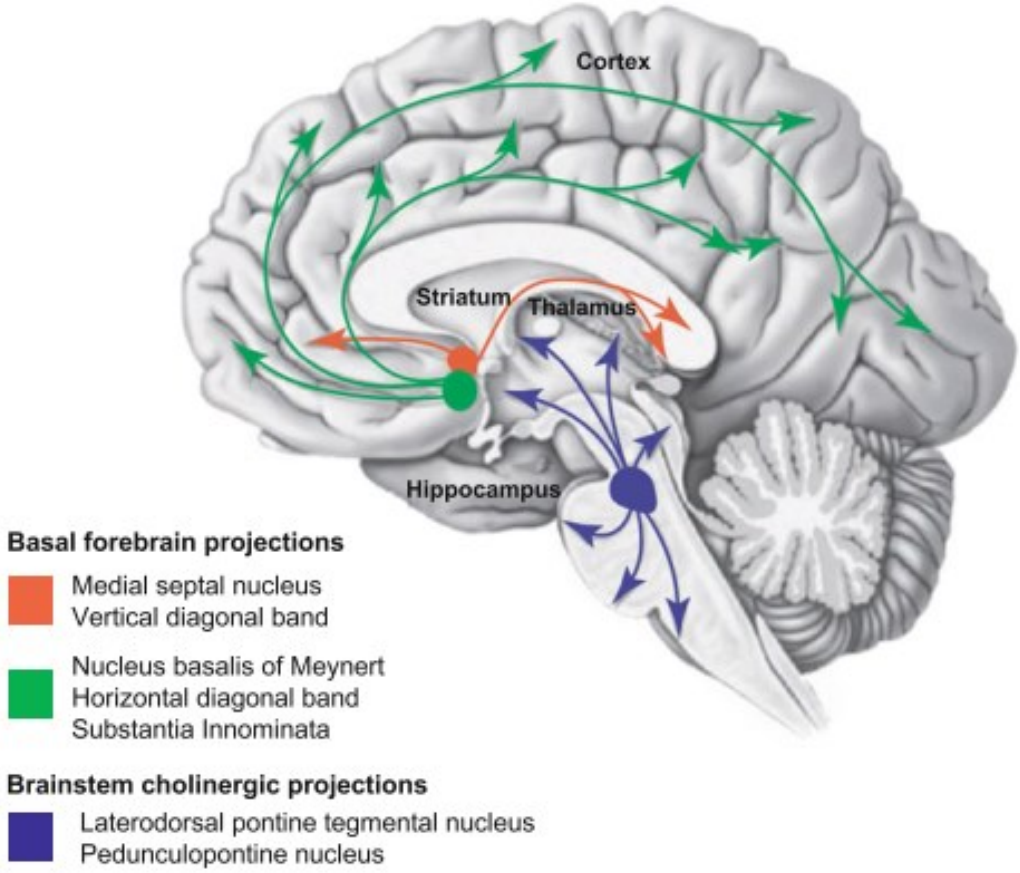


**Figure 8. Schematic representation of the major cholinergic projections in the human brain** (Bertrand & Wallace, 2020).

### 1.6.1. Acetylcholinesterase

According to the International Union of Biochemistry and Molecular Biology (IUBMB), AChE belongs to the group of hydrolases acting on ester bonds of esters of carboxylic acids (3.1.1.7) (Enzyme Nomenclature). The AChE molecule has an ellipsoidal shape with dimensions of approx. 45 × 60 × 65 Å, consisting of 537 amino acids (Walczak-Nowicka & Herbet, 2021). The binding site of AChE consists of several sub-sites catalytic (CAT), acyl pocket, oxyanion hole, anionic site and peripheral anionic site (PAS). The enzyme structure has a deep and narrow gorge, which penetrates halfway into the enzyme (length 20 Å), extending toward the base of the enzyme (Walczak-Nowicka & Herbet, 2021). The enzyme structure has a deep and narrow gorge, which penetrates halfway into the enzyme (length 20 Å) (Walczak-Nowicka & Herbet, 2021). In addition, it contains residues such as Asp-285, Glu-273, Asp-72, Tyr-334 and Glu-199 (Sussman *et al*., 1991;

Taylor, 2017). The residue of Glu-199 is associated with stabilization of the transition state, and the residue of Asp-72 is involved in trapping the ligand within the gorge (Barril *et al*., 2001).

The human gene that encodes AChE is located on chromosome 7q22 (Ehrlich *et al*., 1992; Getman *et al*., 1992). Additional splice variants involving both the 5' and 3' ends of the gene have been reported (Zhang & Greenberg, 2012), generating proteins that possess the same catalytic domain, associated with distinct C-terminal peptides (Massoulié, 2002). Among these splice variants are three distinct AChE variants called the "synaptic"(S), "erythrocytic" (E), and "readthrough" (R) AChE isoforms (Soreq & Seidman, 2001; Meshorer & Soreq, 2006). "Synaptic" $AChE_S$ is the major form of acetylcholinesterase found in the brain and muscle; "readthrough" $AChE_R$ has been reported in embryonic and tumor cells and is induced under psychological, chemical, and physical stress; and, finally, erythrocytic $AChE_E$ are associated with red blood cell membranes (Grisaru *et al*., 1999; Zhang & Greenberg, 2012). These subunits generate a variety of quaternary structures, including homomeric oligomers (monomers, dimers, tetramers) (Massoulié, 2002). At CNS synapses, the functional form is a G4 tetramer that assembles around a proline-rich domain similar to that in ColQ, which is near the NH2-terminus of a protein called proline-rich membrane anchor (PRiMA) (Silman, 2021) (Fig. 9). This protein contains a single trans-membrane sequence that serves to anchor the tetramer to the plasma membrane at the synapse (Perrier *et al*., 2002).

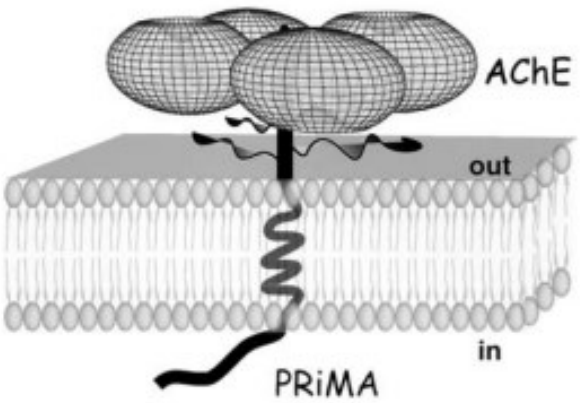


**Figure 9. Schematic Model of the Organization and Membrane Insertion of the AChE-PRiMA Complex.** The spheres represent the catalytic subunit and the depression the

entrance of the catalytic gorge. PRiMA is shown as a black ribbon and the expansions between the catalytic tetramer and the membrane correspond to the putative glycosylation motifs (Obtained from Perrier *et al*., 2002 with authors' permission).

Its principal biological role is the termination of impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter ACh to acetate and choline (Colovic *et al*., 2013). AChE has a remarkably high specific catalytic activity, especially for a serine hydrolase - each molecule of AChE degrades about 25.000 molecules of ACh per second, approaching the rate of a diffusion-controlled reaction (Quinn, 1987; Taylor & Radic, 1994; Colovic *et al*., 2013). Additionally, besides its primary catalytic function, accumulating evidence have been shown that this enzyme has many non-cholinergic functions. These include AChE involvement in cell growth, stem cell differentiation (Sperling *et al*., 2008; Imamura *et al*., 2015; Sogorb *et al*., 2016), cell signaling (Park & Yoo, 2010), synaptogenesis, activation of dopamine neurons (Soreq & Seidman, 2001), regulation of neuritic growth, neuronal network formation, cell adhesion (Soreq & Seidman, 2001; Paraoanu & Layer, 2008; Lazarević-Pašti *et al*., 2017). And some authors suggest that these "novel" functions may depend on protein–protein interactions rather than the enzyme's catalytic activity (Brimijoin & Koenigsberger, 1999; Paraoanu *et al*., 2006; García-Ayllón *et al*., 2011).

In the treatment of degenerative dementias, such as AD, the major benefits in cognitive symptoms have been achieved through the use of Cholinesterase Inhibitors (ChEIs) (Leguizamón *et al*., 2014). ChEIs have been used to reduce ACh breakdown and enhance their levels at the synaptic cleft (Sharma *et al.,* 2021). This subsequently makes the neurotransmitter available to the nicotinic and muscarinic receptors, enabling them to increase cholinergic signaling and thereby reducing AD memory deficits (Lazarević-Pašti *et al.,* 2017; Sharma *et al*., 2021). Currently, three Food and Drug Administration (FDA) ChEIs are available in the market: Donepezil, Galantamine, and Rivastigmine (see table 2). These drugs have been shown to statistically significantly improve cognition, daily and global function, and some behavioral manifestations of AD compared with placebo treatment (Massoud & Gauthier, 2010; Farlow *et al*., 2010; Hampel *et al*., 2018).

**Table 2. Pharmacologic properties of the cholinesterase inhibitors.**

| Drugs | Rivastigmine | Donepezil | Galantamine |
|---|---|---|---|
| Class | Carbamate | Piperidine | Alkaloid |
| Inhibition | Acetilcholinesterasa y Butirilcholinesterasa | Acetilcholinesterase | Acetilcholinesterase |
| Nicotinic modulation | No | No | Yes |
| Union | Pseudo-irreversible | Reversible | Reversible |
| Elimination | Renal | Hepathic | Renal Hepathic |
| Metabolism | Hydrolisis | CYP2D6 y CYP 3A4 | CYP2D6 y CYP 3A4 |
| Protein binding (%) | 40% | 96% | 18% |
| T ½ (hours) | 2 | 70-80 | 5-7 |
| Duration of inhibition (hours) | 10-12 | 50-70 | 7 |

**(**Obtained from Leguizamón *et al*., 2014 with authors' permission)

Donepezil is the most prescribed ChEI. It eases crosses the BBB, being highly selective for the CNS (Colovic *et al*., 2013; Leguizamón *et al*., 2014). The drug is available as a disintegrating tablet and oral solution with a relative oral bioavailability of 100%, reaching peak plasma concentration in 3 to 4 hours (food does not affect the drug absorption) (Leguizamón *et al*., 2014). Donepezil has a linear pharmacokinetic and is metabolized by the hepatic cytochrome P450 system (CYP 2D6 and 3A4), followed by glucuronidation (Leguizamón *et al*., 2014). It has a half-life of about 70 hours, so it can be taken once a day (Colovic *et al*., 2013). The excretion is mainly through the urinary tract with minimal fecal excretion (Leguizamón *et al*., 2014). The drug is available in 5 and 10 mg dose strengths, and treatment is usually initiated at 5 mg per day, and increased after several weeks to 10 mg per day (Colovic *et al*., 2013), with a maximum daily dose of 23 mg once daily used in moderate to severe AD cases (Farlow *et al*., 2010; Tariot *et al*., 2012). The adverse effects are cholinergic reactions: bradycardia, low blood pressure, nausea, vomiting, diarrhea, muscular cramps, headache and sleep disorders (Tayeb *et al*., 2012; Leguizamón *et al*., 2014; Hampel *et al*., 2018).

### 1.7. Hypercholesterolemia as a risk factor for Alzheimer's Disease

Evidence accumulated over the past decades point to an association between hypercholesterolemia and sporadic AD (Sparks *et al*., 1990; Sparks *et al*., 1994; Kuo t al., 1998; Solomon *et al*., 2009). The pathologist Larry Sparks and colleagues (1990) described the first report that cholesterol could be involved in the AD pathogenesis. In their interesting

study published in the Neurobiology of Aging journal, the authors observed that around 70% of the individuals who had died due to coronary artery disease also had amyloid plaques in their brains – one of the pathological features that define AD. On the other hand, age-matched individuals who had died from other causes were far less likely to develop amyloid plaques in their brains, which led Sparks and his colleagues to suspect a link between high plasma cholesterol levels and AD (Sparks *et al*., 1990). Subsequently, the same research group demonstrated that feeding rabbits a hypercholesterolemic diet produces Aβ plaques in the animal's brains (Sparks *et al*., 1994). In a similar manner, Refolo and colleagues (2000) corroborated the previous findings of Sparks and his colleagues by demonstrating that exposure to a hypercholesterolemic diet induces a significant increase in the levels of Aβ in the CNS of PSAPP[10] mice and that this increase is strongly correlated with plasma cholesterol levels (Refolo *et al*., 2000). One year later, the same group observed that the treatment with a cholesterol-lowering drug reduces brain Aβ levels as well as amyloid plaques in this same AD animal model (Refolo *et al*., 2001).

In this sense, clinical and epidemiological studies have indicated that hypercholesterolemic individuals are more susceptible to developing AD (Kivipelto *et al*., 2001; Kivipelto *et al*., 2002; Kivipelto *et al*., 2005). Particularly, a prospective cohort study published in the British Medical Journal by Kivipelto and colleagues (2001) demonstrated that individuals who have high plasma cholesterol levels during midlife have an increased risk of developing mild cognitive impairment (MCI) and AD in later life (a total of 1449 individuals were evaluated with an average of 21 years' follow up). Furthermore, Sparks and colleagues (2005) observed that when hypercholesterolemic individuals receive cholesterol-lowering therapies, i.e., statins, they present a decreased cognitive deterioration and prevalence of AD (Sparks *et al*., 2005). Moreover, Anstey and colleagues (2017) published a meta-analysis comparing data from more than 23.000 patients from 17 different studies They observed that the relative risk of developing AD for adults with high total cholesterol (TC) in midlife was 2.14 times higher than normocholesterolemic individuals (Anstey *et al*., 2017). Similar results were seen in a longitudinal, population-based prospective cohort study, which followed participants without dementia for 13 years and found that higher TC concentrations at baseline were associated with an increased risk of

[10] PSAPP: Transgenic mouse model for AD. They develop amyloid plaques - similar to those seen in AD patients - as the animals age.

AD (Schilling *et al*., 2017). Also, elevated TC concentrations in late life can accelerate cognitive decline (Ma *et al*., 2017; Guo *et al*., 2020). Furthermore, several lines of evidence suggest that cholesterol can modify APP processing. An association has been demonstrated between serum cholesterol levels and cerebral amyloidosis (Reed *et al*., 2014). *In vitro* and *in vivo* data indicate that cholesterol directly modulates APP processing and Aβ production in neuronal cells and peripheral cell lines (Bodovitz & Klein, 1996; Simons *et al*., 1998; Frears *et al*., 1999; Ehehalt *et al*., 2003; Wolozin, 2004). Simons *et al*. (1998) demonstrated that Aβ production in hippocampal neurons is completely inhibited when cell membrane cholesterol levels are reduced by cholesterol-extracting compounds, such as methyl-β-cyclodextrin. Specifically, reducing cholesterol levels by stripping off the membrane cholesterol with methyl-β-cyclodextrin or by treating with HMGCR inhibitors can enhance α-secretase cleavage and decrease BACE-1 activity, thus inhibiting Aβ production (Simons *et al*., 1998; Frears *et al*., 1999; Fassbender *et al*., 2001; Kojro *et al*., 2001; Abad-Rodriguez *et al*., 2004). One possible explanation for the alteration of APP processing through the modification of cellular/membrane cholesterol levels is that the localization of APP and its secretases are differentially affected by lipid composition, thereby altering the cleavage of APP (Maulik *et al*., 2013). Accumulated evidence clearly indicates that α-secretase ADAM10 mostly resides in the low-cholesterol nonraft domains (Kojro *et al*., 2001), whereas a subset of β-secretase BACE1 and γ-secretase are associated with lipid raft domains of the membrane (Wahrle *et al*., 2002; Vetrivel *et al*., 2004; Kalvodova *et al*., 2005; Osenkowski *et al*., 2008; Vetrivel & Thinakaran, 2010). APP, on the other hand, is believed to exist in two distinct pools in the cell membrane, one associated with lipid rafts and the other in the phospholipid-rich domains (Ehehalt *et al*., 2003). These observations raised the possibility that the amyloidogenic processing of APP occurs in cholesterol-rich lipid rafts, while the non-amyloidogenic processing occurs mainly in a more fluid phospholipid-rich region of the membrane outside the rafts and that altering cellular cholesterol levels regulates the processing of APP through these two pathways (Kojro *et al*., 2001; Maulik *et al*., 2013). Under physiological conditions, only very minor amounts of APP appear to be present in detergent-resistant rafts (Bouillot *et al*., 1996; Parkin *et al*., 1999), thereby making α-secretase cleavage the predominant pathway (Maulik *et al*., 2013). However, increasing membrane cholesterol levels may elevate the percentage of rafts in the membranes and consequently enhance the activity/contact between APP and their

processing enzymes BACE1 and γ-secretase, leading to increased production of Aβ peptides (Maulik *et al*., 2013) (Fig. 10). This notion is supported by three distinct lines of elegant experiments: (1) antibodies cross-linking APP and BACE1 showed that these two proteins co-patched with raft markers and dramatically increase the production of Aβ peptides in a cholesterol-dependent manner (Ehehalt *et al*., 2003), (2) fluorescence microscopy techniques have shown that increase in membrane cholesterol level can enhance accessibility of BACE1 to its substrate APP by clustering in lipid rafts, followed by rapid endocytosis and generation of Aβ peptide (Marquer *et al*., 2011), and (3) inhibition or absence of γ-secretase activity can lead to increased accumulation of APP-CTFs in the lipid raft microdomains (Vetrivel *et al*., 2004; Maulik *et al*., 2013).

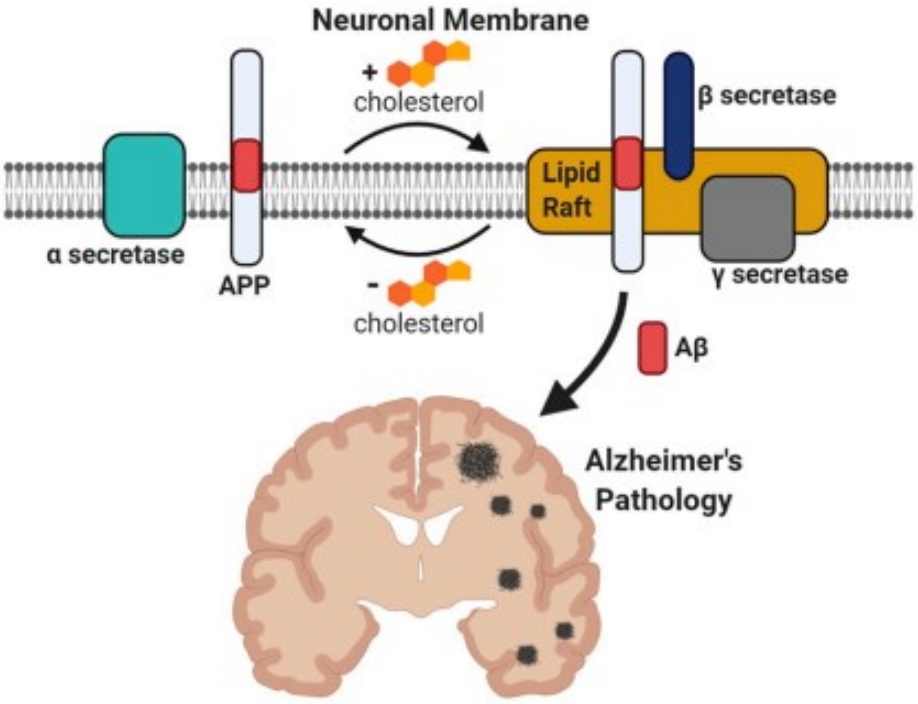


**Figure 10. Cholesterol content in neuronal membranes regulates Aβ production by APP interactions with secretases.** In low-cholesterol membranes (left), APP interacts with α-secretase, generating sAPPα. When neuronal membranes are loaded with cholesterol, APP increasingly interacts with β- and γ-secretases generating Aβ peptides, resulting in brain plaque formation over time (right) (Obtained from Wang *et al*., 2021 with authors' permission).

Moreover, in an attempt to better understand the relation between hypercholesterolemia and cognitive deficits, our research group has previously shown that Swiss mice fed a high cholesterol diet (which was a standard rodent chow supplemented with 1.25% cholesterol and 20% fat) for 2 months increased their total plasma cholesterol levels and this increase resulted in short-term spatial memory impairment (seen by the location index in the object location task), without causing locomotor deficts seen in the number of crossings in the open field test (Moreira *et al*., 2014). One of the most interesting findings in this paper was that a significant increase in the AChE catalytic activity was also observed in two key brain areas that are responsible for learning and memory processes in the CNS, for instance the prefrontal cortex and hippocampus. Well, but is cholesterol indeed responsible for increasing the activity of acetylcholinesterase? In order to have one more indication of it, human neuroblastoma cells from the SH-SY5Y lineage were exposed to LDL cholesterol at 2 different concentrations (50 and 100 ug/mL) for 24 hours, and the activity of the AChE enzyme was subsequently evaluated. It was observed that LDL cholesterol significantly increased, in a dose-dependent manner, AChE activity in these cells (Moreira *et al*., 2014).

Thus, we have one more indication that this association seems to be direct. Nonetheless, despite this previous evidence, the complete whole of hypercholesterolemia in cognitive impairment and AD progression is still far from being fully understood since cholesterol itself cannot cross a healthy/preserved BBB. On the other hand, 27-OH, the main oxysterols in the circulation, is able to freely cross the BBB and it has already been seen that there is a constant flow of this metabolite from the periphery to the CNS (Meaney *et al*., 2002; Björkhem *et al*., 2009). The function of this oxysterol in the brain remains unknown, but it has been suggested that it modulates cholesterol homeostasis in the brain (Loera-Valencia *et al*., 2019a). In this sense, Zhang and colleagues (2015) hypothesized that impaired memory functions are caused by aberrant cholesterol metabolism *via* increased levels of 27-OH in the circulation and increased flux of this oxysterol into the brain. Particularly in this study, rats received daily caudal vein injections of 27-OH at three different doses, 7, 21, and 70 uM for 28 days. The authors observed that these peripheral injections of 27-OH were able to transpass the BBB and resulted in increased total cholesterol, free cholesterol and cholesterol esters within the rat's brains; impairing spatial learning and memory seen by the escape latency in the Morris water maze test; and

modulating brain cholesterol catabolism seen by the reduction of the gene expression and protein density of HMGCR and LDLR, as well as an increase in the CYP46A1, LXRa and LXRb and ABCA1 transporter gene expression (Zhang *et al*., 2015). Furthermore, rabbits on a high cholesterol diet exhibit increased 27-OH within the brain, which was also associated with increased hippocampal neurodegeneration (Brooks *et al*., 2017). In late-stage AD brains, CYP27A1 mRNA levels are significantly increased as are 27-OH concentrations within the frontal and occipital cortex, and CSF, suggesting 27-OH might be involved in AD progression (Wang *et al*., 2016; Testa *et al*., 2016). Additionally, a hypercholesterolemic diet reduced the levels of the "memory protein" Arc (Activity Regulated Cytoskeleton associated protein) in the hippocampus of the wildtype mice but not in the hippocampus of the Cyp27-/- mice, suggesting that 27-OH is the mediator of the adverse effects of cholesterol on cognition (Heverin *et al*., 2015).

## 2. Justification

Previous evidence has pointed to hypercholesterolemia as a potential risk factor for dementia and cognitive impairment (Refolo *et al*., 2000; Sun *et al*., 2015; Loera-Valencia *et al*., 2019a). AD, the leading neurodegenerative disorder resulting in severe cognitive impairment (Sáiz-Vazquez *et al*., 2020), is characterized by a gradual decline in memory and other cognitive and executive functions and progressive development of affective and behavioral disorders (Liu *et al*., 2020). It is well known that acetylcholine-mediated neurotransmission is fundamental to the functioning of the nervous system (Ferreira-Vieira *et al*., 2016), and the close relationship between the cholinergic system and dementia is well established (Van Beek & Claassen, 2011; Pepeu & Giovannini, 2017). AChE is the primary enzyme responsible for hydrolyzing acetylcholine, and its inhibition by acetylcholinesterase inhibitory drugs (AChEIs) (e.g., donepezil and galantamine) is the dominant therapeutic strategy in the management of AD (Marucci *et al.,* 2021).

Maintaining cholesterol homeostasis is essential for normal neuronal functioning, and research in the last decades has indicated that cholesterol metabolism appears to be involved in AD pathogenesis (Kivipelto *et al.*, 2002; Cedazo-Minguez *et al.*, 2011; Loera-Valencia *et al.*, 2019a). Scientific evidence points out that different cholesterol levels influence various steps in neurotoxic Aβ generation (Simons *et al.,* 1998; Frears *et al*., 1999; Refolo *et al*., 2000), including APP proteolysis and the related activities of α-, β-, and γ-secretases (Wang *et al.,* 2019). In addition, cholesterol has been proved to mediate Aβ metabolism, such as its fibrillation (Kakio *et al.*, 2001; Fantini *et al.*, 2013), transportation (Prasanthi *et al*., 2008), degradation (Kakio *et al.*, 2001; Ghareeb *et al.,* 2015), and clearance processes (Wang *et al.,* 2019). Moreover, experimental studies with diet-induced hypercholesterolemic rodents showed tau and phospho-tau accumulation (Ullrich *et al*., 2010), BBB disruption, and neuroinflammation (Kalayci *et al*., 2009; Ullrich *et al*., 2010; de Oliveira *et al*., 2014; Denver *et al*., 2018), decreased number of cholinergic neurons in the basal nucleus of Meynert, and decreased cortical acetylcholine levels (Ullrich *et al*., 2010) as well as cognitive impairments (Ullrich *et al*., 2010; de Oliveira *et al*., 2011; Moreira *et al*., 2012; Moreira *et al*., 2013; Denver *et al*., 2018), which are well-known features of AD.

In a previous study, we showed that Swiss mice fed a high cholesterol diet (1.25% cholesterol and 20% fat) for two months displayed short-term spatial memory impairment,

an event that was correlated with an increased AChE activity in the prefrontal cortex and hippocampus (Moreira *et al*., 2014). Furthermore, we observed that aged LDLr$^{-/-}$ knockout mice, exposed to over three-fold cholesterol levels from early life, displayed working, spatial reference, and procedural memory impairments, without altering motor function, together with a significant increase in the AChE activity in the prefrontal cortex (Moreira *et al*., 2012). Moreover, Machado *et al*., (2018) have observed that LDLr$^{-/-}$ knockout mice presented impaired contextual fear memory retrieval and that boosting cholinergic neurotransmission with a single donepezil administration at the consolidation window led to improved fear memory consolidation. It was also observed a concentration-dependent increase in AChE activity in SH-SY5Y cells after treatment with LDL cholesterol for 24 hours (Moreira *et al*., 2014). In this regard, Borroni *et al*. (2003) demonstrated that high serum cholesterol levels negatively affect the long-term efficacy of treatment with AChEIs in the progression of cognitive functions in AD patients. Therefore, one might suggest a causal relationship between these events, i.e., hypercholesterolemia increases AChE activity in the prefrontal cortex and hippocampus and the lower long-term efficacy of AChEIs in hypercholesterolemic AD patients. Nevertheless, it is unclear how plasma cholesterol could affect brain functions since cholesterol cannot cross the BBB under physiological conditions (Björkhem *et al*., 1998; Dietschy & Turley, 2001). On the other hand, oxysterols, which are oxidized cholesterol metabolites, can freely cross the BBB by diffusion and influence brain cholesterol synthesis (Meaney *et al*., 2002; Björkhem, 2002; Ali *et al*., 2013; Loera-Valencia *et al*., 2021a). Specifically, 27-hydroxycholesterol (27-OH), the most abundant cholesterol metabolite in plasma (Björkhem *et al*., 2009), can cross the BBB (Heverin *et al.,* 2005; Jeitner *et al*., 2011), and it was already reported that there is a net influx of this oxysterol from the circulation into the brain driven by a concentration gradient (Meaney *et al*., 2002; Björkhem *et al*., 2009; Ali *et al*., 2013). Moreover, due to the direct relation between cholesterol and 27-OH in the bloodstream (Babiker *et al.,* 2005), the increase in the plasma cholesterol levels likely results in an increase in the brain 27-OH uptake (Björkhem *et al*., 2009; Shafaati *et al.,* 2011; Parrado-Fernandez *et al*., 2021). Nonetheless, in non-pathological conditions, 27-OH remains at shallow levels in the brain due to a very efficient metabolism (Goldstein *et al*., 2006). The function of this oxysterol in the brain remains unknown. However, it was suggested that it is a modulator of the brain cholesterol homeostasis (Loera-Valencia *et al*., 2019a) and, interestingly, several studies have observed an association between high levels

of 27-OH and memory deficits, AD, and other neurodegenerative processes (Mateos *et al*., 2009; Björkhem *et al*., 2009; Cedazo-Minguez *et al*., 2011; Mateos *et al*., 2011a; Mateos *et al*., 2011b; Heverin *et al*., 2015; Ismail *et al*., 2017). 27-OH is produced by CYP27A1 catalytic activity (Björkhem *et al*., 2009), and it was observed that knocking out the CYP27A1 gene in mice ameliorates memory deficits induced by a high-fat diet (HFD) (Heverin *et al*., 2015). This finding suggests that 27-OH may be a contributor to the memory impairment caused by dietary cholesterol.

In light of this evidence, the present study aimed at elucidating the involvement of 27-OH on the mRNA expression, protein density, and catalytic activity of the AChE enzyme in hippocampal and cortico-hippocampal neurons in culture as well as in cortical and hippocampal cells of CYP27A1 overexpressing mice. Moreover, given the previous evidence that hypercholesterolemia increases the AChE activity in the hippocampus of mice (Moreira *et al*., 2014), we also tested the possibility that an association of a lipid-lowering drug (i.e., simvastatin) with an AChEI drug (i.e., donepezil) would result in a better cognitive performance on a short-term spatial memory evaluation.

## 3. Aims

The main purpose of this study was to investigate the involvement of 27-OH on AChE's mRNA expression, protein density, and catalytic activity in hippocampal and cortico-hippocampal neurons in culture as well as in cortical and hippocampal cells of CYP27A1 overexpressing mice as well as to test the hypothesis that the treatment with the association of a lipid-lowering drug (simvastatin) with an AChEI drug (donepezil) would result in a better outcome in several parameters in mice fed a HFD.

### 3.1 Specific Aims

i. To evaluate the effects of the addition of 27-OH on the total cholesterol concentration, mRNA expression, protein density and catalytic activity of the AChE enzyme in hippocampal and cortico-hippocampal neurons in culture.

ii. To observe whether male and female CYP27A1 overexpressing mice (Cyp27Tg) have constitutive alterations in the mRNA expression and protein density of AChE in cortical and hippocampal cells in comparison to age-and-sex-matched wild-type C57BL/6 mice.

iii. To evaluate whether the treatment with the association of a lipid-lowering drug (simvastatin) with an AChEI drug (donepezil) would result in a better outcome in metabolic (bodyweight, plasma cholesterol, and total proteins levels), inflammatory (TNFα & IL-1β in the liver), enzymatic (AChE activity in the prefrontal cortex and hippocampal cells) and cognitive performance (short-term spatial memory) aspects in mice fed a HFD.

# 4. Methods

## 4.1. Primary Cell Culture

Hippocampal and cortico-hippocampal tissue from 18-day-old wild-type C57BL/6 mice embryos were homogenized in serum-free neurobasal medium supplemented with 2% B27 (Thermo Fisher Scientific). Cells were seeded separately in 12-wells dishes, pre-coated with 10% poly-D-lysine (Thermo Fisher Scientific) in sterile water. Cultures were incubated at 37 °C with 95% air and 5% CO2 for ten days. Experiments with primary cultures were conducted with approval from the regional ethical committee of Karolinska Institutet and by the Swedish Board of Agriculture (Ethical permit 4884-2019).

## 4.2 Animals

### 4.2.1. Cyp27Tg and wild-type C57BL/6 mice

Generation and breeding of the enzyme sterol 27-hydroxylase (CYP27A1) overexpressing mice (Cyp27Tg) have been described previously (Meir *et al*., 2002). CYP27A1 is responsible for converting cholesterol to 27-OH. Cyp27Tg mice have 5–6 times higher levels of 27-OH than control mice (WT) in serum (283 ± 11 vs. 48 ± 2 ng/mL) and in the brain (3.5 ± 0.5 vs. 0.3 ± 0.0 ng/mg) throughout life (Ismail *et al*., 2017). Age-matched (3-month-old) C57BL/6 male and female mice without the transgene (WT), obtained from the animal facility of the Karolinska Institutet, were used as controls. All mice had *ad libitum* access to normal rodent chow and water. The animals were housed in the animal facility in groups of 4–5 with 12 h light/dark cycle in Karolinska Institutet, Stockholm, Sweden. Experimental procedures involving animals were conducted following the European regulation and approved by the Swedish Board of Agriculture (Ethical permit 4884-2019). All efforts were made to minimize suffering or distress to experimental animals.

### 4.2.2 Swiss mice

3-month-old female Swiss mice obtained from the animal facility of the Federal University of Santa Catarina (UFSC, Florianópolis, Brazil) were used and kept in groups of ten mice per cage in a room under controlled temperature (23 ± 1 °C) and subjected to

a 12-h light cycle (lights on 07:00) with free access to food and water. All procedures used complied with the guidelines on animal care of the local Ethics Committee on the Use of Animals (CEUA, UFSC) (Protocol nº 1793080916), which follows the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health. All efforts were made to minimize suffering or distress to experimental animals.

## 4.3. Experimental Design

### 4.3.1. 1st set of experiments

The first set of experiments was designed to observe whether 27-OH would alter total cholesterol concentration, mRNA expression, protein density and catalytic activity of AChE in hippocampal and cortico-hippocampal neurons in culture. After ten days in vitro, cultures were treated with 27-OH (final concentration of 0.5 and 1 μM, Steraloids; Newport, Rhode Island, USA) or dimethyl sulfoxide (DMSO) (final concentration 1μM) for 6 hours. This concentration of 27-OH is similar to that found in the mammalian brain (Heverin *et al*., 2004).

### 4.3.2. 2nd set of experiments

The second set of experiments was designed to observe whether male and female CYP27A1 overexpressing mice (Cyp27Tg) had constitutive alterations in the mRNA expression and protein density of AChE in cortical and hippocampal cells in comparison to age-and-sex-matched wild-type C57BL/6 mice. For mRNA expression and immunoblotting analyses, the animals were euthanized by cervical dislocation; their brains were dissected and immediately frozen on dry ice and stored at –80 °C until further analysis. Different animals were used to perform mRNA expression and immunoblotting analysis.

### 4.3.3. 3rd set of experiments

The third set of experiments was designed to test the hypothesis that the association of a lipid-lowering drug (i.e., simvastatin) with an AChEI drug (i.e., donepezil) would

result in a better outcome in metabolic, inflammatory, enzymatic and cognitive parameters in HFD-fed mice. Thus, we have randomly divided female Swiss mice into two initial groups fed up for nine weeks with a standard diet (SD) (Nuvilab CR1, Nuvital; Quimtia Nutrientes, Colombo, PR, Brazil) (n = 13) or a high-fat diet (HFD) (12 kJ% protein, 27 kJ% carbohydrates, and 61 kJ% lipids; PragSoluções Biociências, Jaú, SP, Brazil) (n = 62). Experiments were performed in blocks of 20 animals per block with 2-3 animals per group per block. After 41 days of diet, mice underwent behavioral tests, such as: open field (41$^{st}$, 42$^{nd}$, and 43$^{rd}$ days), and object recognition task (44$^{th}$ day) (Fig. 11). On the 45$^{th}$ day, mice were randomly subdivided into four daily orogastric (o.g) treated groups: vehicle (isotonic saline solution 0.9% NaCl, SD n = 13 and HFD n = 16), simvastatin (1 mg/kg o.g., EMS®, Brazil, HFD n = 16), donepezil (3 mg/kg o.g., Eurofarma®, Brazil, HFD n = 14), and the association of simvastatin and donepezil (1 + 3 mg/kg o.g, respectively, HFD n = 16). Treatments were performed in the mornings for fifteen days. The animals' body weight was measured weekly using an electronic balance along with the protocol. One day after the last treatment day, mice underwent behavioral tests, such as open field (61$^{st}$ day) and object location test (62$^{nd}$ day) (Fig. 11). At the end of the protocol (63$^{rd}$ day), mice were food-deprived for six hours (from 08:00 to 14:00), anesthetized with xylazine/ketamine, and whole blood was collected via cardiac puncture, immediately centrifuged at 1000 x *g* for 10 min at 4ºC, and the plasma, liver, and hippocampus, were frozen on dry ice and stored at –80 °C until further biochemical analysis.

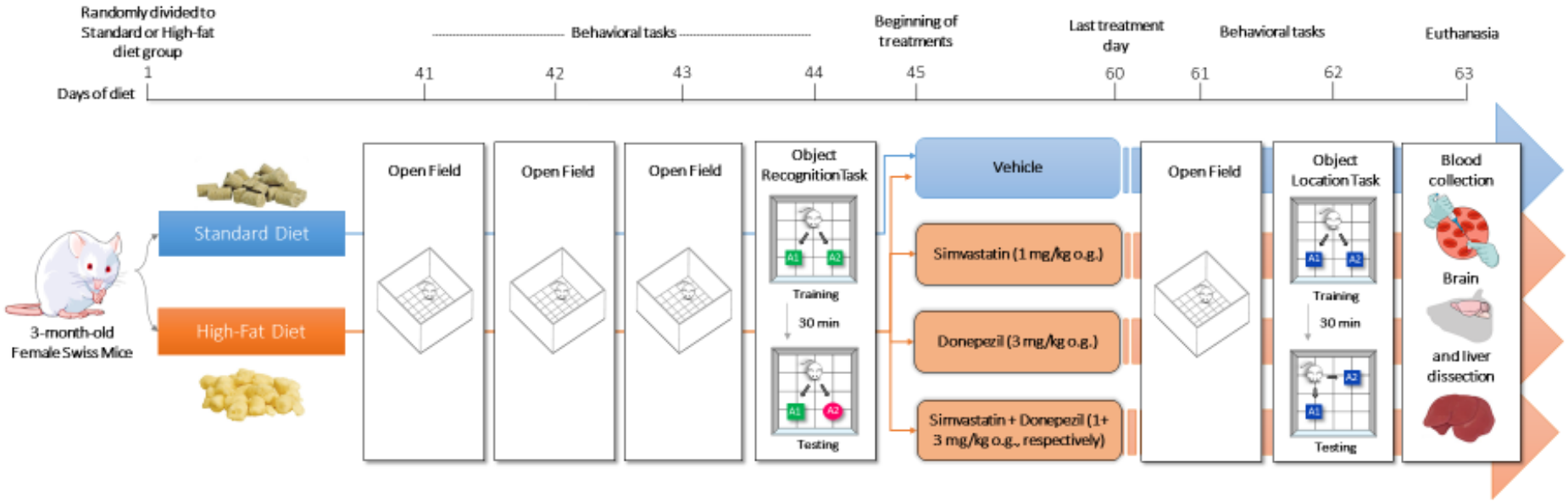


**Figura 11. Illustration of the experimental design of the third set of experiments.** 3-month-old female Swiss mice were randomly divided into either standard or high-fat died fed groups. After 41 days of diet, mice underwent behavioral tests, such as: open field (41$^{st}$, 42$^{nd}$, and 43$^{rd}$ days), and object recognition task (44$^{th}$ day). In the 45$^{th}$ day, mice were again randomly subdivided into four daily orogastric-treated groups: vehicle, Simvastatin (1 mg/kg), Donepezil (3 mg/kg), and the association of Simvastatin and Donepezil (1 + 3 mg/kg, respectively) for fifteen days. After it, mice underwent behavioral tests such as open field (61$^{st}$ day), and object location task (62$^{nd}$ day). At the end of the protocol (63$^{rd}$ day) mice were food-deprived for six hours, anesthetized and had their blood collected, and brain and liver dissected for further biochemical analysis.

## 4.4. Biochemical Analyzes

### 4.4.1. Total proteins levels

Total proteins levels from plasma samples were measured using the enzymatic kit according to the manufacturer's instructions (Química Básica Ltda, Minas Gerais, Brazil – K031).

### 4.4.2. Cholesterol Quantification

Total cholesterol from plasma samples was measured using the enzymatic kit according to the manufacturer's instructions (Química Básica Ltda, Minas Gerais, Brazil - K083). These results are expressed as mg/dL.

In order to quantify the amount of cholesterol present in the neurons after treatment with 27-OH, cells were washed with cold PBS to remove 27-OH. Then it was added 150uL of a working solution (Amplex® Red Cholesterol Assay Kit - A12216, Molecular Probes) and the wells were scratched with a cell scraper and the cells extracts were collected to an Eppendorf tube and stored frozen at -20 °C until further analysis. Cell extracts were then submitted to cholesterol quantification using the Amplex® Red Cholesterol Assay Kit (Molecular Probes), according to the manufacturer's instructions.

### 4.4.3. RNA Extraction and Real-Time Polymerase Chain Reaction (RT-PCR)

The expression levels of CYP27A1 and AChE genes were investigated by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was extracted using the RNeasy lipid tissue mini kit from Qiagen (Palo Alto, CA, USA) following the manufacturer's instructions. RNA was quantified by UV spectrophotometry in a NanoDrop 2000c UV-Vis Spectrophotometer (TermoFisher Scientific), and its purity was confirmed by a ratio value of OD260/OD280 $\geq$ 2. cDNA was synthesized by using the high-capacity cDNA reverse transcription kit (Applied Biosystems™) following manufacturer's instructions. Real-time PCR amplification assay for CYP27A1, AChE, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was performed with a total

volume of 20 µL in each well containing 10 µL of PCR Master Mix (Life Technologies, CA, USA), 2 µL of cDNA corresponding to 10 ng of RNA, 1 µL of each TaqMan® Gene Expression Primer and 7 µL of RNase-free water. To provide quantification, a threshold cycle (Ct) number was defined in the early logarithmic phase of the amplification plot, and the relative expression of gene transcripts was calculated by the Delta-Delta Ct (ΔΔCt) method and converted to the relative expression ratio ($2^{-\Delta\Delta Ct}$) for statistical analysis.

### 4.4.4. Western Blot

Immunoblotting was carried out in mice cortical and hippocampal tissue lysates and cortico-hippocampal cell culture neurons. Protein levels were quantified after separation by acrylamide gel electrophoresis (gradient 10%), transferred to a nitrocellulose membrane (Amersham™ Protran®, GE Healthcare), and blocked with 5% nonfat dry milk diluted in TBST for 1 h at room temperature. Membranes were incubated overnight at 4° C with the following primary antibodies: rabbit anti-AChE (1:1000, Abcam, UK), mouse anti-alpha-tubulin (1:1000; Sigma-Aldrich, USA), and mouse anti-GAPDH (1:1000; Enzo, USA). Incubations with secondary antibodies were done for 2 hours at room temperature with anti-rabbit or anti-mouse immunoglobulin G (IgG) at 1:10000 dilutions (LI-COR Biosciences GmbH, Germany). Immunoreactivity was detected with LI-COR® Odyssey® system (LI-COR Biosciences, USA) by infrared fluorescence and quantified with ImageJ 1.48v software (NIH, MA, USA) by densitometry analysis of the immunoreactive bands.

### 4.4.5. Determination of Acetylcholinesterase (AChE) activity

AChE activity was measured by the Ellman method (Ellman *et al.*, 1961). Neuronal culture and brain lysates were used to measure AChE activity using an AChE colorimetric assay kit (ab138871, Abcam) according to the manufacturer's protocol. The assay uses 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) to quantify thiocholine production by the hydrolysis of acetylthiocholine by AChE. Samples were plated onto a 96-well plate (50 µl/well). Immediately after plating the acetylcholinesterase standards, blank control, and samples onto a 96-well plate (50 µL/well), an acetylthiocholine reaction mixture (containing DTNB and acetylthiocholine; 50 µL/well) was added to each well of the AChE

standard, blank control, and test samples. After 10 min incubation, protected from light at room temperature, the AChE activity was determined by measuring the change in the absorbance at 410 nm using a microplate reader. The data from the samples were normalized using the AChE standard curve according to the manufacturer's protocol provided with the kit.

### 4.4.6. Enzyme-linked Immunosorbent Assay (ELISA)

The liver was removed and stored at - 80°C until preparation for the assay. It was later homogenized with PBS buffer containing 0.05% Tween 20, 0,4M NaCl, 0.1mM benzethonium chloride, 10mM EDTA, 0,5% BSA, 20µL aprotinin and 50µL phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 6900×g for 10 min at 4ºC, and the supernatants were stored at - 80 °C until analyzes were carried out. The levels of tumor necrosis α (TNF-α) and interleukin 1β (IL-1β) were evaluated using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's recommendations (R&D Systems, Minneapolis, MN, USA) and the results are expressed in % relative to control. Protein content was evaluated by the method of Bradford (1976). Bovine serum albumin (Sigma) was used as the standard.

## 4.5. Behavioral tasks

The experiments were conducted during the light phase (08:00 – 12:00) in a controlled-temperature room (23 °C, the humidity was between 40% - 60%), with low-light intensity (12 lx). All apparatus and objects used were cleaned with 10% (v/v) ethanol between test sessions to avoid olfactory cues and sanitized with 70% ethanol (v/v) at the end of the day.

### 4.5.1. Open field

The open field was used to evaluate the locomotor and exploratory activities induced by a new environment (de Souza *et al*., 2021). The animal's natural tendency in a new environment is to explore it, despite the stress and conflict

caused by the new environment (Prut & Belzung, 2003). The apparatus, made of wood and covered with impermeable Formica, had 50 cm wide × 50 cm deep × 40 cm high. Black lines were drawn on the floor to divide it into 16 equal quadrants. Each mouse was placed in the center of the open field and allowed to freely explore the apparatus for 5 min. The total number of crossings was evaluated.

### 4.5.2. Object Recognition Test

The short-term recognition memory was assessed using the object recognition test (ORT). This test is based in the innate preference for novelty, if the mouse recognizes the familiar object, it will spend most of its time exploring the novel object (Lueptow, 2017). Hence, the protocol consisted of two stages: training and test, separated by an inter-trial interval of 30 min between the exposures. At the training phase, mice were put into the open field arena and presented to two identical objects and the time exploring each object was recorded for 5 min. At the testing phase mice were re-exposed to the apparatus but now one of the previous objects was changed to a brand new object. The time spent by the animals exploring the familiar and new objects was recorded over 5 min. Exploring the objects was recorded using a stopwatch when mice sniffed or touched the object while looking at it (i.e., when the distance between the nose and the object was less than 1 cm). A recognition index was calculated as follows: $(T_{new} \times 100)/(T_{new} + T_{familiar})$, where $T_{new}$ is the time spent exploring the brand new object and $T_{familiar}$ is the time spent exploring the familiar object.

### 4.5.3. Object Location Task

The spatial memory of mice was assessed using the object location task (Moreira *et al*. 2013). The task is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects — replaced in a novel location — for a longer time than they explore the non-displaced object (Assini *et al*. 2009). Therefore, mice were reexposed to the open field apparatus for 5 min; however, in this presentation (training), two identical objects were presented to the animals (these objects were different from those used in the ORT). The objects were placed 7 cm away from the

walls of the open field. At this time, the exploration time of each of the objects placed in the box was recorded. After the training phase, the mice were removed from the apparatus for 30 min. After this inter-trial interval, one object was moved to a new location. The time spent by the animals exploring the objects in the new (novel) and the old (familiar) locations was recorded over 5 min. All locations of the objects were counterbalanced among the groups. Exploring the objects was recorded using a stopwatch when mice sniffed or touched the object while looking at it (i.e., when the distance between the nose and the object was less than 1 cm). A location index was calculated as previously described (Assini *et al*. 2009): $(T_{novel} \times 100)/(T_{novel} + T_{familiar})$, where $T_{novel}$ is the time spent exploring the displaced object and $T_{familiar}$ is the time spent exploring the non-displaced object.

### 4.6. Statistical analysis

Data are expressed as mean + standard error of the mean (SEM), with "n" indicating the number of animals. When comparing two groups, the Student *t*-test was used*;* the object recognition and object location tasks ware analyzed by one-sample *t*-test to determine whether the recognition and location index, respectively, differed from the chance performance of 50%. The rest of the statistical analyses were carried out using a one-way analysis of variance (ANOVA). Following significant interaction effects, *post-hoc* tests were performed (Dunnett or Newman Keul's test). Tests were performed using the STATISTICA® software package (StatSoft Inc, Tulsa, OK, USA). Only significant effects of ANOVAs $p \leq 0.05$ were considered an index of significance (F and P values + interaction are shown). All graphs were created using the GraphPad Prism 8.0.2 software (San Diego, CA, USA).

## 5. Results

### 5.1 Treatment with 27-OH increases total cholesterol concentration in cortico-hippocampal neurons

We first observed an increase in the total cholesterol concentration in cortico-hippocampal neurons treated for 6 hours with 27-OH (0.5 and 1μM) in comparison with the DMSO (1 μM) treated group as shown in Fig. 12A [one-way ANOVA indicated a significant effect for the treatment factor, $F_{(2,19)} = 6.453$, $p \leq 0.05$]. Subsequent *post-hoc* comparisons indicated that both groups treated with 27-OH presented an increased total cholesterol concentration ($p \leq 0.05$).

### 5.2 Treatment with 27-OH increases AChE's protein density in hippocampal neurons and its catalytic activity in cortico-hippocampal neurons

The treatment with 27-OH for 6 hours did not alter AChE's copy of mRNA [one-way ANOVA, $F_{(2,9)} = 0.03196$, $p = 0.968$] in cortico-hippocampal neurons (Fig. 12B). Regarding protein density, although we observe a 50% increase in the concentration of AChE in cortico-hippocampal neurons treated with 0.5μM of 27-OH, this difference did not, however, reach significance [one-way ANOVA $F_{(2,9)} = 3.474$, $p = 0.076$] (Fig. 12C). Nevertheless, in hippocampal neurons, one-way ANOVA indicated a significant effect for the treatment factor [$F_{(2,14)} = 9.119$, $p = 0.002$] and Dunnett's *post-hoc* test showed an increase in the AChE protein density in the group treated with 0.5uM of 27-OH ($p = 0.001$) (Fig. 12D). Additionally, regarding AChE catalytic activity, one-way ANOVA indicated a significant effect for the treatment factor [$F_{(2,79)} = 6.609$, $p = 0.002$)] in cortico-hippocampal neurons. Subsequent Dunnett's *post-hoc* test revealed a higher AChE activity in the 27-OH-treated groups in comparison to the DMSO-treated group (*$p \leq 0.05$ and **$p \leq 0.01$) (Fig. 12E).

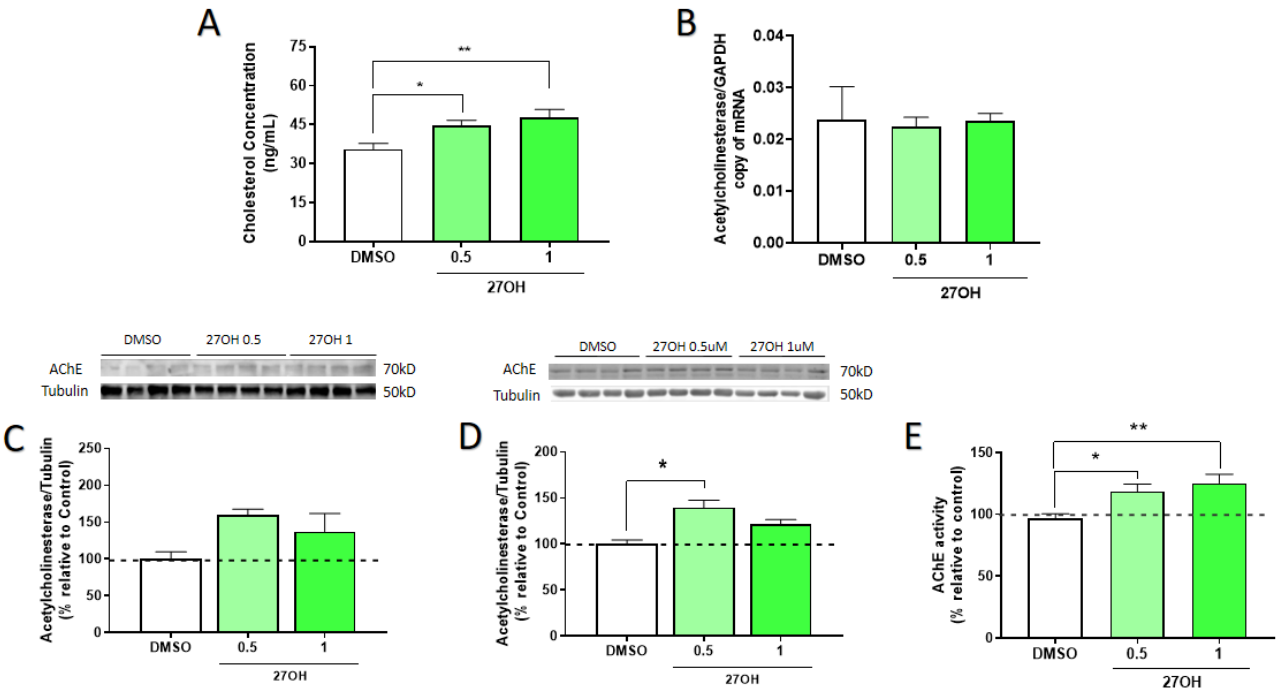


**Figure 12. Effects of treatment with 27-OH (0.5 and 1µM) for 6 hours in hippocampal (D) and cortico-hippocampal neurons (A – C, E).** Total cholesterol concentration (A), AChE copy of mRNA (B), AChE protein density (C and D) and AChE catalytic activity (E). Data are expressed as mean + SEM. *$p \leq 0.05$; ** $p \leq 0.01$ compared to DMSO group (One-Way ANOVA fallowed by Dunnett's *post-hoc* test).

## 5.3 CYP27A1 overexpressing mice have constitutive alterations in gene expression of AChE in the brain

In order to have one more indication that 27-OH modulates AChE's homeostasis within the brain, we used transgenic male and female mice that overexpress the CYP27A1 gene, which is responsible for converting cholesterol to 27-OH, and compared them to sex-and-age-matched wild-type C57BL/6 mice. Hence, as a control experiment, we firstly analyzed the gene expression concentration of CYP27A1 in male and female Cyp27Tg mice. As expected, we observed a great difference in CYP27A1 gene expression between Cyp27Tg and wild-type mice in cortical (Male: $t = 13.07$, $df = 8$, $p < 0.0001$ and Female: $t = 20.9$, $df = 8$, $p < 0.0001$) (Fig. 13A) and hippocampal cells (Male: $t = 8.839$, $df = 8$, $p < 0.0001$ and Female: $t = 4.42$, $df = 8$, $p < 0.0022$) (Fig. 13E).

Subsequently, we investigated the AChE gene expression in male and female Cyp27Tg mice. Although we see no differences in the AChE gene expression in cortical cells of male mice ($t = 0.8806$, $df = 8$, $p = 0.4042$), we observed a tendency of increase in the cortical cells of female Cyp27Tg mice ($t = 2.226$, $df = 8$, $p = 0.056$) (Fig. 13B). Furthermore, we observed an increase in AChE's gene expression in the hippocampal cells of the male ($t = 3.187$, $df = 7$, $p = 0.0153$) and a decrease in the female Cyp27Tg mice ($t = 2.879$, $df = 8$, $p = 0.0205$) in comparison to same-sex wild-type C57BL/6 groups (Fig. 13F). Afterward, we performed a western blot analyses in order to investigate AChE protein density in the cortical and hippocampal cell lysates. Regarding the cortical analyses, although we observe no differences in the protein density of AChE in the cortical cells of the male CYP27Tg mice in comparison to same-sex wild-type C57BL/6 mice ($t = 0.9464$, $df = 7$, $p = 0.375$) (Fig. 13C), we observed a decrease in the protein density of AChE in the cortical cells of the female CYP27Tg mice in comparison to to same-sex wild-type C57BL/6 group ($t = 3.771$, $df = 7$, $p = 0.007$) (Fig. 13D). When we looked at the hippocampal cell lysates we observed a tendency to an increase in the protein density of AChE in the hippocampal cells of Cyp27Tg male mice ($t = 2.253$, $df = 6$, $p = 0.065$), and no differences in hippocampal cells of the female mice ($t = 0.3909$, $df = 8$, $p = 0.706$) in comparison to their respective control group (Fig. 13G and 13H, respectively).

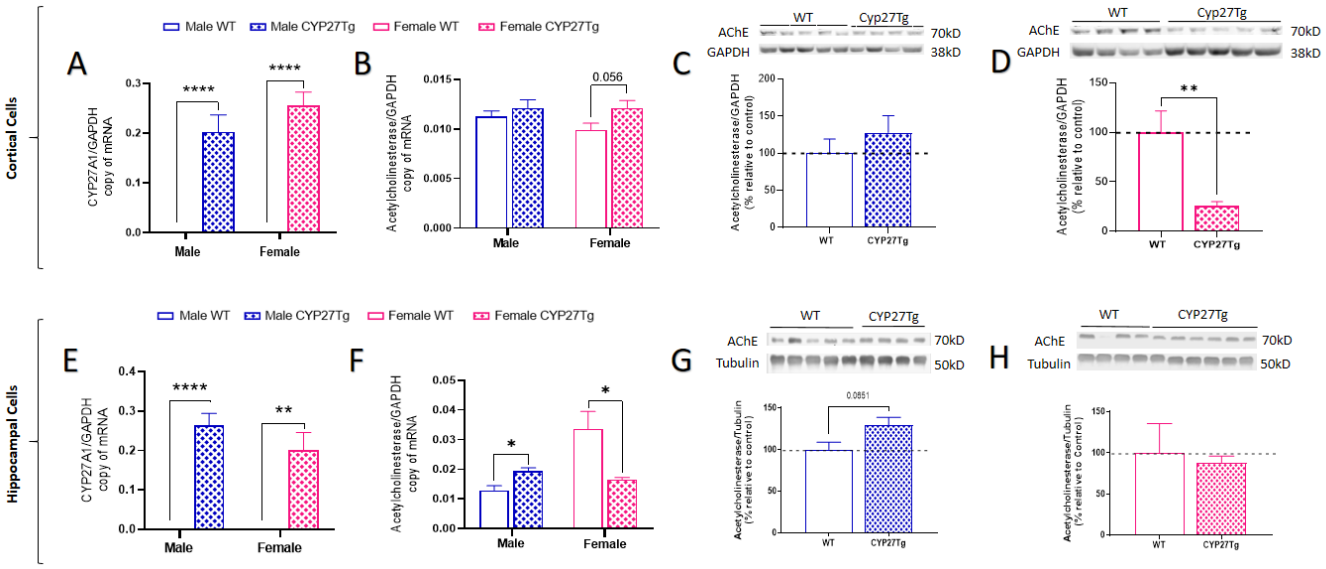


**Figura 13. Effects of 27-OH on gene expression and protein density of acetylcholinesterase (AChE) in CYP27A1 overexpressing mice.** Results in cortical cells: CYP27A1 copy of mRNA (A), AChE copy of mRNA (B), protein density of AChE in male (C) and female (D) mice. Results in hippocampal cells: CYP27A1 copy of mRNA (E), AChE copy of mRNA (F), protein density of AChE in male (G) and female (H) mice. Data are expressed as mean + SEM (n = 4 to 6 animals per group). *$p \leq 0.05$; **$p \leq 0.01$; ****$p < 0.0001$ (unpaired student $t$ test). WT (wild-type); Cyp27Tg (CYP27A1 overexpressing mice).

## 5.4. Short-term recognition memory is impaired in mice fed a HFD

Regarding to the 3rd set of experiments, we first analyzed the locomotor function of the mice. Hence, we observed that there were no differences on the total number of crossings between the groups in the open field test (Fig. 14A). Subsequently, we evaluated the short-term recognition memory of mice using an object recognition task. As we can see in the training phase, both groups explored the two identical objects similarly (around 50% of the exploration time) (Fig. 14B). Nonetheless, in the test section, we observe that only the animals fed a standard diet presented an increase in the recognition index ($t = 7.532$ $df = 12$, $p \leq 0.05$) in the ORT (Fig. 14C).

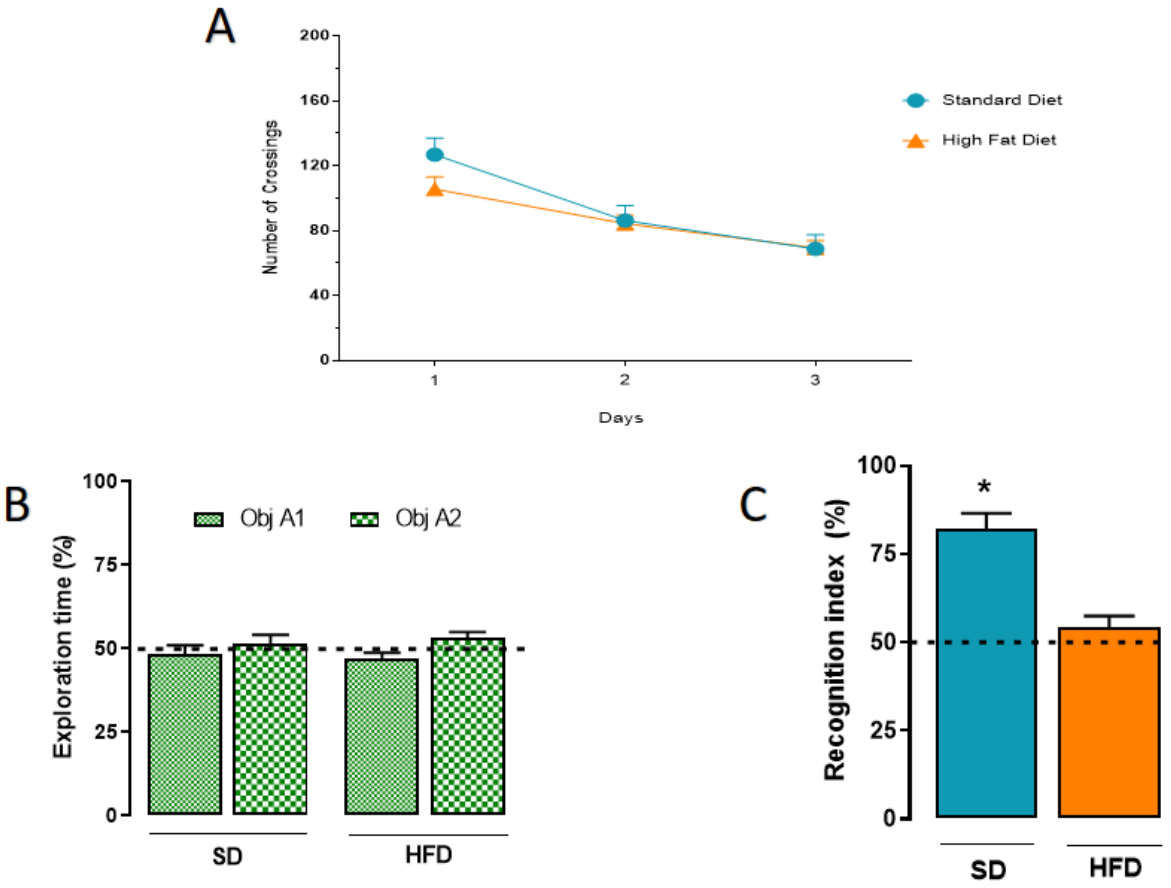


**Figure 14. Short-term recognition memory is impaired in mice fed a HFD.** Total number of crossings in open field test (A). Training (B), and test phase (C) of object recognition task. Data are expressed as mean + SEM (SD n = 13, and HFD n= 62 animals). *p < 0.05 versus 50 % chance level. SD (Standard Diet); HFD (High Fat Diet); OBJ (Object).

## 5.5 High-fat diet increases body weight, plasma cholesterol, and plasma protein levels

Regarding the metabolic analysis, bodyweight increased among groups throughout the study. However, the bodyweight of high-fat-fed mice increased at a greater rate than in standard diet-fed mice (Fig. 15A). The one-way ANOVA with repeated measures indicated significant main effects for diet [$F = 45.214$, $p < 0.05$], and subsequent *post-hoc* comparisons revealed a significant increase in bodyweight of the HFD-fed mice in comparison to the SD-fed mice starting from the second week of diet ($p \leq 0.05$).

Regarding plasma cholesterol levels, one-way ANOVA indicated a significant effect for the treatment factor [$F_{(4,70)}$ = 4.401, $p$ < 0.05], and subsequent *post-hoc* comparisons revealed a significant increase in plasma cholesterol levels of vehicle- and donepezil-treated HFD-fed groups in comparison to the standard diet-fed group ($p \leq 0.05$) which was prevented by the treatment with simvastatin ($p \leq 0.05$) (Fig. 15B). Moreover, regarding the total protein levels, one-way ANOVA indicated a significant effect for the treatment factor [$F_{(4, 70)} = 3.956, p \leq 0.01$], and subsequent *post-hoc* comparisons revealed a significant increase in the total protein levels of the groups fed a HFD in comparison to the standard diet-fed group ($p \leq 0.05$) (Fig. 15C).

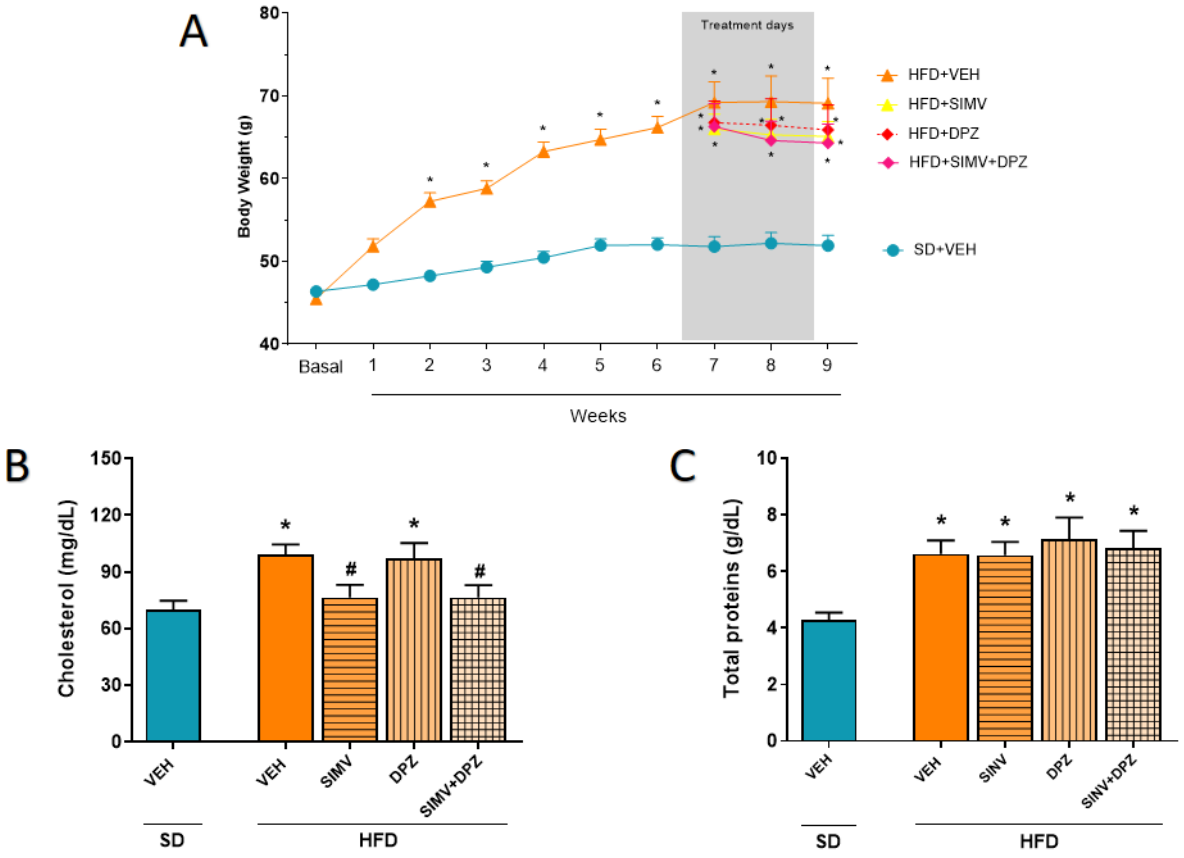


**Figure 15. Effects of high fat diet intake and subsequent treatment with simvastatin and/or donepezil in metabolic parameters.** Female Swiss mice were fed either a standard diet (SD) or high-fat diet (HFD) for 9 weeks. At 45$^{th}$ day, mice were randomly subdivided into four daily orogastric-treated groups: vehicle (VEH), simvastatin (SIMV), donepezil (DPZ) or the association of simvastatin and donepezil for 15 days. (A) Body

weight was measured every week. (B) Plasma cholesterol levels. (C) Total protein levels. Data are expressed as mean + SEM (n = 13 to 16 animals per group). *p ≤ 0.05 (vs. SD+VEH); #p ≤ 0.05 (vs. HFD+VEH) One-way ANOVA, with repeated measures when appropriate, followed by Newman–Keuls *post-hoc* test).

## 5.6 Effects of HFD intake and treatment with simvastatin and donepezil in pro-inflammatory markers in liver HFD

Moreover, to further investigate the effects of HFD intake and subsequent treatment with simvastatin and/or donepezil in pro-inflammatory markers, we measured the levels of TNFα and IL-1β in the liver. No significant differences were observed in the TNFα ($F_{(4,38)}$ = 0.8097, $p$ = 0.526) concentrations (Fig. 16A). Regarding IL-1β, despite observing more than 100% increase in the concentration of this cytokine in the vehicle-treated HFD-fed mice in comparison to the SD-treated group and an apparent prevention with the concomitant treatment of simvastatin and donepezil, these differences, however, almost reach significance ($F_{(4,38)}$ = 2.384, $p$ = 0.068) (Fig. 16B).

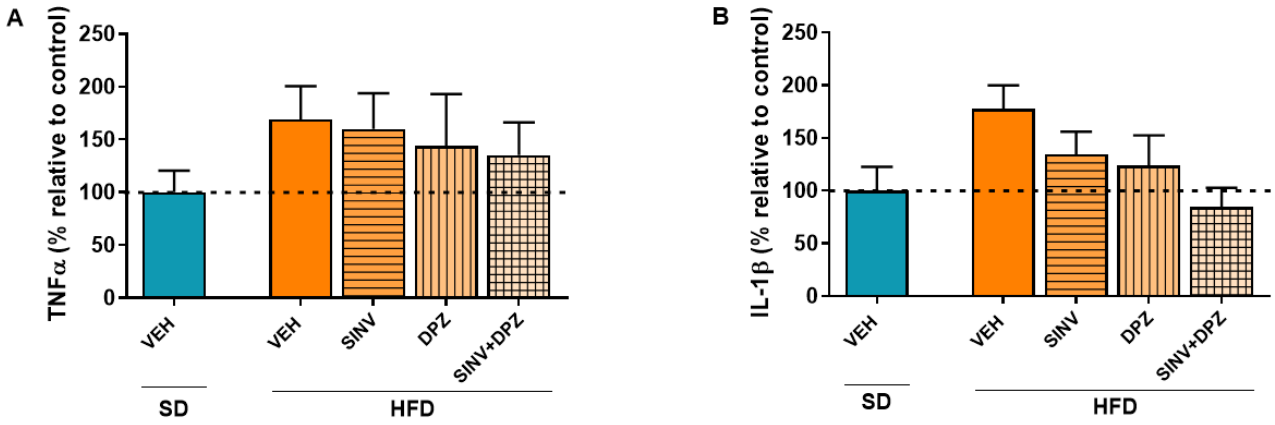


**Figure 16. Effects of high fat diet intake and subsequent treatment with simvastatin and/or donepezil in pro-inflammatory markers in the liver.** Data are expressed as mean + SEM (n = 6 to 11 samples per group). (A) TNFα and (B) IL-1β concentrations. VEH (vehicle); SIMV (simvastatin); DPZ (donepezil).

5.7 Concomitant treatment with simvastatin and donepezil reversed the increased AChE activity in hippocampus of HFD-fed mice

Regarding the AChE activity in the hippocampus, the one-way ANOVA indicated a significant main effect of treatment [$F_{(4,62)} = 3.432$, $p \leq 0.05$], and subsequent *post-hoc* comparisons revealed a lower AChE activity in the HFD-fed mice treated with the association of simvastatin and donepezil compared with the vehicle-treated HFD-fed group ($p \leq 0.05$) (Fig. 17A). No differences were seen in the AChE activity in the prefrontal cortical cells of the evaluated groups (Fig. 17B).

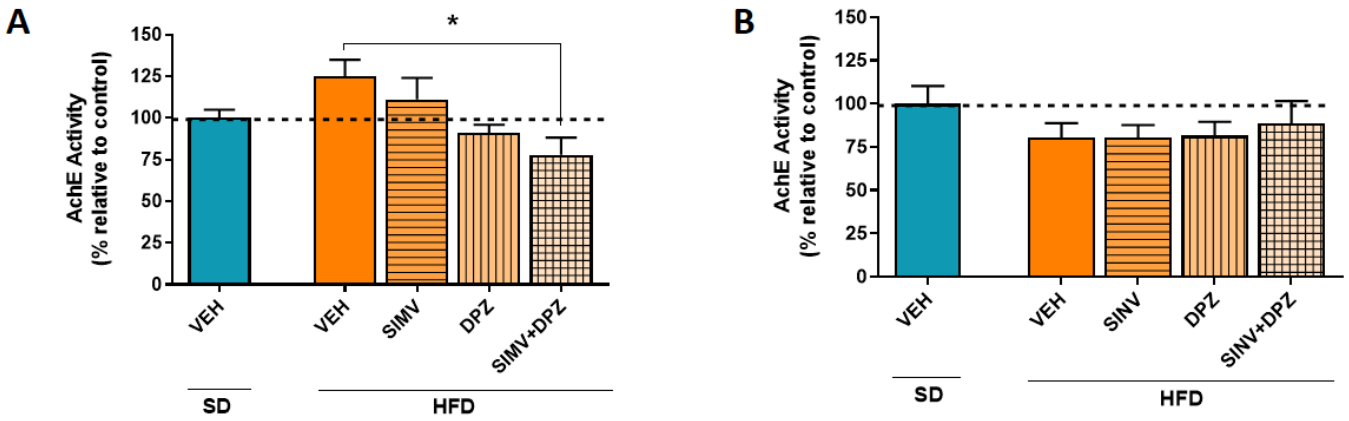


**Figure 17. Effects of the association of simvastatin and donepezil on acetylcholinesterase (AChE) activity in hippocampal (A) and prefrontal cortical (B) cells of mice fed a standard or high-fat diet.** Data are expressed as mean + SEM (n = 10 to 16 samples per group). *p ≤ 0.05 (One-Way ANOVA followed by Newman–Keuls *post-hoc* test). VEH (vehicle); SIMV (simvastatin); DPZ (donepezil).

5.8 HFD intake impairs spatial cognitive performance that is only reversed by concomitant treatment with simvastatin and donepezil

Once again, as a control experiment, we first analyzed whether the HFD intake and subsequent treatment with simvastatin and/or donepezil would affect mice's spontaneous locomotor activity. Hence, we ran an open field test and measured the total number of crossings within five minutes. As shown in Fig. 18A, no significant differences

in locomotor activity were visualized among groups. Subsequently, we evaluated the short-term spatial memory of mice using an object location task. As observed in the training phase, all experimental groups similarly explored the two identical objects (around 50% of the exploration time) (Fig. 18B). However, in the test section, we observed an impairment of the short-term spatial memory observed by the percentage of exploration index in the HFD-fed groups, and this impairment was only reversed in the HFD-fed group concomitantly treated with the association of simvastatin and donepezil ($t = 2.142$, $df = 15$, $p < 0.05$) (Fig. 18C).

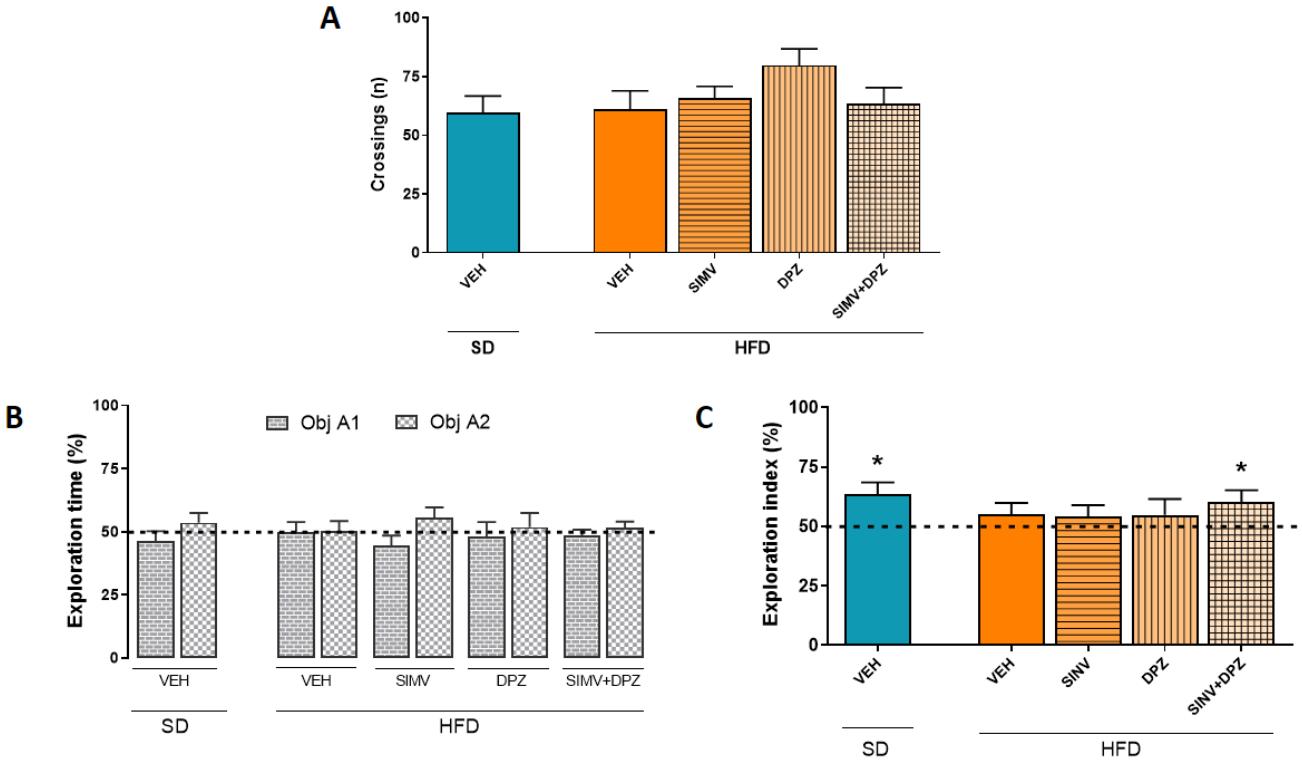


**Figure 18. Effects of the association of simvastatin and donepezil on locomotor and cognitive behaviors in standard and high fat diet fed mice.** Total number of crossings in open field test (A). Training (B), and test phase (C) in the object location task. Data are expressed as mean + SEM (n = 13 to 16 animals per group). *p < 0.05 versus chance level (50 % of displaced object investigation in test trial). VEH (vehicle); SIMV (simvastatin); DPZ (donepezil); OBJ (object).

# 6 Discussion

In the recent decades, prospective epidemiological studies have greatly investigated the relationship between cholesterol and AD (Anstey *et al*., 2008). Several studies point out a relationship between increased risk of developing AD and high cholesterol levels in plasma (Kivipelto *et al*., 2005; Solomon *et al*., 2007; Anstey *et al*., 2008; Solomon *et al*., 2009; Reed *et al*., 2014). Additionally, several reports have looked at patients with altered cholesterol metabolism in the brain and their susceptibility to developing AD (Cedazo-Minguez & Cowburn, 2001; Kivipelto *et al*., 2002; Kivipelto *et al*., 2005; Cedazo-Minguez *et al*., 2011; Orth & Bellosta, 2012). In this regard, in the last decades, BBB-permeable cholesterol metabolites (collectively known as oxysterols) have been identified as possible mediators of cholesterol effects in the brain (Lütjohann *et al*., 1996; Lütjohann *et al*., 2001; Meaney *et al*., 2001; Björkhem, 2006; Björkhem *et al*., 2009; Loera-Valencia *et al*., 2019b). Nevertheless, as far as we know, no one has ever shed light on the relationship between 27-OH and the main enzyme responsible for terminating the cholinergic transmission in the brain, AChE. Moreover, it has long been known that the cholinergic system is critically important for memory, learning, attention, and other higher brain functions (De Bem *et al*., 2021), and it is dramatically affected in AD (Ferreira-Vieira *et al.*, 2016). Therefore, in this study, we aimed to investigate the involvement of 27-OH on the mRNA expression, protein density, and catalytic activity of the AChE enzyme in hippocampal and cortico-hippocampal neurons in culture as well as in cortical and hippocampal cells of CYP27A1 overexpressing mice. We also tested the hypothesis that the association of simvastatin with donepezil would result in a better outcome in several parameters in mice fed a HFD.

In the present study, we first observed an increase in the total cholesterol concentration in cortico-hippocampal neurons treated with 27-OH. Despite not seeing differences in AChE's gene expression, we could observe an increase in its protein density in hippocampal neurons. Later, we observed a concentration-dependent increase in the catalytic activity of AChE in the 27-OH-treated cortico-hippocampal neurons. Furthermore, we also observed that CYP27Tg mice presented constitutive alterations in the brain's AChE homeostasis, which include an increase in the gene expression of AChE

in the hippocampal cells of the male and a decrease in the hippocampal cells of the female CYP27Tg mice in comparison to sex-and-age-matched control groups. We also observed a tendency of an increase in the mRNA expression of the AChE enzyme in the cortical cells of the female CYP27Tg mice, as well as a tendency of increase in AChE's protein density in the hippocampal cells of the male Cyp27Tg mice. Moreover, we observed that female CYP27Tg mice showed a decrease in the protein density of AChE in the cortical cells in comparison to the C57BL/6 female mice. These results are consistent with our hypothesis that 27-OH modulates AChE's homeostasis in the brain. In addition, it is important to mention that we have not performed behavioral tests in the CYP27Tg mice because cognitive deficits have already been explored in this model (Ismail *et al*., 2017). Interestingly, a great body of scientific evidence has already looked at some negative effects of 27-OH over different aspects, such as reduction of Arc levels, as well as NMDAR and Src activities in the hippocampus (Mateos *et al*., 2009; Ismail *et al*., 2017), reduction of glucose uptake by the brain (Ismail *et al*., 2017), decrease in key synaptic protein levels (Ismail *et al*., 2017) and dendritic spine density and dendritic arborization (Merino-Serrais *et al*., 2019), it also alters the renin-angiotensin system in the brain (Mateos *et al*., 2011b), promotes neuroinflammation (Testa *et al*., 2014) and memory deficits in mice (Heverin *et al*., 2015). Notably, high levels of 27-OH have been found in the brains and CSF of early-onset and sporadic AD patients (Heverin *et al*., 2004; Björkhem *et al*. 2006; Shafaati *et al*., 2011), and it was recently shown that high levels of this oxysterol are associated with mild cognitive impairment in the elderly (Liu *et al*., 2016). To our knowledge, this is the first study that directly relates high levels of 27-OH to alterations in mRNA expression, protein density, and catalytic activity of AChE in the brain.

In parallel with our findings, it has been reported that increased cholesterol in neurons exacerbates the production of Aβ throughout an increased β- and γ- secretase activity (Kalvodova *et al*., 2005; Laferla *et al*., 2007; Sun *et al*., 2015), and reduced α-secretase activity (Bodovitz & Klein, 1996; Fonseca *et al*., 2010). Moreover, it has also been shown that cell cultures treated with Aβ enhance AChE expression and that only Aβ peptides that aggregate to form fibrils rather than amorphous amyloid increase AChE levels (Sberna *et al*., 1997). Moreover, *in vivo* studies have reported that $LDLr^{-/-}$ mice

treated with $A\beta_{(1-40)}$ peptide presented increased AChE activity, astrogliosis, oxidative imbalance, and cell permeability within the hippocampus in comparison with $A\beta_{(1-40)}$-treated C57BL/6 wild-type mice (de Oliveira *et al*., 2014). Additionally, studies on brains displaying AD lesions have shown changes in the expression and distribution of AChE forms (Kasa *et al,* 1997; Talesa, 2001). Within the brain, AChE occurs as a globular $G_4$ tetramer (Walczak-Nowicka & Herbet, 2021), however, as AD progresses, there is a reduction or loss of the $G_4$ form in favor of the $G_1$ monomer in the cortex and CSF (Atack *et al.,* 1983; Fishman *et al.,* 1986; Younkin *et al.* 1986; Walczak-Nowicka & Herbet, 2021) and it was observed that the increase in $G_1$ form correlates with the density of amyloid deposits in the cerebral cortex (Schegg *et al*., 1992; Jean *et al*., 2019). Moreover, in agreement with human studies, AChE monomeric species are also increased in brain of the APPC100 and Tg2576 transgenic mice which overproduce human Aβ (Sberna *et al.*, 1998; Fodero *et al.*, 2002). Moreover, it has been shown that AChE can enhance Aβ aggregation and amyloid fibril formation, and, in fact, when AChE is infused stereotaxically into the CA1 region of the rat hippocampus, novel plaque-like structures are formed (Chacón *et al.,* 2003). These amyloid plaques formed share features with the lesions found in AD brains, including amyloid deposits reactive to anti-Aβ antibodies (Chacón *et al.,* 2003). In fact, in AD brains, AChE is predominantly associated with the amyloid core of mature senile plaques and pre-amyloid diffuse deposits (Geula & Mesulam, 1989; Ulrich *et al.,* 1990; Kalaria *et al..,* 1992; Geula & Mesulam, 1994), and those brain areas where senile plaques are present are strongly AChE positive (Ulrich *et al.,* 1990). Interestingly, when AChE became associated with the amyloid fibrils, the enzyme changed some of its characteristics, including resistance to low pH, high substrate concentrations, and lower sensitivity to AChEIs (Alvarez *et al*., 1998), under these conditions the AChE-Aβ complexes are more toxic than the Aβ fibrils alone (Inestrosa *et al*., 2005).

Neuropathological changes generated by human Aβ fibrils and AChE-Aβ complexes have also been compared in rat hippocampus *in vivo (*Inestrosa & Reyes, 1998; Reyes *et al.,* 2004; Inestrosa *et al*., 2005*).* Results showed that AChE-Aβ complexes trigger a more dramatic response *in situ* than Aβ fibrils alone as characterized by the following features observed 8 weeks after treatment: (1) amyloid deposits were larger

than those produced in the absence of AChE; (2) an extensive astrocytosis revealed by both GFAP immunoreactivity and number counting of reactive hypertrophic astrocytes and; (3) a stronger neuronal cell loss in comparison with only Aβ injected animals (Inestrosa & Reyes, 1998; Reyes *et al.,* 2004; Inestrosa *et al*., 2005). These results are consistent with the notion that AChE-Aβ complexes are more toxic than Aβ fibrils and that AChE triggered some of the neurodegenerative changes observed in AD brains (Reyes *et al,* 2004; Inestrosa *et al*., 2005). Moreover, studies have also reported that the toxicity of the AChE-Aβ complex is dependent on AChE concentration in these complexes (Muñoz & Inestrosa, 1999; Muñoz *et al,* 2002; Chacón *et al.,* 2003). In line with this evidence, Rees *et al*., (2003) developed a double transgenic mice overexpressing both the human APP containing the Swedish mutation and the human AChE and observed that these double AChE-APP transgenic mice started to form amyloid plaques around three months earlier than mice expressing only the APP transgene as well as to present more plaques and bigger ones than control. Remarkably, Silveyra and colleagues (2012a) extended the knowledge in this regard by observing that genetic modulation of AChE expression can influence the levels of PS1. In this study, among other things, they observed that AChE knockdown with siRNA end up decreasing PS1 protein density, while AChE overexpression exerted opposing effect; moreover, AChE-deficient mice also had decreased PS1;.and finally, mice administered with tacrine or donepezil displayed lower levels of brain PS1 (Silveyra *et al.,* 2012a). Nonetheless, the authors report that the mechanisms employed by AChE to influence PS1 processing remain unknown (Silveyra *et al*., 2012a).

Interestingly, Silveyra and co-workers published another paper in that same year (2012b), where they showed for the first time that overexpression of mutant hyperphosphorylated tau (P-tau) led to an increase in the gene expression, protein density and catalytic activity of AChE in the brain of Tg-VLW mice, without affecting the same parameters of ChAT. Moreover, they also observed a colocalization of the P-tau with the AChE in the brain of these mice (Silveyra *et al*., 2012b) And, finally, by transfecting mutant tau to SH-SY5Y lineage, they observed that the enzymatic activity of AChE also increased, adding, then, one more evidence in the close relation to mutant tau and this enzyme (Silveyra *et al*., 2012b).

Furthermore - and very interestingly - a study published two decades ago in the *Cell Death and Differentiation journal*, revealed that AChEs is involved in apoptotic processes in various cell types, including cells that do not originate from the nervous or hematopoietic systems (Zhang *et al*., 2002). To date, more than 40 different types of cells from primary cultures or cell lines have shown increased AChE expression or activity during apoptosis events after apoptotic stimuli *in vitro* and *in vivo* (to see all the cell lines in which AChE expression or activity were increased by apoptotic stimuli with various apoptotic inducers, please see Zhang & Greenberg 2012). It has been demonstrated that AChE participates in the formation of apoptosome by influencing the interaction between apoptotic protease activating factor-1 (Apaf-1) and cytochrome-c, which consequently activate procaspase-9 (Park *et al*., 2004; Park *et al*., 2008; Lazarević-Pašti *et al*., 2017). Generally, mitochondrial apoptotic signaling leads to the release of cytochrome-c that binds to Apaf-1 and procaspase-9 making an apoptosome formation (Lazarević-Pašti *et al*., 2017). The apoptosome activates caspase-9 and proceeds down the same common pathway as the extrinsic pathway, which leads to activation of executioner caspases (caspase-3) and thus triggers apoptosis (Lazarević-Pašti *et al*., 2017). Park and colleagues (2004, 2008) tried to elucidate the molecular role of AChE in apoptosome formation. They have shown that AChE plays a pivotal role in the oligomerization of Apaf-1 as well as that the interaction between AChE and caveolin-1 and subsequently cytochrome-c is indispensable for the interaction between cytochrome-c and Apaf-1 and apoptosome formation (Park *et al*., 2004; Park *et al*., 2008; Lazarević-Pašti *et al*., 2017). Indeed, cells in which AChE is overexpressed undergo apoptosis more easily than controls (Zhang & Greenberg 2012; Walczak-Nowicka & Herbet 2021). Moreover, some tumor cells do not express AChE, but when AChE is introduced into a tumor cell, the cells cease to proliferate and undergo apoptosis more readily (Zhang & Greenberg 2012). In addition, it has been observed that that apoptosis is attenuated by knockdown of its expression either by antisense RNA, small interfering RNA, or by heterozygous deletion of the AChE gene (Zhang et a., 2002; Jing *et al*., 2008; Ye *et al*., 2010; Pegan *et al*., 2010; Du *et al*., 2015). Moreover, it was later tested whether AChEs derived from other species also possessed DNA cleavage activity, and, in this sense, Du and his colleagues (2015) took a mouse AChEs (mAChE-T548) and similarly to human AChEs (hAChE-T547), mAChE-

T548 cleaved both plasmid and naked genomic DNA efficiently, demonstrating that mAChE also possesses DNAse activity. In the same paper they investigated whether the cholinesterase active center of AChEs also contributed to its DNase activity by using AChEIs (10 μM huperzine, 1 μM tacrine and 13 μM donepezil). The AChE activity of mAChE-T548 was markedly inhibited by AChEIs, but its DNA cleavage activity was not (Du *et al*., 2015). These data suggested that the functional domain responsible for the DNase activity of AChES is distinct from that for its cholinesterase activity (Du *et al*., 2015). To further confirm this suggestion, AChEs residues S234, E365 and H478 of the catalytic triad contributing to its cholinesterase activity were all mutated to alanine (A) to generate pEGFP–NLS–AChES (S234A, E365A, H478A), and they observed that this mutant AChEs (mtAChES), as expected, presented a completely abolished catalytic activity; however it still stimulated cell apoptosis to a similar degree to that induced by wild-type (wt)-AChEs (Du *et al*., 2015). These results demonstrate that the catalytic triad of AChEs is indispensable for its cholinesterase activity, but is irrelevant to its DNase activity (Du *et al*., 2015).

Nevertheless, these previous findings raised an intriguing question on how cholinergic neurons, which physiologically present high basal levels of AChEs enzyme, are not affected by this apoptotic function of this protein and present long-term growth and normal morphology? The answer to this question may lie on the localization of the protein in the cell. Despite abundant AChEs expression in cholinergic neurons, under normal conditions the protein is localized outside of the nucleus and on the outer surface of the cell membrane, being then inaccessible to the chromosomal DNA; consequently, AChEs is unable to act as a DNase, which makes it understandable that the neurons with high basal AChEs levels survive normally (Du *et al*., 2015). In this sense, AChEs might perform its canonical function of terminating neurotransmission by hydrolyzing ACh. On the other hand, it is well-established that the hippocampal neurons are particularly affected by the neurotoxicity of Aβ in the progression of AD. Interestingly, Du and colleagues (2015), observed that in response to Aβ-induced neurotoxicity, primary hippocampal neurons showed nuclear translocation of AChEs and chromosomal DNA cleavage. In fact, nuclear translocation and subsequent cleavage of chromosomal DNA is thought to be one of the functions of AChEs in neuronal loss during AD progression,

although this speculation requires further investigation. (Du *et al*., 2015). Hence, Du and colleagues (2015) conclude that the stepwise events, including upregulated expression, nuclear translocation, subsequent binding with and digestion of chromosomal DNA, constitute the mechanism by which AChEs mediates cell apoptosis. Nonetheless, it is very important to emphasize that the whole mechanisms by which AChEs are activated during apoptosis and how AChEs and other DNases are coordinated and recruited into apoptotic machinery remain to be fully understood (Du *et al*., 2015).

Therefore, taken all together, one can assume that the high levels of 27-OH can play a critical role in the worsening of the neurodegenerative process observed in AD, since besides promoting the already well-established worsening of the amyloid pathology, we have observed in the present study that it also increases the activity and concentration of AChE in critical brain regions, which, in turn, might aggravate some of the neurodegenerative and apoptotic processes seen in AD brains. In fact, among the neurons that degenerate in AD, there are populations that do not contain ChAT, and - yet - still express AChE, suggesting that a major characteristic of degenerating neurons could be the expression of AChE (Inestrosa *et al*., 2005). These findings may help us understand why AD patients with high cholesterol levels have a low long-term efficacy of the treatment with AChEIs seen by Borroni *et al*. (2003). Hence, additional therapies aimed at reducing high cholesterol levels may represent an alternative strategy to improve AChEIs' efficacy and slow down disease progression over time (Borroni *et al*., 2003).

Therefore, our third set of experiments aimed at observing whether the association of a lipid-lowering drug with an AChEI drug would result in a better outcome in different parameters in mice fed a HFD. In this set, we used Swiss mice exposed to a HFD and treated them with simvastatin, donepezil, or the association of both drugs. Nowadays, rodents exposed to HFDs are widely used to evaluate the impact of diet on the brain (Arnold *et al*., 2014; Underwood & Thompson, 2016; Nakandakari *et al*., 2019), and several studies have shown that the consumption of a HFD impairs critical brain areas that are involved in cognition, which are affected in AD (Arcego *et al*., 2016; Lizarbe *et al*., 2018; Nakandakari *et al*., 2019). Thus, the study of rodents fed a HFD becomes an interesting approach for investigating many aspects of neurodegenerative diseases (De Bem *et al*., 2021). In addition, HFDs increase the flux of oxysterols to the brain (Mateos

*et al*., 2009; Guo *et al*., 2014), and it was already observed that increased AChE activity within the prefrontal cortex and the hippocampus is an early event associated with hypercholesterolemia-induced cognitive impairment in mice (Moreira *et al*., 2014).

In the present study, we observed bodyweight gain among groups over the time course of the study, even though the bodyweight of high-fat-fed mice increased at a greater rate than in SD-fed mice. Bodyweight gain is a normal outcome when feeding mice with hypercaloric diets, and it has widely been seen in other studies with HFD-fed mice (Ayabe *et al*., 2018; Denver *et al*., 2018). In addition, HFD-fed mice presented increased in plasma protein levels in comparison to the SD-fed mice. This result was expected since the HFD had an increased protein concentration. Moreover, mice fed a HFD presented a significant increase in total plasma cholesterol concentrations, and, as expected, the treatment with the lipid-lowering drug simvastatin reduced the cholesterol levels.

Along with the previous findings, diets, and particularly the fat content of the diets, can modulate inflammatory and immune responses (Paschos *et al*., 2004; Baer *et al*., 2004), and it is known that the liver plays a key role in the inflammatory response evoked by dietary constituents (Kleemann *et al*. 2007). Li *et al*. (2005) showed that excess cellular cholesterol induces inflammation and the release of inflammatory cytokines. In the protocol used in this study, we observed no differences in the concentrations of TNF-α in the liver. This might be due to the innate characteristic of this cytokine, which is more activated in the initial stages of the inflammatory response. However, we observed a tendency to increase the concentration of the IL-1β cytokine in the liver of the HFD-fed group treated with vehicle. This might be due to the time course of the protocol here used. However, the liver is not the only important player in diet-induced peripheral inflammation. The adipose tissue (AT) is an important site of inflammatory events in obese individuals. It contains various cell types that contribute to the inflammatory process (Heijden *et al*., 2015). In addition to regulating fat mass and nutrient homeostasis, adipocytes mediate the inflammatory response through the secretion of adipokines, cytokines, and chemokines that enhance the recruitment of immune cells, especially macrophages, to the AT (Cinti *et al*., 2005). Interestingly, Lee and colleagues (2011) observed that mice fed a HFD significantly increased AT mass, adipocyte size, as well as

AT cytokines within one week of feeding. However, the increase in the cytokines mRNA levels in the liver were only seen after 16 weeks of feeding a HFD (Lee *et al*., 2011). These findings are supported by Heijden and colleagues (2015) whose work showed that AT inflammation is established prior to the development of hepatic inflammation. However, this is a limitation of the present work, since we have not taken the AT for analysis. Nevertheless, it is known that the inflammation produced in these sites are not restricted to these tissues, producing then a peripheral inflammation that might travel through the circulation and affect the CNS. In this sense, it has been demonstrated that peripheral inflammation can disrupt the BBB by various pathways, such as (1) promoting changes in tight junctions; (2) damaging the endothelial cells; (3) promoting astrocytes and microglia activation; (4) altering multiple transport pathways and receptors; (5) promoting the penetration of peripheral immune cells, which ultimately end up resulting in different CNS diseases (Huang *et al*., 2021). Noteworthy, a paper published in the beginning of the current year in the *Journal of Alzheimer's Disease Reports* proposed a new hypothesis for AD, called "*the lipid invasion model*". It argues that AD results from external influx of free fatty acids and lipid-rich lipoproteins into the brain, following disruption of the BBB (Rudge, 2022). This new hypothesis states that a key role of the BBB is protecting the brain from external lipid access (Rudge, 2022). Moreover, it is worth mentioning that the rate of flux of 27OH into the brain is also dependent upon the integrity of the BBB (Leoni *et al.,* 2003), and a damaged barrier can thus be expected to lead to a higher influx of this oxysterol into the brain (Ali *et al*., 2013). Interestingly, in a paper published last year in the *Frontiers in Neuroscience* journal, de Paula and colleagues (2021), observed that Swiss mice fed a HFD – exactly the same diet/composition/supplier used in this thesis – presented impaired hippocampal BBB permeability, as well as learning and memory impairment, depressive-like behavior, and reduced synaptic density within one week of feeding. Moreover, authors also showed mitochondrial dysfunction and astrocytic activation after 4 weeks of HFD consumption (de Paula *et al*., 2021). In addition, Loera-Valencia and colleagues (2021b) observed that HFD increases the gene expression and protein density of alarmins (specifically S100A8) - which are endogenous pro-inflamatory mediators released during tissue damage - in hippocampal neurons. These alarmins are increased in the brains of AD patients (Loera-

Valencia *et al*., 2021b). Later, they explored whether the high levels of S100A8 in the HFD-fed mice were mediated by 27-OH. Hence, they took CYP27Tg (the same mice model used in this thesis) and observed that they also had an increase in the gene expression and immunostaining of S100A8 in hippocampal cells (Loera-Valencia *et al*., 2021b). In order to confirm that these effects seen in the CYP27Tg mice were in fact mediated by 27-OH, they also injected 27-OH intracerebroventricularly into the lateral ventricle of WT mice at a concentration of 10 μM in artificial cerebrospinal fluid (27-OH aCSF), and observed that these mice presented increased RNA expression and protein levels of S100A8 (Loera-Valencia *et al*., 2021b). And, finally, authors also observed increased protein levels of RAGE[11] receptors in the CYP27Tg mice in comparison to the WT mice (Loera-Valencia *et al*., 2021b). In addition, Ullrich and colleagues (2010) observed that hypercholesterolemia decreased the number of cholinergic neurons in the basal nucleus of Meynert and the cortical acetylcholine levels in rodents. In this sense, it seems like the cholinergic system is particularly sensitive to diet-induced neurotoxicity. Moreover, Moreira and colleagues (2014) extended this observation by reporting increased AChE activity within the prefrontal cortex and hippocampus of hypercholesterolemic mice. It was also observed that LDL cholesterol significantly increased in a concentration-dependent manner the activity of AChE in SH-SY5Y cells (Moreira *et al.,* 2014). In the present study, we already observed a cognitive impairment seen by the recognition index in object recognition test in the group fed a HFD after 45 days of diet. Noteworthy, De Paula and colleagues (2021) observed that Swiss mice fed a HFD displayed object recognition memory impairment seen within one week of diet intake. Later, at the end of the third set of experiments, we observed no differences among groups regarding the AChE activity in the prefrontal cortical cells. However, we could observe statistical differences in enzymatic activity of AChE in the hippocampal cells of

[11] RAGE is a multiligand receptor in the immunoglobulin (IgG) superfamily, which binds soluble Aβ and mediates pathophysiologically relevant cellular responses consequent to ligation by a variety of ligands (Deane *et al.,* 2004). Interactions of RAGE with Aβ at the BBB results in transcytosis of circulating Aβ into the brain (Deane *et al*., 2003) and is associated with oxidative stress and expression of nuclear factor κB transcription factor (Yan *et al*., 1996), which may promote apoptosis or inflammatory responses including expression of adhesion molecules vascular cell adhesion molecule and intercellular adhesion molecule-1 (Stern *et al*., 2002), cytokines (e.g., TNF-α IL-6), and endothelin-1 resulting in neuroinflammation and suppression of the cerebral blood flow, respectively (Deane *et al*., 2003; Deane *et al.,* 2004).

the groups fed a HFD. Notably, it appears that hippocampus is particularly susceptible to damage by dietary factors (Morris *et al*., 2006; Davidson *et al*., 2007; Francis & Stevenson, 2011; Kanoski & Davidson, 2011; Gibson *et al*., 2013; Baym *et al*., 2014; Hao *et al*., 2016; Attuquayefio *et al*., 2017). Moreover, the hippocampus is a key brain structure to spatial learning and memory (Broadbent *et al*., 2004) and is critical for object–place associations (Mumby *et al*., 2003; Gilbert & Kesner, 2004). In addition, object location test has been used as a model of hippocampal-dependent spatial memory (Ferguson & Sapolsky, 2007; Murai *et al*., 2007; Assini *et al*., 2009), and there is evidence that acetylcholine has a role in the hippocampal modulation of spatial memory processes (Rogers & Kesner, 2003). In fact, the muscarinic cholinergic receptor antagonist, scopolamine, impaired object location test in mice in a dose-dependent manner (Murai *et al*., 2007). On the other hand, the acetylcholinesterase inhibitor donepezil enhanced object location memory (Murai *et al*., 2007). In addition, Gil-Bea and colleagues (2011) extended the knowledge in this regard by showing that a hippocampal denervation of cholinergic fibers, achieved by using the selective cholinergic immunotoxin 192IgG-saporin, led to significant decrease in Arc protein and mRNA as well as in BDNF. Lesioned rats showed a worse spatial memory acquisition performance on the morris water maze (MWM) that was reversed after galantamine treatment (Gil-Bea *et al*., 2011). Recovery of memory acquisition was accompanied by normalization of Arc and BDNF levels in hippocampus (Gil-Bea *et al*., 2011).

Here, we observed that only the association of a lipid-lowering drug (simvastatin) and an AChE inhibitory drug (donepezil) decreased the catalytic activity of the AChE enzyme in the hippocampus of HFD-fed mice. Following this finding, we observed that mice fed a HFD demonstrated cognitive impairment of short-term spatial memory seen by the exploration index in the object location task without presenting any locomotor deficits seen by the number of crossings in the open field test. Only concomitant treatment with simvastatin and donepezil could reverse this cognitive impairment. In this sense, it is known that there is a close relationship between the disruption of the cholinergic system and cognitive deficits, such as spatial reference memory impairments (Deiana *et al*., 2011). It is also well-established that statins, HMG-CoA reductase inhibitors, have pleiotropic effects that are independent of their cholesterol-lowering actions and that may

contribute to a healthier brain environment, which may – ultimately - end up improving higher-order brain functions and memory performance, such as: enhancing blood perfusion by increasing the production of vasodilators such as prostaglandin I2 and nitric oxide (NO) and decreasing the production of vasoconstrictors (Bayorh *et al*., 2005); prevent the release of pro-inflammatory matrix metalloproteinases (MMP) and cytokines, including TNF-α, IL-1β and IL-6 (Wanamaker & Swiger, 2015), thus producing an overall anti-inflammatory effect (Stuve *et al*., 2003; Loera-Valencia *et al*., 2019a); endothelial function improvement (Wassmann & Nickenig, 2003; McGuinness *et al*., 2016); antioxidant effect (by inhibiting NADPH oxidase) (Vaughan & Delanty, 1999; Loera-Valencia *et al*., 2019a); and the treatment with simvastatin have been reported to ameliorate inflammation and memory deficits in APP/PS1 transgenic mice (Huang *et al*., 2017). Interestingly, simvastatin effectively reduced $A\beta_{1-42}$ protein levels in a dose-dependent manner in yeast cells, and the authors hypothesize that this reduction may be due to protein clearance (Dhakal *et al*., 2019). In this sense, in brain capillary endothelial cells, simvastatin treatment significantly increased intracellular apoJ levels (Wu *et al*., 2022). ApoJ can bind Aβ, thus promoting Aβ clearance through BBB and reducing Aβ uptake (Zandl-Lang *et al*., 2018; Wu *et al*., 2022). Of note, simvastatin is the most lipophilic statin and has the greatest ability to cross the BBB and exert its effects on the brain, and, in fact, among nine statins, simvastatin was proposed to be the best statin for use in neurodegenerative diseases (Sierra *et al*., 2011). Furthermore, simvastatin appears to be the best candidate for regulating oxysterols (Gamba *et al*., 2021).

Here, we observed the synergic effect of two different drugs, simvastatin and donepezil that together resulted in a decreased AChE activity and, ultimately, led to an improvement of the cognitive function of mice fed a HFD. The underlying mechanisms by which simvastatin could mediate effects on AChE activity remain elusive. However, Roensch *et al.* (2007) observed that human neuroblastoma cells from the SH-SY5Y lineage decreased AChE and BuChE catalytic activity after treatment with simvastatin. Furthermore, treatment with simvastatin for seven days has been observed to significantly decrease AChE activity in rats (Lane & Farlow, 2005). In addition, Ghodke *et al*., (2012) reported that brain AChE activity was found to be decreased after treatment with simvastatin in mice.

In this regard, one may assume that the treatment association of simvastatin with donepezil represents an interesting therapeutic strategy that might be useful in the treatment management of cognitive impairment in AD, especially in dyslipidemic patients. Nonetheless, additional studies are necessary to better understand the underlying mechanisms by which 27-OH modulates AChE's homeostasis within the brain. One hypothesis to this lies on the lipid rafts that are wide spread in the cell surface of the cell membrane. It is known that AChEs anchored to PRiMA is mainly localized at these cholesterol microdomains (Hicks *et al*., 2011). Given our results here, one may posit that 27-OH can alter the cholesterol composition of these microdomains on the cell surface and end up altering the AChE homeostasis. Hence, one way to explore the relationship of AChE and the lipid raft would be using the methyl-β-cyclodextrin, which is cell membrane cholesterol sequestrant, and observe if the cell membrane cholesterol depletion would alter the AChE parameters seen in this thesis. Moreover, it is known that oxysterols help maintain membrane cholesterol homeostasis by activating LXRs to increase cholesterol efflux, accelerating HMGCR degradation to reduce cholesterol biosynthesis, and stimulating ACAT to promote cholesterol esterification (Brown *et al*., 2021; Ormsby *et al*., 2022). Hence, it would be interesting to observe whether silencing the LXRα and LXRβ would alter the mRNA expression and protein density of AChE.

Overall, the findings presented in this thesis may have important implications since they provide an opportunity for clinical interventions such as control of vascular risk factors (especially cholesterol concentrations) that might worsen the overall clinical condition and the response to AChEI medications.

## 7. Conclusions

In the present study, we demonstrated that (A) treating hippocampal and cortico-hippocampal neurons with 27-OH increases total cholesterol levels, AChE's protein density, and catalytic activity; moreover, we observed that (B) Cyp27Tg mice have constitutive alterations in gene expression and protein density of AChE in cortical and hippocampal cells in comparison to C57BL/6 mice (C) HFD-fed mice presented body weight gain, as well as an increase in plasma total protein levels and plasma cholesterol concentrations. Furthermore, we observed that the concomitant treatment of simvastatin and donepezil resulted in a decrease of AChE's catalytic activity in the hippocampal cells of the HFD-fed mice which ended up resulting in a better performance in a short-term spacial memory task.

Overall, the present findings suggest that 27-OH modulates the AChE homeostasis within the brain. We propose that the treatment with the association of simvastatin and donepezil might be an interesting approach to managing cognitive impairment in AD, especially in dyslipidemic patients.

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