ROLE OF *TOMM40'523 – APOE* HAPLOTYPES IN ALZHEIMER'S DISEASE ETIOLOGY – FROM CLINICS TO MITOCHONDRIA

RÈMY CARDOSO

Tese para obtenção do grau de Doutor em Envelhecimento e Doenças Crónicas

Doutoramento em associação entre:

Universidade NOVA de Lisboa (Faculdade de Ciências Médicas | NOVA Medical School - FCM|NMS/UNL)

Universidade de Coimbra (Faculdade de Medicina - FM/UC)

Universidade do Minho (Escola de Medicina - EMed/UM)

Novembro, 2020

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PROGRAMAS DE DOUTORAMENTO FCT

Novembro, 2020

This thesis was conducted at the **Center for Neuroscience and Cell Biology (CNC.CIBB) of University of Coimbra and Coimbra University Hospital (CHUC)** and was a collaboration of the following laboratories and departments with the supervision of **Catarina Resende Oliveira MD, PhD, Full Professor of FM/UC** and the co-supervision of **Duarte Barral PhD, Associated professor of Nova Medical School, Universidade Nova de Lisboa**:

- **Neurogenetics laboratory** (CNC.CIBB) headed by **Maria Rosário Almeida PhD**
- **Neurochemistry laboratory** (CHUC) headed by **Inês Baldeiras PhD**
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This thesis also included the collaboration of **Bárbara Cecilia Bessa dos Santos Oliveiros Paiva PhD** from the **Laboratory of Biostatistics and Medical Informatics of FM/UC** and **Carolina Lemos PhD** from the **UnIGENe group of I3s**

Remy Cardoso was a student of the **Inter-University Doctoral Programme in Ageing and Chronic Disease (PhDOC)**, a collaboration between the Faculty of Medicine of the University of Coimbra, the NOVA Medical School of Universidade NOVA de Lisboa and the School of Medicine of the University of Minho and their associated research centers, and a recipient of the fellowship **SFRH / BD / 51995 / 2012 from the Portuguese Foundation for Science and Technology (FCT).**

The scientific content of the present dissertation is based on the following international scientific publication:

Cardoso R, Lemos C, Oliveiros B, Rosário Almeida M, Baldeiras I, Fragão Pereira C, Santos A, Duro D, Vieira D, Santana I, Resende Oliveira C. **APOEε4-TOMM40L Haplotype Increases the Risk of Mild Cognitive Impairment Conversion to Alzheimer's Disease**. J Alzheimers Dis. 2020 Sep 25. doi: 10.3233/JAD-200556. Epub ahead of print.

The procedures in this thesis involving human subjects have been approved by the Ethics Board of Faculty of Medicine, University of Coimbra **(refª CE-013/2017)** and NOVA Medical School of Universidade NOVA de Lisboa **(nr. 02/2017/CEFCM).**

Dance," said the Sheep Man. "You gotta dance. As long as the music plays. You gotta dance. Don't even think why. Start to think, your feet stop. Your feet stop, we get stuck. We get stuck, you're stuck

Haruki Murakami, Dance Dance Dance

Science should serve man and not man science
The Immortal Life of Henrietta Lacks, Rebecca Skloot

Never forget that behind each sample there is a person and a family
The author

To my wife

To my parents and grandparents

AGRADECIMENTOS

O caminho até aqui foi um caminho bastante árduo e cheio de obstáculos que não conseguiria ter ultrapassado sem os meus dois orientadores: Professora Catarina Oliveira e Professor Duarte Barral. Ambos foram orientadores no sentido literal da palavra apoiando e guiando-me nos momentos mais complicados. Ambos um exemplo de grandes cientistas e extraordinários seres humanos.

Gostaria também de agradecer à doutora Cláudia Pereira, à doutora Inês Baldeiras e à doutora Maria Rosário Almeida por me terem acolhido nos seus laboratórios e por terem feito parte do trabalho aqui presente.

Um muito obrigado à professora Isabel Santana, responsável pela consulta de demência da unidade de neurologia do Hospital Universitário de Coimbra, à Dra. Diana Duro, também colega de doutoramento, e à restante equipa da consulta de demência sem os quais este trabalho não seria possível. Foi também uma grande aprendizagem ter a oportunidade de colaborar com esta equipa, que é, sem dúvida, uma referência nacional no cuidado de doentes com demência. Igualmente gostaria de agradecer à equipa de enfermagem do hospital de dia da unidade de neurologia.

Um enorme obrigado à doutora Bárbara Oliveiros e à doutora Carolina Lemos que sem hesitações aceitaram colaborar neste projeto e cuja ajuda foi imprescindível.

Gostaria também de agradecer à doutora Charlotte Teunissen, à doutora Marta del Campo do VUmc (Amsterdão) e também ao doutor Tiago Fleming Outeiro do *University Medical Center Goettingen*, por terem aceite colaborar comigo no projeto inicial de doutoramento que, por razões de maior, não pode ir em frente.

Um especial obrigado a todos os colegas do laboratório *Membrane Traffic In Disease* do professor Duarte Barral e restantes colegas do CEDOC, que foi a minha casa no início do doutoramento.

Gostaria de agradecer também a todos os elementos do grupo *Cell Signaling And Metabolism In Disease* do CNC, nomeadamente à doutora Rosa Resende, por me terem guiado e auxiliado no trabalho laboratorial que aqui exerci.

Um forte abraço aos meus colegas de laboratório e gabinete no CNC: Daniel, Emanuel, Judite, João, Patrícia e Tiffany que tornaram os meus dias sempre mais divertidos e a quem desejo o maior sucesso no seu percurso de doutoramento.

Contudo o maior agradecimento vai para a minha família, principalmente para os meus pais, irmão e avós que foram um pilar fundamental durante todo este percurso. Um especial obrigado ao meu pai e tia Charlotte que sempre me incentivaram a seguir ciência e acreditaram na minha carreira como cientista. Esta tese é dedicada a todos vocês.

O último, mas o mais importante agradecimento, vai para a minha namorada, noiva e agora esposa Filipa, por todo o carinho, apoio e paciência que teve durante este longo percurso. As palavras não são suficientes para tão enorme agradecimento e, por isso, esta tese é também dedicada a ti!

Este trabalho foi realizado ao abrigo da Bolsa (SFRH / BD / 51995 / 2012), com o apoio da FCT (Fundação para a Ciência e a Tecnologia), do FSE (Fundo Social Europeu) e do POCH (Programa Operacional Capital Humano).

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Abbreviations list

Aβ – Amyloid - β peptide
ABAD – Aβ-related alcohol dehydrogenase
Ac-DEVD-pNA – Acetyl-Asp-Glu-Val-Asp p-nitroaniline
ACh – Acetylcholine
AChE – Acetyl cholinesterase enzyme
AChEIs – Acetyl cholinesterase inhibitors
AD – Alzheimer's disease
ADAS-Cog – Alzheimer's Disease Assessment Scale-Cognitive
AICD – APP intracellular domain
AIF – Apoptosis-inducing factor
aMCI – amnestic mild cognitive impairment
AOO – Age of onset
Apaf 1 – Apoptotic protease factor 1
ApoE – Apolipoprotein E
APOE – Apolipoprotein E gene
APP – Amyloid precursor protein
APPsα – Soluble APP α
APPsβ – Soluble APP β
ATP – Adenosine triphosphate
$A\beta_{42}/_{40}$ – 42/40 amino acids form of Aβ
BACE-1/2 – Beta-secretase 1/2
Bak – Bcl-2 homologous antagonist killer
Bax – Bcl-2-associated X protein
BCA – Bicinchoninic acid
Bcl-2 – B-cell lymphoma 2
BLAD – Lisbon Battery for Dementia Assessment
bp – base pair
BSA – Bovine serum albumin
Ca^{2+} – Calcium
CAD – Caspase-activated deoxyribonuclease
Cat – Catalase
CCCP – Carbonyl cyanide 3-chlorophenylhydrazone
CDR – Clinical Dementia Rating scale
CHAPS – 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid
CHUC – Coimbra University Hospital
CI – Confidence interval
CJD – Creutzfeldt- Jakob Disease
COX – Cytochrome c oxidase
CSF – Cerebrospinal fluid
CT – Computed tomography
CTFα – C-terminal fragment α
CTFβ – C-terminal fragment β
DAD – Disability Assessment for Dementia
DLB – Dementia with Lewis Bodies

DMTs – Disease modifying therapies
DNA – Deoxyribonucleic acid
Drp1 – Dynamin related protein 1
DSM – Diagnostic and Statistical Manual of Mental Disorders
DSM-IV-TR – Diagnostic and Statistical Manual of Mental Disorders – fourth edition text review
DTT – Dithiothreitol
e^- – Electron
ECF – Enhanced chemifluorescence
EDTA – Ethylenediaminetetraacetic acid
EGTA – Ethyleneglycol- bis(β-aminoethyl)-N,N,N',N'-tetraacetic Acid
EM – Electron microscopy
EOAD – Early onset AD
ER – Endoplasmic reticulum
ERC – Entorhinal cortex
ETC – Electron transport chain
FBS – Fetal bovine serum
FDA – Food and Drug Administration
Fis1 – Fission protein 1
FTLD – Fronto-Temporal Lobar Degeneration
GSH – Glutathione
GWAS – Genome-wide association studies
H^+– **Proton**
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP60 – Heat Shock Protein 60 kD
HSP70 – Heat Shock Protein 70 kD
IAP – Inhibitor of apoptosis
IATI – Innotest Amyloid-Tau Index
ICAD – Caspase-activated deoxyribonuclease inhibitor
IMM – Inner mitochondrial membrane
IMS – Intermembrane space
IP_3R – Inositol 1,4,5- trisphosphate receptor
kDa – Kilodalton
KM – Kaplan-Meyer
L – Long
LATE – Limbic-predominant age-related TDP-43 encephalopathy
LBC1936 – Lothian Birth Cohort 1936
LD – Linkage disequilibrium
LOAD – Late onset AD
MAM – Mitochondrial-associated ER membrane
MCI – Mild cognitive impairment
MCI-AD – MCI patients that converted to AD
MCI-S – MCI patients that remained cognitively stable
MCU – Mitochondrial calcium uniporter
Mfn1/2 – Mitofusin 1/2
MMSE – Mini Mental State Examination
MnSOD – Manganese superoxide dismutase

MoCA – Montreal Cognitive Assessment
mPTP – mitochondrial permeability transition pore
MRI – Magnetic resonance imaging
mRNA – messenger RNA
mtDNA – mitochondrial DNA
NaF – Sodium fluoride
naMCI – non-amnestic mild cognitive impairment
NFL – Neurofilament light protein
NFTs – Neurofibrillary tangles
NGS – Next generation sequencing
NIA-AA – National Institute on Aging and Alzheimer's Association
NINCDS - ADRDA – National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders
NMDA – N-methyl-D aspartate receptor
NPI – Neuropsychiatric Inventory
NRF1 – Nuclear respiratory factor 1
NRF2 – Nuclear factor erythroid-derived 2-related factor 2
OMM – Outer mitochondrial membrane
OPTN – Optineurin
OR – Odd ratio
OXPHOS – Oxidative phosphorylation
p-Tau – phosphorylated Tau
p^0 cells – mtDNA depleted cells
PBMCs – Peripheral blood mononuclear cells
PBS – Phosphate saline buffer
PCR – Polymerase chain reaction
PCR-RFLP – Polymerase chain reaction-restriction fragment length polymorphisms
PD – Parkinson's Disease
Pen/Strep – Penicillin-Streptomycin
PET – Positron-emission tomography
PGC1α – Proliferator-activated receptor γ coactivator-1α
PINK1 – PTEN-induced kinase 1
PMSF – Phenylmethanesulfonyl fluoride
pNA – p-nitroaniline
PreP – Mitochondrial peptide processing enzyme
PS1/2 – Presenilin 1 and 2 protein
PSEN 1/2 – Presenilin 1 and 2 gene
PVDF – Polyvinylidene fluoride
RNA – Ribonucleic acid
RNS – Reactive nitrogen species
ROS – Reactive oxygen species
rRNA – ribosomal RNA
RT – Room temperature
RyRs – Ryanodine receptors
S – Short
SDS – Sodium dodecyl sulfate
Ser – Serine

SMase – Sphingomyelinase
SNPs – Single-nucleotide polymorphisms
T – Thymidine
t-Tau – total Tau
TBS -T – Tris - buffered saline - Tween 20
TDP-43 – Transactive response DNA binding protein of 43kDa
TEM – Transmission electron microscopy
TFAM – Mitochondrial transcription factor A
TIM – Translocase of the inner membrane
TMRM – Tetramethylrhodamine methyl ester perchlorate
TOM – Translocase of the outer membrane
TOM40 protein – Translocase of outer membrane 40 kDa
TOMM40 – Translocase of the Outer Mitochondrial Membrane 40 homolog gene
***TOMM40'*523** – *TOMM40* rs10524523 polymorphism
tRNA – transfer RNA
UPRmit – Mitochondrial Unfolded Protein Response
VaD – Vascular Dementia
VDAC – voltage-dependent anion-selective channel protein
VDAC1 – voltage-dependent anion channel 1 protein
VL – Very Long
WB – Western blot
ΔΨm – Mitochondrial membrane potential
FDG-PET – fluorodeoxyglucose positron emission tomography

Abstract

TOMM40'523 is a poly-T polymorphism of the gene *TOMM40* which was reported by Roses *et al.* to be associated with risk and age of onset (AOO) of Alzheimer's Disease (AD) nearly a decade ago. Meanwhile, based on the distribution behavior of the number of thymine (T) residues, three categories (alleles) of repeat length were established: short (S, ≤ 19), long (L, 20– 29) and very long (VL, ≥ 30).

Since the original discovery by Roses *et al.* multiple studies found associations between *TOMM40*' 523 and LOAD-related features, such as AOO and risk of AD, brain structure and cognition, while other studies could not replicate these associations. Moreover, this polymorphism has been poorly addressed in mild cognitive impairment cohorts (MCI) and as far as we know, none of these studies fully addressed the connection between *TOMM40*' 523 polymorphism and the risk and time of conversion from MCI to AD.

Therefore, in the first part of this work our aim was to investigate the relationship between *TOMM40*' 523 polymorphism with the risk and conversion time from MCI to AD, and replicate the association of *TOMM40*'523 polymorphism with AOO and risk of AD. Secondly, the association between *TOMM40*' 523 genotype and AD cerebrospinal fluid (CSF) biomarkers, particularly $A\beta_{42}$, t-Tau and p-Tau, was also explored. For this purpose, 147 AD patients, 102 MCI patients and 105 cognitively normal controls were genotyped for poly-T polymorphism. MCI patients were subdivided into 2 groups, the group of patients that converted to AD (MCI-AD) and the group of those that remained stable (MCI-S).

We first demonstrated that MCI non-converters (MCI-S) and converters (MCI-AD) had a different poli-T distribution, where the L allele was significantly more frequent in the MCI-AD group. We further evaluated how this difference impacted the risk of conversion and found that having at least one L allele significantly increased the risk of conversion from MCI to AD. However, when adjusted for the presence of APOE ε4 allele, both the L allele and ε4 allele lost significance in the model ($p > 0.05$). We then analysed the *APOE* ε4-TOMM40' 523 L haplotype and observed that patients carrying this haplotype had significantly higher risk and mean lower times of conversion to AD. This haplotype was also significantly associated with a biomarker profile compatible with AD namely,

significantly lower levels of $A\beta_{42}$ and higher levels of t-Tau and p-Tau. Similar results were observed for AD where ε4-L haplotype carriers where associated with a significantly higher risk of AD and lower AOO of AD patients.

TOMM40 gene encodes the Tom40 protein (translocase of the outer mitochondrial membrane, 40 kD) which forms the channel subunit of the outer mitochondrial membrane protein complex through which the majority of nuclear-encoded proteins enter mitochondria. As the import of mitochondrial proteins into mitochondria is essential for biogenesis and functioning of mitochondria, it is not surprising that TOM40 is essential for life in eukaryotic organisms and that modifications in this protein could lead to mitochondrial dysfunction. Mitochondria dysfunction is a well characterized event in AD. Considering this, it was hypothesized that *TOMM40*' 523 polymorphism could have a role on AD through mitochondrial dysfunction. However, the few studies performed so far did not reach clear conclusions. Using Peripheral Blood Mononuclear Cells (PBMCs) from AD patients, we addressed the impact of this polymorphism on different mitochondrial features, such as function, structure and apoptosis. We obtained PBMCs from patients homozygous for S, L and VL TOMM40' 523 polymorphisms, which are thereafter called S, L and VL groups.

In this study, we observed that VL group had significant higher levels of TOM40 than S group, similar mitochondrial membrane potential, higher mitochondrial fission protein levels and lower caspase activation. Although these two last parameters did not reach statistical significance, our data reinforce the hypothesis that increased levels of TOM40, as have been observed by Zeitlow *et al.* seem to be protective to mitochondria, however these experiments should be replicated.

In summary in this work we aimed to study the potential role of *TOMM40*'523 as a risk gene for AD and its involvement in AD pathophysiology through mitochondrial dysfunction, under the perspective "from clinics to mitochondria".

Resumo

TOMM40'523 é um polimorfismo poli-T do gene *TOMM40* que foi descrito por Roses *et al.* como estando associado com o risco e idade de início da doença de Alzheimer (DA). Entretanto, e com base na distribuição do número de timinas, três tipos de alelos foram estabelecidos: *"short"* (S, ≤ 19), *"long"* (L, 20– 29) e *"very long"* (VL, ≥ 30).

Desde a descoberta inicial de Roses *et al.* vários estudos encontraram associações entre o polimorfismo *TOMM40*' 523 e características relacionadas com a DA entre elas, idade de início e risco de DA, alterações na morfologia cerebral e cognição, enquanto outros estudos não conseguiram replicar estas associações. Além do mais, este polimorfismo foi pouco estudado em populações com défice cognitivo ligeiro (DCL) e tanto quanto sabemos nenhum destes estudos se debruçou na relação entre o polimorfismo *TOMM40*' 523 e o risco e tempo de conversão de DCL para DA.

Assim, na primeira parte do trabalho aqui apresentado o nosso principal objetivo foi investigar a relação do polimorfismo *TOMM40*' 523 com o risco e tempo de conversão de DCL para DA, bem como replicar a associação inicial descrita por Roses *et al.* com o risco e idade de início da DA. Em segundo lugar, explorámos a relação entre este polimorfismo e os níveis de biomarcadores de DA no líquido cefalorraquidiano (LCR), nomeadamente $A\beta_{42}$, Tau total e fosfo-Tau. Para este efeito, procedemos à genotipagem para o polimorfismo *TOMM40*' 523 em 147 pacientes com DA, 102 pacientes com DCL e 105 controlos cognitivamente normais. Os pacientes DCL foram subdivididos em dois grupos distintos: um grupo de pacientes DCL que converteram para AD (DCL-C) e um grupo de pacientes que permaneceu cognitivamente estável (DCL-NC).

Começámos neste estudo por demonstrar que os doentes DCL que não converteram (DCL-NC) e os doentes que converteram (DCL-C) apresentavam uma distribuição distinta do número de timinas neste polimorfismo, sendo o alelo L significativamente mais frequente no grupo DLC-C. Posteriormente, avaliámos como é que esta diferença afetava o risco de conversão de DCL para DA e verificámos que a presença de pelo menos um alelo L aumentava significativamente o risco de conversão de DCL para DA. Contudo, quando tido em conta o alelo ε4 do gene *APOE,* verificámos que tanto o alelo L como alelo ε4 perdiam significância no modelo de conversão ($p > 0.05$). Então

decidimos estudar o haplótipo *APOE* ε4-*TOMM40*' 523 L e observámos que doentes com este haplótipo apresentavam um risco significativamente maior de conversão de DCL para DA e menores tempos de conversão. Verificou-se também uma associação significativa entre este haplótipo e um perfil de biomarcadores compatível com DA, nomeadamente menores níveis de $A\beta_{42}$ e níveis mais altos de Tau total e fosfo-Tau. Adicionalmente, verificamos que o haplótipo *APOE* ε4-*TOMM40*' 523 L estava significativamente associado com um maior risco de desenvolver DA e com menor idade de início da doença.

O gene *TOMM40* codifica a proteína TOM40 (Translocase da membrana externa, 40 kD) que forma o canal na membrana externa da mitocôndria através do qual a maioria das proteínas codificadas no citoplasma entram na mitocôndria. Sendo a importação de proteínas essencial para a biogénese e funcionamento da mitocôndria, não é de surpreender que a proteína TOM40 seja essencial para os seres eucarióticos, e que modificações nesta proteína possam eventualmente levar a disfunção mitocondrial. A disfunção mitocondrial é um evento bem caracterizado na DA. Tendo isto em conta, colocámos a hipótese de que o polimorfismo *TOMM40*' 523 poderia ter um papel importante na disfunção mitocondrial e patogénese da DA. Utilizando células mononucleares do sangue periférico (PBMCs) de doentes com DA, estudámos o impacto deste polimorfismo em diferentes parâmetros mitocondriais como função, estrutura e apoptose. As PBMCs foram obtidas de doentes homozigóticos para os alelos S, L e VL, sendo denominados posteriormente como grupos S,L e VL.

Neste estudo, podémos observar que o grupo VL apresentava níveis significativamente mais elevados da proteína TOM40, semelhante potencial de membrana mitocondrial, maiores níveis de proteínas de fissão mitocondrial e uma menor ativação da caspase 3 quando comparado com o grupo S. Apesar destes dois últimos parâmetros não alcançarem significância estatística, estes dados reforçam a hipótese de que o aumento nos níveis de TOM40, tal como observado também por Zeitlow *et al*., poderão ser protetores para mitocôndria. Contudo estes dados têm de ser replicados em experiências futuras.

Em jeito de sumário, neste trabalho pretendemos estudar o potencial papel do polimorfismo *TOMM40*'523 no risco de desenvolver DA, bem como o seu envolvimento

na fisiopatologia desta doença via disfunção mitocondrial, numa perspetiva " da clinica para a mitocôndria".

Chapter 1
General Introduction

1.1 – Alzheimer's disease: an overview

1.1.1 – Social and economic impact of Alzheimer's Disease

Alzheimer's disease (AD) was described for the first time by the German psychiatrist Alois Alzheimer as a "Strange Disorder of the Brain". At a conference in Tubingen in 1906, Alois Alzheimer described a patient and the clinical picture of the dementia (progressive cognitive impairment, focal symptoms, hallucinations, delusions, and psychosocial incompetence), as well as the histological findings in the brain (amyloid plaques and neurofibrillary tangles) found at necropsy **(see 1.1.5)** [1]. Today, AD is the most common form of dementia [2], being estimated that 60%–80% [3] of 50 million people living with dementia worldwide suffer of AD. In other words, over the world there will be one new diagnosis of dementia every 3 seconds, leading to a number of cases that is expected to increase to 83 million in 2030 and 152 million in 2050. Indeed, the number of people who have dementia is rising rapidly in low- and middle-income countries, as people live longer, whereas in high-income countries the incidence rate shows a tendency to decrease, probably due to the improvement in cardiovascular health, nutrition and education in these countries **(see 1.1.8).** Regarding dementia costs, the total estimated worldwide cost of dementia in 2018 was 1 trillion of dollars and is expected to double to 2 trillion dollars in 2030 [4].

Despite the economic impact, there is also an enormous social impact of dementia and AD. The long duration of illness before death contributes significantly to the public health impact of AD, because much of that time is spent in a state of disability and dependence. AD is a very burdensome disease, not only to the patients but also to their families and informal caregivers. In 2017, just in the US, it was estimated that caregivers of people with AD or other dementias provided 18.4 billion hours of informal (that is, unpaid) assistance, a contribution to the nation valued at $232.1 billion [3]. On the other hand, a meta-analysis study found dementia family caregivers to be significantly more stressed than non- dementia caregivers and to suffer more serious depressive symptoms and physical problems. Furthermore, another meta-analysis found overall prevalence rates of 34 and 44%, respectively, of elevated depressive and anxiety symptoms [5].

Considering the facts aforementioned, AD is considered one of the great health-care challenges of the 21st century. In December 2013, the G8 Alliance stated that dementia should be made a global priority, aiming that a cure or a disease-modifying therapy should be available by 2025 [2].

1.1.2 – What is Dementia?

Dementia is a syndrome (group of symptoms) caused by a disease of the brain, usually chronic, characterized by a progressive, global deterioration in intellect including memory, language, problem-solving and other cognitive skills that affect a person's ability to perform everyday activities. These difficulties occur because neurons in areas of the brain involved in cognitive function have been damaged or destroyed. As previously stated, AD is the most common form of dementia in the elderly, but dementia is linked to a very large number of other underlying brain pathologies with distinct symptom patterns and brain abnormalities such as: Vascular Dementia (VaD), Dementia with Lewis Bodies (DLB), Fronto-Temporal lobar degeneration (FTLD), Parkinson's Disease (PD), Creutzfeldt-Jakob disease (CJD) and Normal pressure hydrocephalus **(Table 1.1)**

It is important to highlight that the boundaries between these subtypes are indistinct, and mixed forms may be the norm. Pathological studies in post-mortem brain indicate that mixed pathologies are much more common than 'pure' pathologies. In fact, mixed dementia is a form of dementia characterized by the hallmark abnormalities of more than one cause of dementia, most commonly AD combined with VaD, followed by AD with DLB, and AD with VaD and DLB, whereas the combined form of VaD with DLB is less common. Recent studies suggest that half of older people with dementia have pathologic evidence of more than one cause of dementia and that the likelihood of having mixed dementia increases with age and becomes the highest in the elderly (people age 85 or older) [3]. Recently, another form of dementia was described: Limbic-predominant age-related TDP-43 encephalopathy (LATE) [6]. The neuropathology of this disease is characterized by the presence of transactive response DNA binding protein of 43kDa (TDP-43) proteinopathy in limbic brain structures and is commonly observed in

subjects past 80 years of age. This type of dementia has been associated with substantial cognitive impairment that mimics AD [6].

Table 1.1 – Causes of dementia.

Dementia subtype	Characteristic symptoms	Neuropathology
Alzheimer's Disease (AD)	Difficulty remembering recent conversations, names or events is often an early clinical symptom; apathy and depression are also often early symptoms. Later symptoms include impaired communication, disorientation, confusion, poor judgment, behaviour changes and, ultimately, difficulty speaking, swallowing and walking **60 percent to 80 percent of cases**.	**Progressive accumulation of amyloid plaques outside neurons and neurofibrillary tangles inside neurons.** These changes are eventually accompanied by the damage and death of neurons.
Vascular dementia (VaD)	Impaired judgment or impaired ability to make decisions, plan or organize is more likely to be the initial symptom, as opposed to the memory loss often associated with the initial symptoms of AD. In addition to changes in cognition, people with VaD can have difficulty with motor function, especially slow gait and poor balance. **10 percent of the cases**	**Vessel blockage or damage leading to infarcts (strokes) or bleeding in the brain.** The location, number and size of the brain injuries determine whether dementia will result and how the individual's thinking and physical functioning will be affected.
Dementia with Lewy Bodies (DLB)	Some of the symptoms are common with AD but are more likely to have initial or early symptoms of sleep disturbances, well-formed visual hallucinations and slowness, gait imbalance or other parkinsonian movement features. These features, as well as early visuospatial impairment, may occur in the absence of significant memory impairment.	**Abnormal aggregation of the protein alpha-synuclein (Lewy bodies) in neurons.** When they develop in a part of the brain called the cortex, dementia can result.
Fronto-temporal lobar degeneration (FTLD)	Typical early symptoms include marked changes in personality and behaviour and/or difficulty with producing or comprehending language. Unlike AD, memory is typically spared in the early stages of disease. The brain changes of FTLD may occur in those age 65 years and older, similar to AD, but most people with this form of dementia develop symptoms at a younger age. About 60 % of people with FTLD have ages from 45 to 60. **10 percent of cases.**	Nerve cells in the front (frontal lobe) and side regions (temporal lobes) of the brain are especially affected, and these regions become markedly atrophied (shrunken). In addition, the upper layers of the cortex typically **become soft and spongy and have protein inclusions (usually tau protein or the TDP-43 protein).**
Parkinson's disease (PD)	Problems with movement (slowness, rigidity, tremor and changes in gait) are common symptoms of PD. As PD progresses, it often results in dementia secondary to the accumulation of Lewy bodies in the cortex (similar to DLB) or the accumulation of beta-amyloid clumps and tau tangles (similar to AD).	**Alpha-synuclein aggregates appear in an area deep in the brain called the substantia nigra.** The aggregates are thought to cause degeneration of the nerve cells that produce dopamine.
Creutzfeldt-Jakob disease (CJD)	**Very rare** and rapidly fatal disorder impairs memory and coordination and causes behaviour changes. May be hereditary, sporadic (unknown cause) or caused by a known prion infection.	**Misfolded protein (prion)** that causes other proteins throughout the brain to misfold and malfunction.

Normal pressure hydrocephalus	Difficulty walking, memory loss and inability to control urination. **Less of 5 percent of cases**	**Impaired reabsorption of cerebrospinal fluid** and the consequent build-up of fluid and increasing pressure in the brain.

Adapted from 2018 Alzheimer's Disease Facts and Figures [3].

On the other hand, in some cases individuals with symptoms of dementia do not actually have dementia, but instead have a condition whose symptoms mimic those of dementia, such as, depression, delirium, side effects from medications, thyroid problems, certain vitamin deficiencies and excessive use of alcohol, among others. Unlike dementia, these conditions often may be reversed with treatment [3].

1.1.3 – Symptoms and diagnosis of Alzheimer's Disease

AD is a progressive disorder that begins with a gradual decline of memory and then increases in severity until the symptoms eventually become incapacitating **(Table 1.2).** These symptoms reflect the degree of damage to neurons in different parts of the brain. The pace at which the symptoms advance from mild to moderate to severe varies from person to person**.** In early stages, differences between typical age-related cognitive changes and early signs of AD can be subtle. These symptoms include: memory loss; difficulty completing familiar tasks at home, at work or at leisure; challenges in planning or solving problems; confusion with time or place; problems with words in speaking or writing; decreased or poor judgment; withdrawal from work or social activities and changes in mood and personality. However, in late stage of disease other clinical problems appear such as: blood clots, skin infections due to bed bound and aspiration pneumonia, which is a contributing cause of death among many individuals with AD. Aspiration pneumonia is caused by the difficulty in eating and drinking, which in turn can result in food swallowing into the trachea instead of the esophagus. Ultimately, food particles may be deposited in the lungs and cause lung infection [3].

Table 1.2 – Alzheimer's disease stages and its characteristics.

Disease Stage	Characteristics
Mild Stage	• Most people are able to function independently in many areas but are likely to require assistance with some activities to maximize independence and remain safe. • May still be able to drive, work and participate in favorite activities.
Moderate Stage	• Become confused about where they are and begin wandering. • Start having personality and behavioral changes, including suspiciousness and agitation
Severe stage	• Individuals require help with basic activities of daily living, such as bathing, dressing and using the bathroom. • Their ability to verbally communicate is limited. • Individuals become bed-bound. • Difficult to eat and drink.

Adapted from 2018 Alzheimer's Disease Facts and Figures [3].

There is no single test for AD. Instead, physicians use a variety of approaches and tools to make the AD diagnosis. These tools include: medical history with the patient and caregiver; general physical, neurological, and psychiatric examination; cognitive instruments; blood tests and brain imaging to rule out other potential causes of dementia symptoms, such as a tumor or certain vitamin deficiencies. In more specialized centers, cerebrospinal fluid (CSF) biomarkers, imaging tools such as positron-emission tomography (PET), and genetic studies are used [3,7].

Dementia is diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders (DSM) and AD, according to the 2011 National Institute on Aging and Alzheimer's Association (NIA-AA) criteria [8]. A novel NIA-AA framework was launched in 2018 defining a biological rather a syndromal definition of AD [9]. In these recommendations, AD is defined by its underlying pathologic processes, which can be documented by postmortem examination or *in vivo* using biomarkers. This framework thus focuses on the diagnosis of AD with biomarkers in living people. Biomarkers are grouped in β-amyloid (Aβ) deposition (A), pathologic Tau (T) and neurodegeneration (N) in a system denominated [AT(N)]. It was determined that these recommendations should be cast as "research framework" and not as diagnostic criteria or guidelines.

Thus, unlike the 2011 NIA-AA criteria, the 2018 research framework is not intended for general clinical practice [9].

1.1.4 – Mild Cognitive Impairment

Mild cognitive impairment (MCI) represents an intermediate state of cognitive function between the changes seen in aging and those fulfilling the criteria for dementia, namely AD. Since persons with MCI are at increased risk for developing dementia, this entity has been receiving considerable attention in clinical practice and research settings [10].

MCI is characterized by cognitive decline not fulfilling the criteria of dementia and is classified into two main subtypes: amnestic and non-amnestic. In amnestic MCI (aMCI) there is a memory impairment, although other cognitive capacities, such as executive function, use of language and visuospatial skills are relatively preserved. On the other hand, the non-amnestic MCI (naMCI) is characterized by a subtle decline in functions not related to memory, affecting attention, use of language, or visuospatial skills. aMCI and naMCI can be further classified as single domain or multiple domain [10,11] **(Figure 1.1).** MCI can be diagnosed with the following criteria: (1) cognitive complaint by subject or informant; (2) notable decline in cognition; (3) cognitive deficits not normal for subject's age; (4) normal or near-normal functional activities; and (5) cognitive and functional difficulties not severe enough to yield diagnosis of dementia [11,12]. The nonamnestic type of MCI is less common than the amnestic type and may be the forerunner of dementias that are not related to AD, such as FTLD or DLB. On the other hand, aMCI is more likely to convert to AD [10]. A meta-analysis of 42 MCI studies identified the annual progression rate to dementia from MCI as 10% in clinical settings (8% of the entire sample progressed to AD) [13] whereas the remaining percentage of MCI patients stay clinically stable or even revert to normal cognition [11].

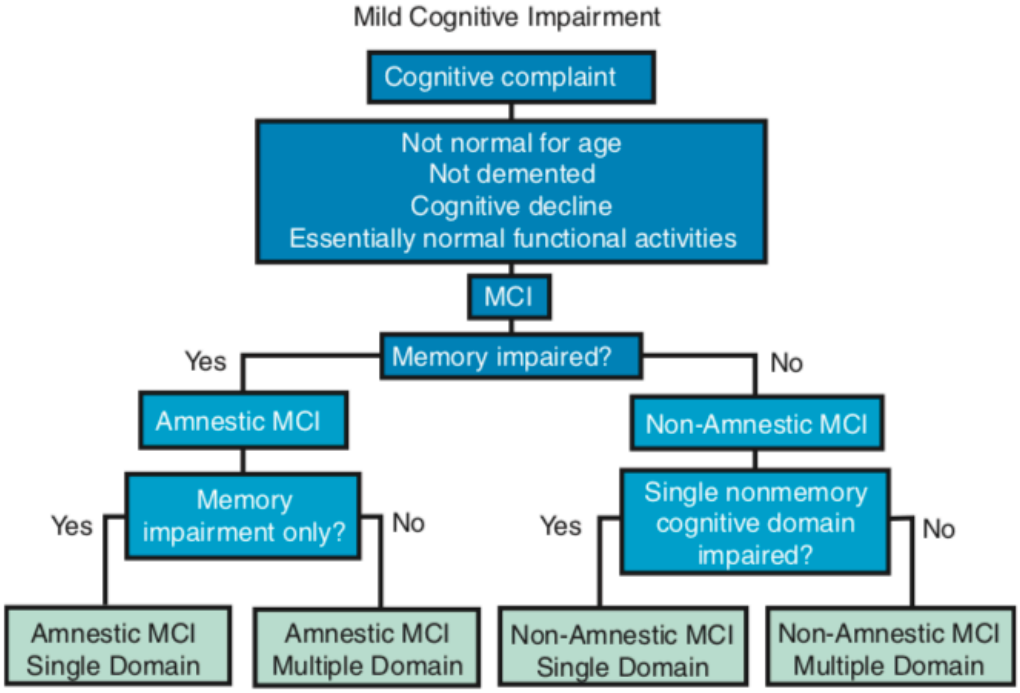


Figure 1.1 – Diagnostic algorithm for Mild Cognitive Impairment. Adapted from Petersen *et al.* [14].

1.1.5 – Neuropathological hallmarks

The neuropathologic hallmarks of AD brains are extracellular accumulation of diffuse and neuritic amyloid plaques, composed of aggregated Aβ peptide, and the intra-neuronal accumulation of neurofibrillary tangles (NFTs) composed of hyperphosphorylated protein tau (p-tau). These pathologic features are accompanied by gliosis and the loss of neurons and synapses [15,16].

Aβ peptide is the product of amyloid precursor protein (APP) cleavage in the amyloidogenic pathway. In this pathway, APP is cleaved by β-secretase (BACE 1 or 2) resulting in smaller ectodomain, soluble APP β (APPsβ), which is released into the extracellular space, and APP C-terminal fragment β (CTFβ) or C99, which remains embedded in the plasma membrane. CTFβ is further cleaved by γ- secretase releasing Aβ into the extracellular space and the AICD (APP intracellular domain) into the cytoplasm. Different Aβ forms are produced ($Aβ_{38}$, $Aβ_{39}$, $Aβ_{40}$, $Aβ_{42}$, etc.) depending on the γ- secretase cleavage site [17,18]. However, the 42 amino acid form of Aβ ($Aβ_{42}$) is the most associated and studied in AD. On the other hand, in the non-amyloidogenic pathway, APP is cleaved by α-secretase, releasing soluble APP (APPsα) into the

extracellular space. The other product C-terminal fragment α (CTFα) or C83, which remains embedded in the plasma membrane is further cleaved by γ-secretase, releasing a small p3 fragment into the extracellular space and the AICD into the cytoplasm **(Figure 1.2)** [18–20].The APP fragments aforementioned are known to have a physiological role in mammalian central nervous system (reviewed by Müller *et al*. [18]).

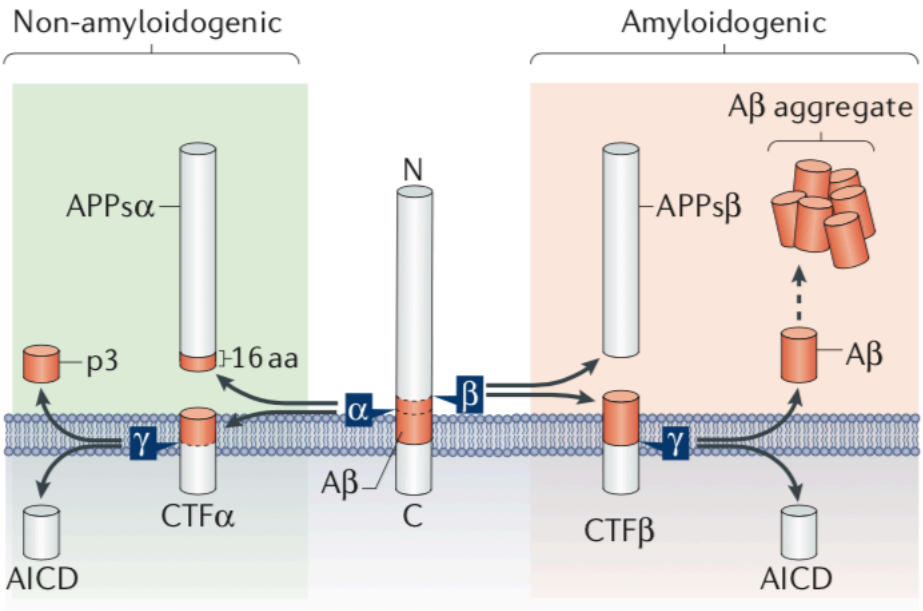


Figure 1.2 – Amyloid Precursor Protein processing. In the nonamyloidogenic pathway (green background) APP is processed through sequential cleavages by α- and γ-secretases generating P3 and AICD. In the amyloidogenic pathway (red background). APP is processed through sequential cleavages by β- and γ-secretases generating Aβ and AICD. Adapted from Müller et al [18].

Tau is a microtubule-associated protein that polymerizes tubulin into microtubules and participates in maintaining the complex neuronal cell microarchitecture, such as microtubule assembly and stabilization, particularly in the axon. In adult human brains, high heterogeneity of the Tau protein is apparent. There are six different Tau isoforms, all of which are derived from a single gene by alternative messenger RNA (mRNA) splicing. The six isoforms of Tau protein differ from each other in the number of microtubule-binding domains (3R/4R) [21,22]. In AD, natively unfolded Tau becomes hyperphosphorylated and, by losing its affinity for microtubules, tends to aggregate and form NFTs [22].

Tau and Aβ forms are used in diagnostic tools. $A\beta_{42}$, which shows cortical amyloid deposition; total Tau (t-Tau), which reflects the intensity of neurodegeneration; and phosphorylated tau (p-tau), which correlates with neurofibrillary pathological changes are used as core CSF biomarkers for AD [2]. Tracers for Aβ and Tau are also used on amyloid and Tau PET respectively [2,23].

1.1.6 – Current pharmacological therapies for Alzheimer's Disease

Only six drugs were so far approved for AD: four acetyl cholinesterase inhibitors (AChEIs), one N-methyl-d aspartate (NMDA) antagonist and one orexin receptor antagonist. Although these medications appear to be able to produce moderate symptomatic benefits, they do not stop or delay disease progression [24].

Treatment with AChEIs is mainly based on the "Cholinergic hypothesis of AD". According to this hypothesis, the selective destruction of cholinergic neurons in the basal forebrain and the resulting deficit in the central cholinergic transmission contribute substantially to the characteristic cognitive symptoms observed in the patients [25,26]. Acetyl cholinesterase enzymes (AChE) are involved in the degradation of acetylcholine (ACh) and consequently, inhibition of AChE would be expected to lead to an increase in the ACh concentration in the synaptic cleft and would thus be expected to ameliorate the cholinergic deficit. In 1993, Tacrine, the first AChEI was approved by Food and Drug Administration (FDA). However, Tacrine is no longer used because of hepatoxicity [27]. Later on, FDA approved Donepezil (1996), Rivastigmine (2000) and Galantamine (2001) for the treatment of mild to moderate AD and these currently represent the standard and first-line treatment for AD. Donepezil and Galantamine are selective inhibitors of AChE, while Rivastigmine also inhibits butyrylcholinesterase that can also hydrolyze ACh in the brain and possibly plays a role in cholinergic transmission [24].

Another characteristic of AD is the increase of extracellular glutamate, which is thought to lead to excessive activation of NMDA receptors and consequently to intracellular accumulation of calcium (Ca^{2+}) [28]. This intracellular accumulation of Ca^{2+} then initiates a cascade of events that results in further neuronal death [29] **(see 1.3.6)**. Memantine, a non-competitive, moderate affinity NMDA antagonist can protect

neurons from excitotoxicity without preventing the physiological activation of the NMDA receptor. Memantine was approved in 2002 for the treatment of moderate to severe AD.

In February 2020, Suvorexant, an orexin receptor antagonist became the first medication to be approved for treating sleep disorders in AD (https://www.alzforum.org/therapeutics/suvorexant ; accessed: 29th September 2020)

1.1.7 – The Quest for an Alzheimer Therapy

As stated before, the drugs so far approved for AD do not stop or delay the disease progression. The increasing awareness that dementia, and in particular AD, represents one of the major challenges to health systems in coming years has led to an unprecedented emphasis on the need for an effective therapy, now considered as a priority for science and society. The year 2025 has been set by world leaders as the target for the availability of an effective therapy or prevention of AD [2]. It has been estimated that the overall frequency of the disease would be decreased by nearly 50% if the onset of the disease could be delayed by 5 year [30]. However, despite the enormous efforts and costs in search for new and effective agents for AD treatment in the past decade, there are still no new drugs on the market [31].

In the last decade, a major focus was put on disease modifying therapies (DMTs). DMTs are agents that prevent, delay, or slow progression and target the underlying pathophysiologic mechanisms of AD (*e.g*. amyloid targeted therapies) [30,32]. Up to date, 251 therapies for AD have been tested or are currently on clinical tests**.** From those, 6 therapeutics were approved **(see 1.1.6)**, 106 are inactive or discontinued, 1 is not regulated and 129 still on clinical trials (https://www.alzforum.org/therapeutics; accessed: 29th September 2020). From the 129 drugs actually on clinical trials (Phase 1 to 3), the majority are directed to amyloid, whereas the remaining therapies are focused on other targets such as: cholesterol, cholinergic system, inflammation, metals, neurotransmitters, Tau and other/ unknown mechanisms **(Figure 1.3).**

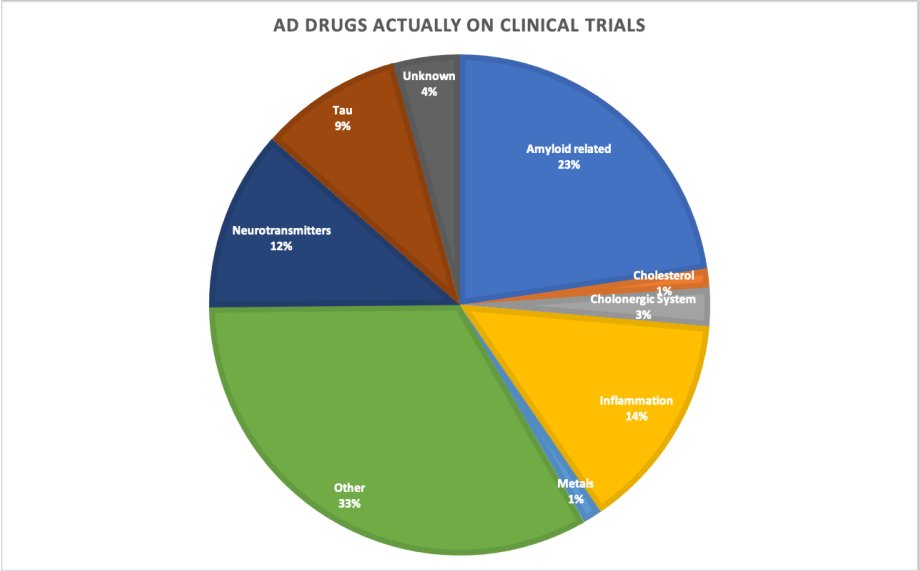


Figure 1.3 – AD drugs currently on clinical trials. (https://www.alzforum.org/therapeutics; accessed: 29th September 2020)

The majority of the drugs tested so far on clinical trials were designed to target Aβ and based on the "amyloid cascade hypothesis". This hypothesis proposed that deposition of Aβ was the causative agent of AD pathology and that the NFTs, cell loss, vascular damage, and dementia followed as a direct result of this deposition [33]. However, in recent years, this hypothesis has been modified and challenged principally due to substantial failures in clinical trial drugs based on it [17,34–37]. Nevertheless, in July 2020 Biogen submitted Aducanumab for FDA approval. Aducanumab is a therapeutic antibody developed to clear amyloid plaques from the brain. If approved, this would be the first biologic, first anti-amyloid treatment—and the first new drug for AD in more than 16 years in the U.S. (https://www.alzforum.org/news/research-news/biogen-asks-fda-approve-aducanumab ; accessed: 29th September 2020).

With the recent failures of clinical trials directed to amyloid, smaller companies are focusing on AD inflammation. The immune system is an important mediator in the pathogenesis of AD [38], so new and already approved (for other conditions) anti-inflammatory agents are being tested for AD. Drugs targeting different types of neuronal receptors involved in neuronal plasticity and signal transduction (neuronal transmitters) are another type of AD drug on clinical trials. These include: serotonin

receptors ligands, glutamate receptors ligands and ligands of other receptors and ionic channels. Tau is the component of one of the main pathological hallmarks of AD (NFTs), so drugs have also been designed against this molecule, namely Tau immunotherapies. Drugs that target metals, cholesterol and cholinergic system **(see section 1.1.6)** are less studied in actual clinical trials. However, the major number of drugs actually on clinical trials does not fit one of the categories aforementioned and are represented in **figure 1.3** in a miscellaneous group denominated as "others". This group is composed mainly by small molecules which target a variety of signalling pathways involved in AD.

To complete the picture, there are also other therapies in clinical trials for AD with unknown mechanisms such as deep brain stimulation or young plasma transfusion (https://www.alzforum.org/therapeutics, reviewed by Bachurin et *al*. [31]). New targets such as vascular function, epigenetics, neurovascular junction, blood-brain barrier targets and the role of gut microbiota are also being studied (reviewed by Loera-Valencia [39]). The multifactorial nature of AD is commonly recognized, implying the involvement of a number of neurobiological mechanisms in the etiopathogenesis of this neurodegenerative disease. In this context, the concept of multitarget drugs having an integrated action on a number of biological targets involved in pathogenesis of the disease appears to be highly promising in the design of new drugs for treating AD [31].

1.1.8 – Risk factors for Alzheimer's Disease

With the exception of cases of AD caused by genetic mutations (see below), experts believe that AD, like other common chronic diseases, develops as a result of multiple factors rather than a single cause [3]. Age is considered the greatest risk factor for developing AD. Accordingly, AD can be classified as Early Onset AD (EOAD, <65 years) and late-onset AD (LOAD, ≥65 years) depending on its age of onset (AOO) [40,41]. Of all AD patients, around 10% are diagnosed with EOAD. EOAD patients present with their first symptoms between 30 and 65 years with most of the EOAD patients being diagnosed between 45 and 60 years. These can present different symptoms compared to LOAD [40]. It is important to note that AD is not a normal part of aging, and older age alone is not sufficient to cause it.

Other risk factor is family history. Individuals who have a parent, brother or sister with AD are more likely to develop the disease than those who do not have a first-degree relative with the disease. When diseases run in families, heredity (genetics) and shared environmental and lifestyle factors (for example, access to healthy foods and habits related to physical activity) may play a role. Another risk factor for AD is carrying one or two ε4 alleles of apolipoprotein E (*APOE*) gene [42,43] **(see section 1.1.9).** Although risk factors such as age, family history and number of ε4 alleles cannot be changed, others can be changed or modified, to reduce the risk of cognitive decline **(Table 1.3)**. There is strong evidence, from a population-based perspective, that regular physical activity and management of cardiovascular risk factors (specially diabetes, obesity, smoking and hypertension), a healthy diet and lifelong learning/cognitive training may reduce the risk of cognitive decline [44]. Livingston *et al*. estimated that as much as 35% of dementia cases could be prevented by targeting nine modifiable risk factors (early life education; midlife hypertension, obesity, hearing loss; old-age smoking, depression, physical inactivity, diabetes, and social isolation) [45].

Table 1.3 – Modifiable risk factors for Alzheimer's Disease.

Modifiable risk factors	Characteristics
Alcohol	Moderate alcohol consumption is a modifiable protective factor, while heavy alcohol consumption may promote dementia.
Blood Pressure	There is a suggestion of an age-dependent relationship, i.e., hypertension may be harmful in midlife and protective in late-life.
Diabetes	There is an association between diabetes diagnosis and increased risk of AD, suggesting that measures to prevent diabetes such as exercise, weight reduction and diet control will likely provide some protective benefit.
Dietary pattern	There is an inverse relation between AD and a healthy dietary (diet high in fruits and vegetables, low in red and processed meats, and favouring mono- and polyunsaturated fats over saturated fat)
Education	People with more years of formal education are at lower risk for AD and other dementias. Some researchers believe that having more years of education builds "cognitive reserve."

Head Injury	There is a harmful effect of head injuries, with risk especially elevated among those whose head injuries occur later in life or are more severe.
Homocysteine	Higher levels of plasma total homocysteine may be associated with an increased risk of incident AD
Obesity	There is some evidence suggesting that obesity may be associated with AD and total dementia.
Physical Activity	There is an inverse association between physical activity and risk for both AD and dementia
Social and Cognitive Engagement	Studies suggest that remaining socially and mentally active throughout life may support brain health and possibly reduce the risk of Alzheimer's and other dementia

Table based on information of Alzrisk Database [46].

1.1.9 – Alzheimer's Disease genetics: an overview

Both EOAD and LOAD have a strong genetic component. Whereas in contrast to LOAD, which is a complex disorder with a heterogeneous etiology and an heritability of 70 to 80%, EOAD is an almost entirely genetically determined disease with a heritability ranging between 92% to 100% [40]. The first genes associated with AD (*APP*, *PSEN1*, *PSEN2*, *APOE* and *ADAM10*) have been unveiled using linkage studies [41]. These studies have been performed in the early 1990s and played a major role in identifying the genes associated with AD. Moreover, they aimed to define independence of the transmission between disease and genetic markers using the joint segregation of disease, as well as genetic markers in a series of AD families [41,47].

APP was the first discovered gene associated with AD. In 1991 Goat *et al*. identified a mutation associated with AD in the *APP* gene (V717I; London mutation) [48]. *APP* encodes the APP protein, which generates Aβ through amyloidogenic pathway **(see section 1.1.5)**. In 1995, further studies unveiled another gene locus harbouring AD associated mutations: *PSEN 1* [49] and *PSEN2* [50]. *PSEN1* and *PSEN2* encodes the aspartyl proteases Presenilin 1 and 2 (PS1 and PS2), which constitute the catalytic part of γ – secretase complex [20,51]. Mutations in these three genes are thought to affect AD by increasing the $A\beta_{42}/A\beta_{40}$ ratio (reviewed by Bekris and Cacace *et al*. [40,52]). To date, 51, 219 and 16 different mutations have been identified in *APP, PSEN1* and *PSEN2*

genes, respectively (http://www.molgen.ua.ac.be/ADMutations) [53] **(Table 1.4)**. *APP*, *PSEN1* and *PSEN2* mutations are associated with EOAD. However, these mutations only explain 5-10% of EOAD patients, leaving a large group of autosomal dominant pedigrees genetically unexplained (reviewed by Cacace *et al*. [40]) **(Figure 1.4).** The frequencies for the three genes mutations are 1% for APP, 6% for PSEN1, and 1% for PSEN2 in EOAD cohort [40].

Table 1.4 – Causal Early Onset Alzheimer's Disease genes.

Gene	Chromosome location	Inheritance	Mutation spectrum	Mutations (N)
APP	21q21.1–21q21.3	Autosomal dominant Autosomal recessive	• Missense • Gene Duplication • Amino acid deletion	51
PSEN1	14q24.3	Autosomal dominant *de novo*	• Missense • Small indels • Genomic deletions	219
PSEN2	1q31–q42	Autosomal dominant	• Missense	16

Adapted from Cacace *et al*. [40].

In 1993 the ε4-allele of the *APOE* gene was identified on chromosome 19q13.2 as a genetic risk factor for LOAD [43,54,55]. To date, the ε4 allele of *APOE* is the most highly replicated genetic risk factor for LOAD [42,56,57], being also associated with EOAD (reviewed by Cacace *et al*. [40]). There are three common alleles of *APOE*: ε2, ε3, and ε4 alleles, which have a worldwide frequency of 8.4%, 77.9% and 13.7%, respectively. However, the frequency of the ε4 allele is dramatically increased to about 40%, in patients with AD [58]. *APOE* ε4 increases LOAD risk, whereas *APOE* ε2 is associated with decreased LOAD risk [56]. The presence of one or two copies of the APOE ε4-allele increased the risk to develop LOAD by a factor of 3 to 15-fold in a dose-dependent manner [42,58] . The presence of the ε4 allele of the *APOE* gene, not only dose dependently increases the risk for AD, but also lowers the AOO [42,56]. The frequency of AD and mean age at clinical onset are 91% and 68 years in ε4 homozygotes, 47% and 76 years in ε4 heterozygotes, and 20% and 84 years in ε4 noncarriers [42]. Moreover, the impact of the *APOE* ε4 could only account for about 27% of the disease heritability [41]. *APOE g*ene encodes the Apolipoprotein E (ApoE) protein, which is a

glycoprotein that regulates lipid homeostasis by mediating lipid transport from one tissue or cell type to another [56,57]. ApoE has also a role on Aβ metabolism and clearance, Tau phosphorylation, brain activity and atrophy, brain cholesterol transport, synaptic plasticity, inflammation and brain neurogenesis, as reviewed by Liu *et al*. [57].

The last gene unveiled by linkage studies was *ADAM10* on chromosome 15q21.3. In 2009, two variants (Q170H and R181G) of this gene have been reported to be associated with AD. ADAM10 is the catalytic component of α-secretase complex, which mediates the cleavage of APP in the non-amyloidogenic pathway [20]. It is thought that these two variants contribute to the shift of APP proteolysis toward the amyloidogenic pathway, by disrupting α-secretase activity [59] **(see section 1.1.5).**

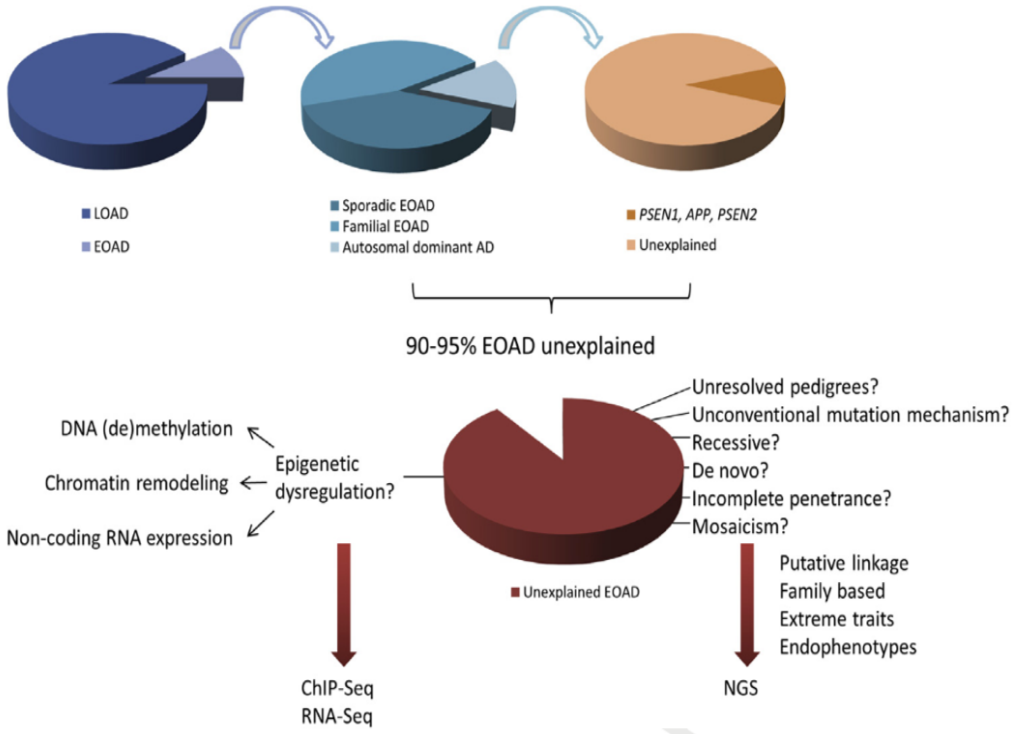


Figure 1.4 – Missing genetic etiology of Early Onset Alzheimer's Disease. The pie charts indicate the distribution of EOAD and LOAD, the fraction of sporadic and familial EOAD patients with the sub-fraction of autosomal dominant patients (light emerald). The orange pie chart depicts the fraction of unexplained autosomal dominant families. The possible mechanisms that may explain the missing genetic etiology of EOAD are divided in two groups arising from the red pie chart: (1) possible undetected genetic alterations due to different causes, listed arising from the red pie chart (right side) and (2) possible undetected epigenetic dysregulation (left side). For both scenarios, some examples of study designs (e.g., family based, extreme trait design and so forth or investigation of DNA(de) methylation and so forth) and technological approaches such as next-generation sequencing (NGS), chromatin immunoprecipitation assay combined with sequencing (ChIP-Seq) and RNA sequencing (RNA-Seq) are schematically suggested. Adapted from Cacace *et al*. [40].

More than a decade after the identification of *APOE* ε4, large European and international genome-wide association studies (GWAS) uncovered novel risk genes for AD. Until now, more than 20 genetic loci have been associated with AD by using GWAS. Similar to other genetic association studies, GWAS are also used to identify the association between genetic variants and AD or AD-related phenotype in different populations. Nevertheless, since with this methodology millions of single-nucleotide polymorphisms (SNPs) are evaluated in thousands of samples, its success depends on sample size, frequency of risk alleles, and individual effect sizes [41,60]. The main gene hits discovered by GWAS according to the Alzgene database (http://www.alzgene.org/) [61] are described on **Table 1.5** and reviewed by Giri and Zhu *et al.* [41,60]. Other genes such as: *SORL1, HLA-DRB5-DRB1, PTK2B, CASS4, INPP5D, CELF1, MEF2C, SLC24A4-RIN3, ZCWPW1, NME8, FERMT2* have also been unveiled using GWAS [41,62]. Despite the utility of GWAS to unveil new risk genes for AD, genetic heritability, frequently caused by rare genetic variants with large effects for AD cannot be captured by GWAS.

Other risk variants such as: *TREM2, PLD3, UNC5C, AKAP9, TM2D3* have been unveiled using Next Generation Sequencing (NGS), reviewed by Giri and Zhu *et al*. [41,60]). All the genes described above and respective pathways in AD are depicted in **Figure 1.5.**

Table 1.5 – Risk variants for Alzheimer's Disease.

Gene	Location	Polymorphism	Known function	Pathways
1. APOE*	19q13.2	ε4/ε4 ε3/ε4	Lipids transport	Cholesterol
2.BIN1	2q14.3	rs7562528 rs744373	Synaptic vesicle endocytosis	Endocytosis
3.CLU	8p21-p12	rs11136000, rs2279590 rs93318	Molecule Chaperone Lipid transport	Cholesterol, immune response
4.ABCA7	19p13.3	rs3764650 rs3752246	Transportation of phospholipids and phagocytosis	Cholesterol
5.CR1	1q32.2	rs6656401 rs3818361 rs1408077	Modulation of complement system	Immune response

6.PICALM	11q14.2	rs3851179 rs541458	Clathrin-mediated endocytosis	Endocytosis
7&9.MS4A6A/E	11q12.2	rs610932(A) rs670139(E)	Signal transduction	Immune response
9.CD33	19q13.41	rs3865444	Cell-cell interactions,	Immune response
CD2AP	6p12.3	rs9296559 rs934940	Regulation actin cytoskeleton, vesicle movement	Endocytosis

Table based on AlzGene Top Results [62].In this table the top risk genes are ranked based on Hugenet interim guidelines for the assessment of genetic association studies [63]. *All genes in this table have been discovered using GWAS methodology with exception for *APOE* gene.

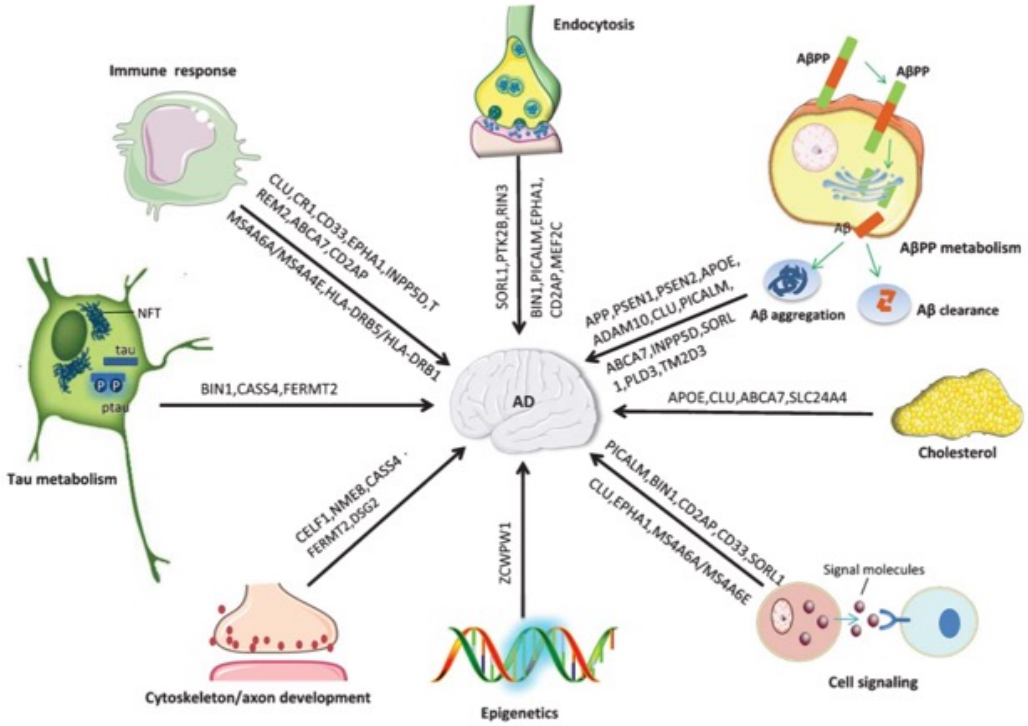


Figure 1.5 – Potential pathways of susceptibility genes involved in the pathogenesis of Alzheimer's Disease. Adapted from Zhu et *al.* [41].

1.2 – *TOMM40* as a risk gene for Alzheimer's Disease

1.2.1 – *TOMM40* rs10524523 polymorphism

The most relevant discoveries in fine-mapping and GWAS studies for LOAD are detected within a linkage disequilibrium (LD) region of Chr:19q13.32 that incorporates *APOE*, *TOMM40* and *APOC1* genes [64–66] **(Figure 1.6).** Within this region, multiple polymorphisms have been related with LOAD risk, namely within the *TOMM40* gene. This gene encodes the Tom40 protein (translocase of the outer mitochondrial membrane, 40 kD) which forms the channel subunit of the outer mitochondrial membrane through which the majority of nuclear-encoded proteins enter mitochondria [67,68] **(See section 1.3.3)**. Different polymorphisms in this gene, such as rs11556505, rs17664883 and rs157584 [69]; rs157580 [70] and rs2075650 [71] have been associated with AD risk and other AD-related features. Nevertheless, in this chapter I will focus in the rs10524523 polymorphism.

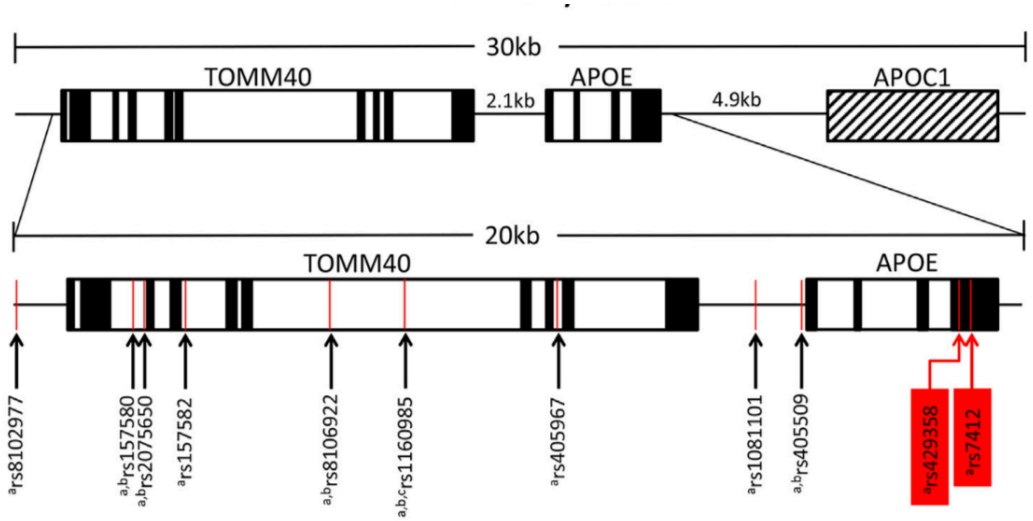


Figure 1.6 – *TOMM40-APOE-APOC1* linkage disequilibrium region. Linkage disequilibrium occurs when a particular marker allele is associated with the disease-trait locus at a greater than expected frequency across multiple families. Adapted from Roses *et al.* [72].

Using deep sequencing and phylogenetic analysis, Roses *et al*. discovered a polymorphic poly-T variant, rs10524523 in the *TOMM40* gene, which was associated with AOO and risk of AD [73] *TOMM40* rs10524523 polymorphism (hereafter

*TOMM40'*523) is a variable length, deoxythymidine homopolymer located in chromosome 19 at position 45403049 (Genome Build 37.1) within intron 6 of the *TOMM40* gene (Ensembl: ENSG00000130204).

In the human reference sequence, the number of thymidine (T) residues in the homopolymer is 35, and the variant allele described by rs10524523 is a 19-base pair (bp) deletion (*i.e*. the variant allele is 16 T residues). However, a wide range of lengths have been described for this homopolymer (11-54 T residues) in different ethnicities, where the longest homopolymers were noted in the African-American (54 T residues) and the shortest in Japanese and Korean cohorts [74] **(Table 1.6)**. Meanwhile, based on the modes of distribution of the number of T residues, three categories (alleles) of repeat length were established: short (S, ≤ 19), long (L, 20–29) or very long (VL, ≥ 30) [75] **(Figure 1.7)**. Similar to the variation in the number of T residues, the frequency of S, L and VL alleles also varies across ethnicities. Far-Eastern cohorts (Japanese, Korean and Han Chinese) are enriched for VL allele with a concomitant decrease of S allele when compared to Caucasian cohort. In contrast, African-American and Ghanaian cohorts have an enrichment of the S allele and concomitant decrease of VL allele when compared to Caucasian cohort. Caucasian and Hispanic have a similar allele distribution **(Table 1.6).** These data show the importance of studying *TOMM40'* 523 polymorphism in different ethnicities worldwide.

In the Caucasian population it has been demonstrated in different studies that the *APOE* ε4 allele is almost exclusively linked to an L allele, whereas the ε3 allele is linked to either a S or a VL allele. **(Figure 1.7)** [73,75]. The same was observed for the Hispanic population [74]. Unlike Caucasian and Hispanic cohorts, African American and Ghanaian population showed a significant number of ε4 alleles linked to S alleles. Interestingly, the Japanese population had a frequent occurrence of ε4-S haplotypes similar to African-American and Ghanaian populations. On the other hand, Korean and Han Chinese demonstrated a similar allelic distribution to that observed in Caucasian and Hispanic cohorts [74]. A later study confirmed some of the results above and concluded that the poly-T distribution observed in African-Americans is an admixture of West Africans (Yoruban) and Caucasian populations [76]. A subsequent study investigated the effect of ε4- *TOMM40* '523 haplotype variations on the risk of incident AD dementia among older Caucasians and African Americans [77]. Since the studies referred next

were performed in Caucasian populations, I will pay attention to the alleles occurring in this population. According to the relation observed between *APOE* and *TOMM40* genes there are six different genotypes: S/S, S/VL and VL/VL for *APOE* ε3/ε3; S/L; L/VL for *APOE* ε3/ε4 and L/L for *APOE* ε4/ε4.

Table 1.6 – *TOMM40*'523 allele frequencies in different ethnicities.

Ethnicity	Subjects	S (%)	L(%)	VL (%)	Poli-T range
Korean	60	20	8	72	11-38
Japanese	60	24	18	58	11-35
Han Chinese	60	38	10	52	12-36
Hispanic	179	43	9	48	14-39
Caucasian	177	45	11	44	14-39
African Americans	370	65	10	25	14-54
Ghanaian	40	71	8	21	12-36

Table based on Linnertz *et al*. [74].

1.2.2 – *TOMM40*' 523 and Alzheimer's Disease Risk and Age of Onset

In the same study referred above, where Roses *et al*. described the *TOMM40' 523* polymorphism the authors also demonstrated that individuals with longer poly-T repeats (> 27 T) linked to ε3 developed LOAD on average of 7 years earlier than individuals with shorter poly-T repeats (< 27 T) linked to ε3 (70.5±1.2 years versus 77.6±2.1 years) in a ε3/ε4 cohort [73]. However, other independent studies failed to reproduce this result. In a larger cohort than the initial Roses' cohort, Cruchaga *et al*. found a significant association between *TOMM40'523* and risk of LOAD in the opposite direction that Roses reported [78]. When analysis was restricted to *APOE* ε3/ε3 the authors' found that VL allele was underrepresented in AD cases vs. controls, which

shows a protective role of VL allele. Moreover, in the whole population VL carriers show a higher, but not statistically significant different AOO than short allele carriers. Furthermore, the same was found for ε3/ε4 patients (same population addressed in the study by Roses *et al*. [73]).

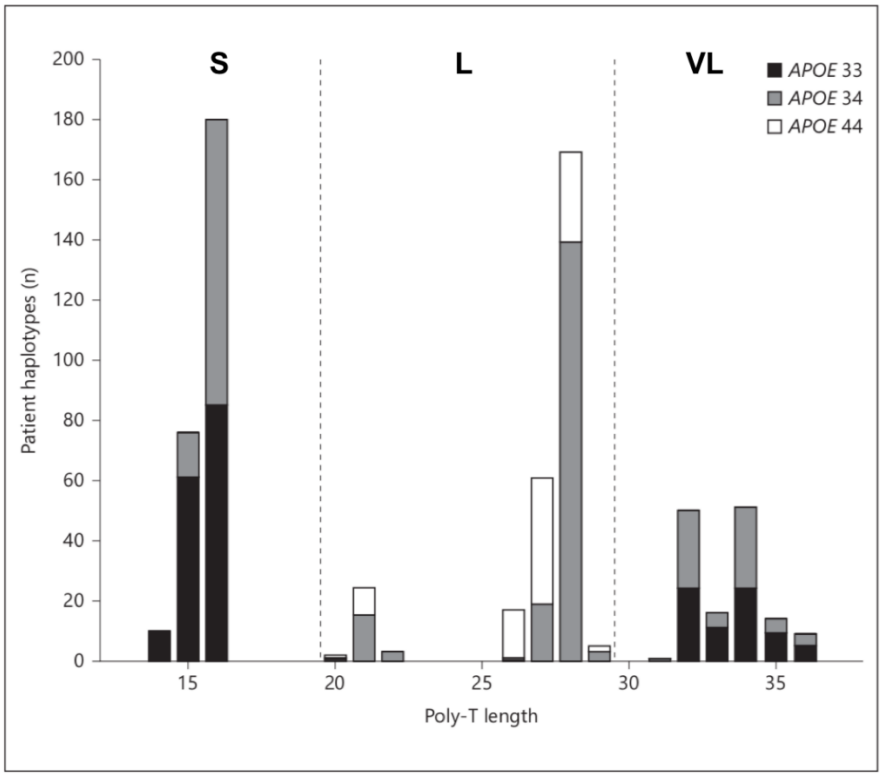


Figure 1.7 – Example of a poly-T distribution in a Caucasian cohort. *TOMM40*' 523 alleles are classified into: short (S, ≤ 19), long (L, 20–29) or very long (VL, ≥ 30). *APOE* ε3/ε3 genotypes are associated with S and/or VL alleles whereas *APOE* ε4/ε4 genotypes are almost exclusively associated with L alleles. As a consequence, *APOE* ε3/ε4 have an admixture of S, L and VL alleles. Adapted from Helisalmi *et al.*[79].

In line with the study by Cruchaga *et al.* [78], Maruszak *et al*. also reported significantly lower frequency of VL allele in LOAD cases compared to controls and centenarians [80]. It is important to emphasise that in both studies referred, the *TOMM40*' 523 polymorphism was classified as S, L, and VL, where VL allele has ≥ 30 T residues. In contrast, in the Roses *et al*. study [73] the cut-off for "longer" alleles was > 27 T residues, thus making comparisons difficult. To study the relation of AOO and *TOMM40*' 523 and replicate Roses *et al*. findings, Chu *et al*. fitted models using as predictor variables: the count of *APOE* ε4 and a binary variable with a division between

small and long repeats (long>27), as Roses *et al*. described [73] or an ordinal factor based on the poly-t allele distribution. After accounting for the ε4 allele counts, neither one of the poly-T predictors was significant [81]. Similarly, the association found by the effect of L allele both in risk and AOO by Jun *et al.* [82] and Helisalmi *et al*. [79]. was attributed to ε4 allele.

Bernardi *et al*. investigated the association between *TOMM40'* 523 and AOO, in patients with the *PSEN1* M146L mutation in a large familial AD Calabrian kindred, and found that VL/VL patients had a tendency for an earlier AOO compared to those with VL/S and S/S in *APOE* ε3/ε3 patients [83]. On the other hand, in a cohort of homozygous carriers of *APP* A713T mutation, Conidy *et al.* demonstrated that the the large span of AOO in this cohort of patients was not influenced by *TOMM40'* 523 polymorphism [84]. In *APOE* ε3/ε3 individuals Li *et al*. observed that the presence of a long poly-T was associated with an earlier AOO in patients with *PSEN2* mutation, whereas in families with a *PSEN1* mutation or in patients with LOAD, this association was not found [85]. Note that in this study poly-T length was dichotomized into short (<20) and long (>20), where the vast majority of the alleles (98%) that were considered long had 34 or more T repeats, consistent with VL alleles.

Lastly, Crenshaw *et al*. presented a stratification, by *TOMM40' 523* and *APOE* genotype of the AOO cognitive impairment for cognitively normal individuals followed in the prospective Joseph Bryan ADRC cohort in order to create a risk algorithm for clinical trial enrichment **(Figure 1.8)** [86]. which was further evaluated in other studies [87,88].

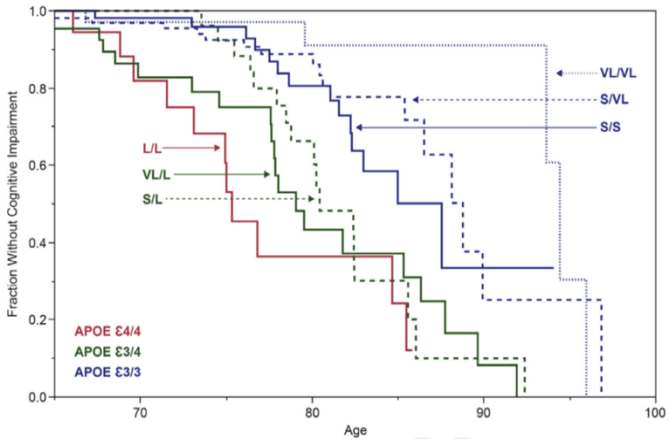


Figure 1.8 – Age of onset of cognitive impairment as a function of *TOMM40'* 523 and *APOE* genotype. Adapted from Crenshaw *et al*. [86]

1.2.3 – *TOMM40*' 523 and Cerebrospinal fluid biomarkers

To determine a possible mechanism underlying the observed disease risk associated with the *TOMM40*' 523 polymorphism, Cruchaga *et al*. evaluated if CSF $A\beta_{42}$, $A\beta_{40}$, t-Tau, or p-Tau levels were associated with this polymorphism. However, no association was found between *TOMM40*' 523 polymorphism and these CSF biomarkers. The authors argued that the *TOMM40*' 523 polymorphism may affect AD risk through another mechanism [78]. These results were confirmed in an independent study by Helisalmi *et al*. [79], in a smaller cohort of LOAD patients and by Pomara *et al*. in cognitive intact elderly individuals [89]. The differences observed in $A\beta_{42}$ by Helisalmi *et al*. were attributed to L/L group, which is strongly linked to *APOE* ε4/ε4, thus not attributed to *TOMM40'523* genotype [79]. In the same line Pomara *et al*. described significant reductions of $A\beta_{42}$ levels in ε4 carriers compared to non ε4 carriers, but no differences were detected across *TOMM40'523* variants [89]. Pomara *et al*. also addressed if *TOMM40'523* variants affected or mediated the effects of *APOE* ε4 in the CFS levels of cortisol [90] and Neurofilament light (NFL) protein [91] in healthy, cognitively individuals. Cortisol is a glucocorticoid released upon stressful events and is essential for human survival and for the ability of individuals to cope with stress. Evidence also suggests an association between higher brain concentrations of glucocorticoids and AD hippocampal pathology (reviewed by Pomara *et al*. [92]). This author demonstrated that the increase in CSF cortisol, associated with the presence of the *APOE* ε4 allele, was only detected when the S '523 allele was not present [90]. Similarly, Pomara *et al*. also found that individuals with *APOE* ε4 had higher CSF NFL levels than non-ε4 carriers, only when they do not carried a S allele of *TOMM40'523* [91]. NFL proteins in CSF are a marker of neuronal damage, especially subcortical axonal injury and white matter disease [93]. Patients with AD have shown elevated levels of CSF NFL as compared to controls [94]. In summary, these results suggest that S *TOMM40*' *523* allele might have a ''protective'' effect against the deleterious effects of *APOE* ε4 on cortisol and NFL levels.

1.2.4 – *TOMM40*'523 and brain structure

In a group of asymptomatic late middle-aged ε3/ε3 individuals, a dose-dependent increase in the VL *TOMM40*' 523 polymorphism (from no VL alleles, to S/VL heterozygous, to VL/VL homozygous) was associated with decreasing gray matter volume in the ventral posterior cingulate and medial ventral precuneus, which are regions of the brain associated with AD [95] . In *PSEN1* and *PSEN2* mutation carriers and LOAD *APOE* ε3/ε3 patients, Li *et al*. demonstrated that individuals with a higher number of long poly-t alleles (zero vs. one vs. two) were found to exhibit high levels of neuritic tangles (Braak stage of V– VI) and higher frequency of pathological defined AD. In this study poly-T length was dichotomized into short (<20) and long (>20), where the vast majority of the alleles (98%) that were considered long had 34 or more T repeats, which is consistent with VL alleles. However, in this study there was not an association with the presence of amyloid pathology, cerebrovascular damage or Lewy body pathology [85]. Using as sample the Lothian Birth Cohort 1936 (LBC1936), which is a longitudinal ageing sample of a generally healthy community-dwelling older adults [96], the impact of *TOMM40*' 523 on different brain structure aspects has been addressed. In a first study, using brain structural magnetic resonance imaging (MRI), no significant effects of *APOE* or *TOMM40'*523 genotype were found on hippocampal volumes when analyzed raw, or when adjusted for either intracranial or total brain tissue volume [97]. Nevertheless, other study performed in the same cohort demonstrated deleterious effects of the *TOMM40'523* S allele (vs L or VL) on specific tracts of white matter integrity [98]. In another study, Lyall *et al*. analyzed the association between *TOMM40' 523* and white matter hyperintensities and cerebral microbleeds, which commonly occur in people with AD [99] but no significant effects were found [100]. White matter tracts are thought to relate to cognitive functions [100]. Burggren *et al*. demonstrated that in older, normal control individuals who do not carried the *APOE* ε4 genotype, longer *TOMM40* poly-T lengths were significantly associated with thinner entorhinal cortex (ERC) [101]. Substantial work has shown that the first brain changes in AD begin in ERC [102]. In line with this finding, Laczo *et al*. demonstrated that VL/VL group had thinner left entorhinal cortex and left posterior cingulate cortex than the S/S group in aMCI patients [103]

1.2.5 – *TOMM40*' 523 and cognition

Similar to the findings on the impact of *TOMM40*' 523 polymorphism on grey matter reported above, Johnson *et al*. also demonstrated that the VL/VL group had a lower performance than the S/S *TOMM40* group on a test of primary retrieval from a verbal list learning task in a group of asymptomatic late middle-aged ε3/ε3 individuals [95]. Hayden *et al*. showed that in cognitively normal participants, the S/S homozygotes had better cognitive performance than those with the VL allele and that subjects with L and VL *TOMM40*'523' genotypes had mild deficits in cognitive domains that are typically affected in early AD [104]. In a cohort of cognitively normal individuals aged 21 to 97 years, Caselli *et al*. found that those homozygous for the VL were associated with accelerated memory decline using the test-retest flattening [105]. In another study, using the Lothian Birth Cohort 1921, Schiepers *et al.* found that only the L *TOMM40'523* allele variant, which is linked to the *APOE* ε4 allele, was predictive of the rate of cognitive change in non- demented older individuals [106]. Using the LBC1936 (described above), Lyall *et al*. demonstrated that *TOMM40'*523 was not associated with cognitive ageing [107]. Among a sample of cognitively normal Jewish elderly with type 2 diabetes, Greenbaum *et al*. described that carriers of the *TOMM40'*523 S/S genotype performed significantly better than individuals with the *TOMM40'*523 VL/VL genotype on executive function and episodic memory tasks, when controlling for multiple relevant demographic and cardiovascular variables [108]. In a cohort of community-dwelling elderly volunteers who had been followed for changes in cognitive functioning over a period of 14 years, Payton *et al*. showed that the shorter poly-T variant was significantly associated with reduced vocabulary ability and a slower rate of vocabulary decline with age, compared to the very long poly-T variants [109]. Using annual cognitive data from community-based older caucasian Americans followed for up to 21 years, Yu *et al*. demonstrated that participants with *TOMM40'*523 S/S genotype had faster decline in global cognition than participants with S/VL or VL/VL *TOMM40'*523 genotypes and that this effect was driven primarily by episodic and semantic memory [110]**.** In a group of ε3/ε3 homozygotes with aMCI, Laczo *et al*. demonstrated that the S/S group performed better on allocentric navigation than S/VL and VL/VL groups [103]. On the

other hand, in a cohort of cognitively normal, aMCI and AD subjects, Watts *et al*. demonstrated that participants with two *TOMM40* S alleles showed lower baseline performance compared to those with two very long alleles, regardless of clinical status using attention, verbal memory, and executive function tests [111].

1.2.6 – *TOMM40*' 523 risk alleles and biological insights

As reported above, several studies show an independent association of *TOMM40*'523 variant with different AD-related features such as: AD risk and AOO; biomarkers; brain structure and cognition **(Table 1.7),** while others did not. A number of methodological aspects that may account for the contradictory results and requirements for the replication this type of studies are reviewed by Roses *et al*. [112]. Watts *et al*. further suggest that future research would benefit from harmonization of the cognitive domains studied, and more consistent methods of combining multiple test scores to enable better comparisons [111]. However, other authors defend that *TOMM40* poly-T associations with LOAD-related phenotypes are simply a consequence of LD with the *APOE* [113]. On the other hand, in studies where an independent association of *TOMM40* poly-T was found, the identity of the TOMM40 poly-T risk allele has been controversial (S vs VL; reviewed by Chiba-Falek *et al*.[114]). While several studies have suggested that VL allele has a risk effect, other studies have suggested a beneficial effect **(Table 1.7)**. Chiba-Falek *et al*. suggest that the identity of the *TOMM40* poly-T risk allele depends on the phenotype being evaluated, the age of the study individuals at the time of assessment, and the context of the *APOE* genotypes [114].

Another example of alleles that display both risk and protective effects that depend on the evaluated phenotypes and/or age, are *APOE* alleles. In the context of neurodegenerative disorders, *APOE* ε4 is associated with higher risk for LOAD, and *APOE* ε2 is considered as a protective allele **(see 1.1.8),** but with respect to the lipoprotein disorder type III dysbetalipoproteinemia, homozygosity for *APOE* ε2 is the primary causal genetic factor [115]. On the other hand, it was also suggested that the *APOE* ε4 has beneficial effects on cognition and other traits in early ages (reviewed by Zetterberg *et al*. [116]). To gain more insights on the risk associated with *TOMM40*' 523 alleles, there is an unmet need to translate these findings to biological mechanism. Moreover,

Linnertz *et al*. found that *TOMM40* mRNA levels were dose-dependent on the number of VL alleles in brain samples from cognitively normal individuals [117]. These results were confirmed in the same study and other two studies using luciferase model constructs [69,109].

Understanding, in depth, the mechanistic role of the TOMM40 poly-T will provide insights on the phenotypic outcome of *TOMM40* poly-T. Toward this goal, current research directions should focus on developing strategies, including *in vitro* and *in vivo* models (reviewed by Chiba-Falek *et al*. [114]).

Table 1.7 – Independent associations of *TOMM40* poly-T with LOAD-related features.

Study	Type of Study	Associated Phenotype	Sample size	Age years (mean ± SD)	APOE	TOMM40 risk allele
Roses [73]	AD Risk/AOO	AOO	34	71.5 ± 6.7	3/4	VL
Cruchaga [78]	AD Risk/AOO	AOO	495	75.0 ± 8.5	3/3	S
Crenshaw [86]	AD Risk/AOO	AOO	232	74.8 ± 9.6	3/3	S
Crenshaw [86]	AD Risk/AOO	AOO	161	71.4 ± 8.7	3/4	VL
Johnson [95]	Brain Structure	Gray Matter Volume	117	55.5 ± 6.0	3/3	VL
Burggren [101]	Brain Structure	Hippocampal thickness	41	63.5 ± 9.2	3/3	VL
Johnson [95]	Cognition	Primacy retrieval from a verbal list	117	55.5 ± 6.0	3/3	VL
Hayden [104]	Cognition	Memory and executive function	101	80.6 ± 6.0	3/3	VL
Caselli [105]	Cognition	Test-retest flattening	336	57.8 ± 12.1	3/3	VL
Greenbaum [108]	Cognition	Memory and executive function	331	72.0 ± 4.9;	3/3	VL
Payton [109]	Cognition	Rate of Vocabulary decline	1613	65	All	VL
Payton [109]	Cognition	Vocabulary–single time point	1613	65	All	S
Yu [110]	Cognition	Rate of cognitive decline	1170	78.5 ± 7.4	3/3	S
Watts [111]	Cognition	Attention and Executive Function	170	72.34 ± 7.6	3/3	S

Adapted from Chiba-Falek *et al*. [43].

1.3 – Mitochondria in health and disease – an overview of mitochondria role in Alzheimer's Disease.

1.3.1 – An overview of mitochondria structure and function

Mitochondria are essential for the viability of eukaryotic cells and are commonly seen as the powerhouses of the cell. However, despite the production of adenosine triphosphate (ATP), many other functions performed by mitochondria are known, such as: ion homeostasis, small-molecule biosynthesis, cell cycle and growth, cell survival, cellular metabolism, cell signalling, cellular redox status maintenance, Ca^{2+} homeostasis, apoptosis, proteostasis and innate immunity [68,118,119]. In this section, I will review the main structure of mitochondria, its main functions and how unhealthy mitochondria can be involved in disease, focusing on AD.

In 1948, Eugene Kennedy and Albert Lehninger discovered that oxidative phosphorylation (OXPHOS) occurs in mitochondria. Mitochondria consist of two membranes: the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM). Moreover, mitochondria have two aqueous compartments: the intermembrane space (IMS) and the matrix. The OMM is permeable to small molecules and ions, whereas the IMM is impermeable to most small molecules and ions, including protons (H^+). The surface area of the IMM is several-fold larger than that of the OMM, forming numerous folds named cristae. Cristae width and length determine the efficiency of the electron transport chain (ETC) [120]. ETC comprises a group of four multimeric protein complexes (I to IV) embedded in the IMM and two membrane-permeable electron (e^-) carriers (ubiquinone and cytochrome c). Movement of e^- through the ETC to O_2, its terminal acceptor, is accompanied by H^+ pumping across the IMM by complexes I, III and IV which in turn leads to an electrochemical gradient of H^+, also referred to as mitochondrial membrane potential ($\Delta\Psi m$). The resulting transmembrane potential and concentration of H^+ in the intermembrane space drives H^+ flow through the ATP synthase to provide the rotational energy for ATP synthesis [121] **(Figure 1.9).**

During ATP production, e^- can leak out of the ETC, in particular from complex I and III and react with oxygen to produce O_2^-, which in the end leads to the production of

reactive oxygen species (ROS), with superoxide anions, hydroxyl radicals, and hydrogen peroxide being the predominant forms [122,123]. To prevent oxidative damage by O_2^-, cells have different antioxidant defences such as: glutathione (GSH), manganese superoxide dismutase (MnSOD) and catalase (Cat) (reviewed by Andreyev *et al*. [124]) **(Figure 1.9)**. The nuclear factor erythroid-derived 2-related factor 2 (NRF2) is a regulator of a variety of antioxidant and detoxifying enzymes and has been shown to directly affect mitochondrial homeostasis via its regulation of nuclear respiratory factor 1 (NRF1) [125] **(see 1.3.2)**.

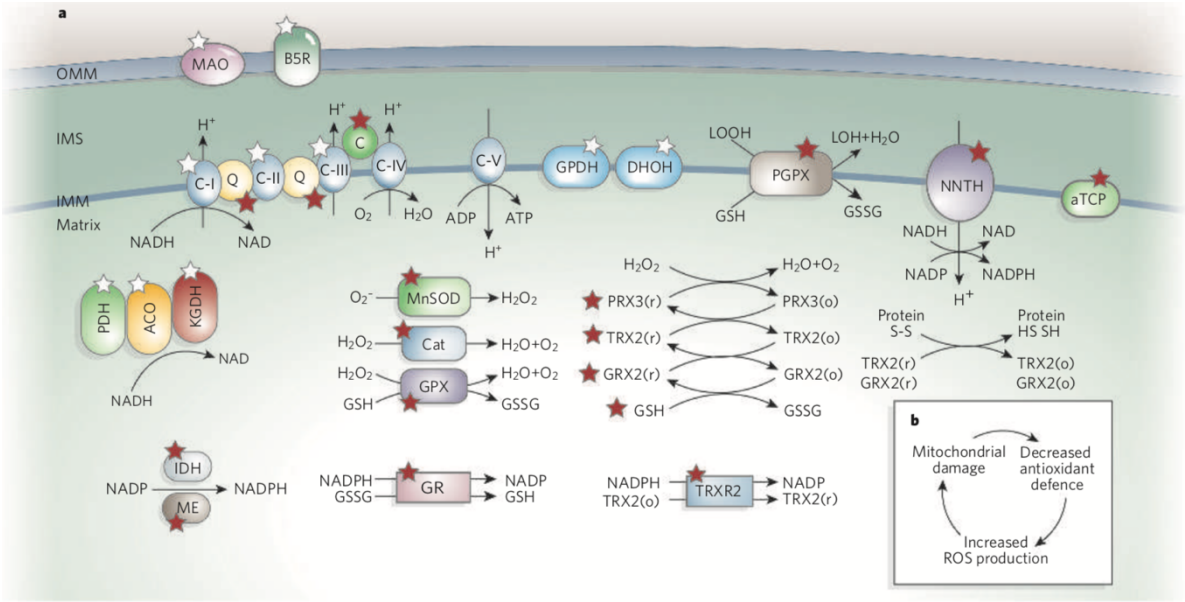


Figure 1.9 – **Antioxidant defences of mitochondria.** In this figure the components of electron transport chain (ETC) are presented. White stars indicate the processes and components involved in reactive oxygen species (ROS), while red stars mark the components of antioxidant defence. The mitochondria enzymes known to generate ROS include the tricarboxylic acid (TCA) cycle enzymes, aconitase (ACO) and α-ketoglutarate dehydrogenase (KGDH); the electron-transport chain (ETC) complexes I, II and III; dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH); glycerol-3-phosphate dehydrogenase (GPDH); dihydroorotate dehydrogenase (DHOH); the monoamine oxidases (MAO) A and B; and cytochrome b5 reductase (B5R). Cells have different antioxidants. Nonenzymatic components of the system include α-tocopherol (aTCP), coenzyme Q10 (Q), cytochrome c (C) and glutathione (GSH). Enzymatic components include manganese superoxide dismutase (MnSOD), catalase (Cat), glutathione peroxidase (GPX), phospholipid hydroperoxide glutathione peroxidase (PGPX), glutathione reductase (GR); peroxiredoxins (PRX3/5), glutaredoxin (GRX2), thioredoxin (TRX2) and thioredoxin reductase (TRXR2). In structurally and functionally intact mitochondria, a large antioxidant defence capacity balances ROS generation, and there is little net ROS production. Mitochondrial damage with decrease of antioxidant defence capacity is a prerequisite for net ROS production. Once this occurs, a vicious cycle (inset) can ensue, whereby ROS can further damage mitochondria, causing more free-radical generation and loss or consumption of antioxidant capacity. Adapted from Lin *et al*. [126].

Mitochondria also play a central role on apoptosis. Apoptosis is an active mechanism of programmed cell death in response to stress-inducing or regulatory signals. When active, Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) can form oligomers in the OMM, leading to the release of cytochrome c and other proapoptotic molecules such as Smac/Diablo and Omi/Htr2. Bax and Bak proteins belong to the B-cell lymphoma 2 family (Bcl-2), which is divided in tree functional groups: antiapoptocic, proapoptotic multidomain and proapoptotic BH3 only. The fate of the cell is determined by the balance of activity of proapoptotic and antiapoptotic family members, which act to regulate one another. [127]. Cytochrome c is an essential component of the respiratory chain that facilitates the transfer of electrons from complex III to complex IV, as reported earlier. However, the release of cytochrome c to the cytosol leads to the activation of the key initiator caspase (caspase-9), forming the apoptosome complex by binding to the apoptotic protease factor 1 (Apaf 1). Caspase-9 then activates caspase-3 by proteolytic cleavage, which in turn liberates the caspase-activated deoxyribonuclease (CAD) from its inhibitor, ICAD, resulting in apoptotic features of deoxyribonucleic acid (DNA) fragmentation and chromatin condensation. On the other hand, release of Smac/Diablo and Omi/Htr2 promotes cell death by inhibiting the action of IAPs (Inhibitor of apoptosis), which are inhibitors of caspases [127]. **(Figure 1.10)**. Cytochrome c is normally associated with cardiolipin in the IMM membrane. Thereby, the oxidation of cardiolipin results in both mitochondrial membrane permeabilization and cytochrome c dissociation and release. Mitochondria can also trigger a caspase-independent mechanism of apoptosis trough the release of proapoptotic proteins, such as apoptosis-inducing factor (AIF), which can travel further into the nucleus where it causes DNA fragmentation and chromatin condensation [122,127,128].

Another important role of mitochondria is Ca^{2+} homeostasis, by which mitochondria can function as a Ca^{2+} storage organelle. Under normal physiological conditions, Ca^{2+} is continuously shuffled between the endoplasmic reticulum (ER) and the mitochondria through the mitochondrial-associated ER membrane (MAM). MAM is a lipid-raft-like ER membrane domain where mitochondria are adjacent to the ER (10-30 nm), communicating both physically and biochemically [129,130] **(Figure 1.11).** At MAM, Ca^{2+} sorts the ER via IP_3R (inositol 1,4,5- trisphosphate receptor) and enters in

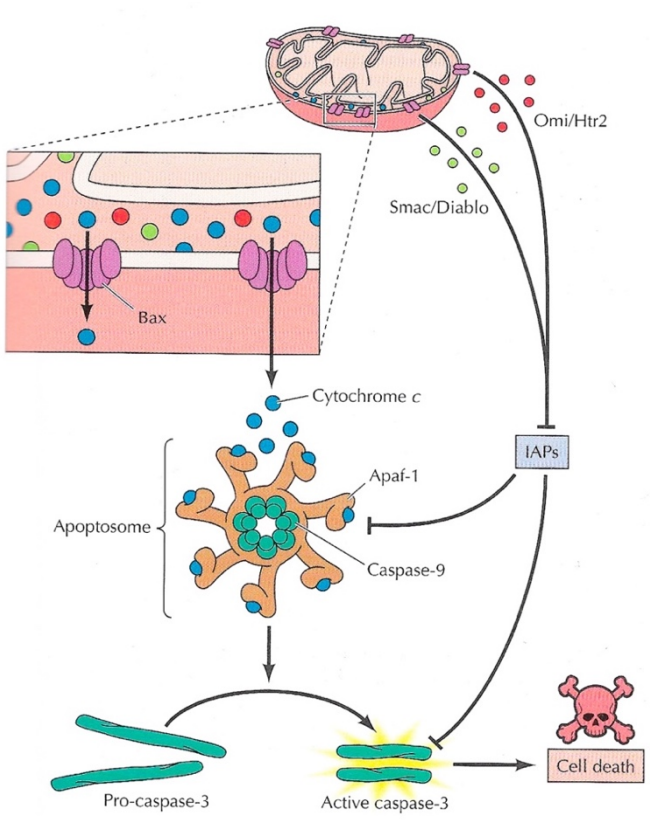


Figure 1.10 – The mitochondrial pathway of apoptosis. When active, the proapoptotic multidomain BCL-2 family (e.g Bax and Bak) form oligomers in the OMM of mitochondria, resulting in the release of cytochrome c and other proapoptotic molecules. Release of cytochrome c leads to the formation of the apoptosome and activation of caspase-9. Caspase-9 then activates caspase-3 by proteolytic cleavage which leads to cell death. On the other hand, release of Smac/Diablo and Omi/Htr2 promotes cell death by inhibiting the action of IAPs (Inhibitor of apoptosis), which are inhibitors of caspases. Adapted from Cooper *et al*. [127].

via voltage channel protein (VDAC) in the OMM and then is taken up to the mitochondrial matrix through a highly Ca^{2+} selective -dependent anion-selective Ion conductance channel called the mitochondrial calcium uniporter (MCU) [129] **(Figure 1.11)**. Mitochondria can also uptake ER Ca^{2+} released through the ryanodine receptors (RyRs). An increase in Ca^{2+} transfer into the mitochondria promotes mitochondrial bioenergetics, but mitochondrial Ca^{2+} overload may suppress normal mitochondrial functions [131] **(see 1.3.6)**. MAM is also involved in lipid and cholesterol metabolism, lipid transfer between the ER and maintenance of mitochondrial function and morphology [130].

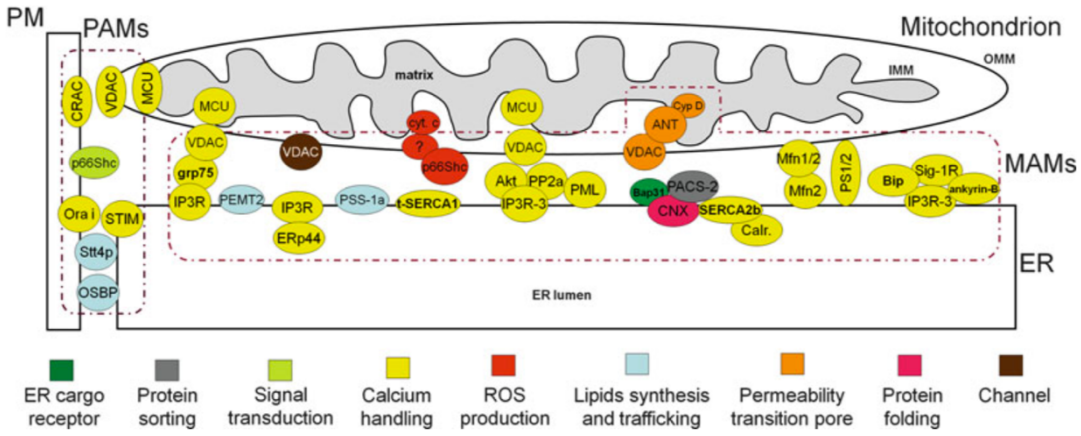


Figure 1.11 – Mitochondrial-associated ER membrane structure. Schematic view of the inter-organelle interactions and protein composition of the membranes contact sites. Possible contact sites are marked in dotted brown line. ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PAMs, plasma membrane-associated membranes; PM, plasma membrane. Adapted from Bononi *et al*. [132].

1.3.2 – Mitochondrial dynamics

Mitochondria are very dynamic organelles. In normal physiological conditions, mitochondria continuously go through fusion, fission, mitophagy, and biogenesis **(Figure 1.13).** These tightly regulated processes are crucial for maintenance of mitochondrial homeostasis and therefore, proper functioning of the cell [119,122].

Mitochondrial fusion is a process in which two or more mitochondria fuse together in an attempt to reduce mitochondrial stress. Fusion enables damaged mitochondria to repair their function and prevent the accumulation of mitochondrial DNA (mtDNA) mutations. In this process, mitofusins (Mfn1 and Mfn2) mediate mitochondrial OMM fusion, whereas (Opa1) mediates mitochondrial IMM fusion. Fission occurs when mitochondrial fusion is unable to restore mitochondrial homeostasis, leading to removal of damaged mitochondria. Mitochondrial fission compartmentalizes damaged mitochondrial components into daughter organelles to be removed and targeted for elimination by mitophagy. Mitochondrial fission occurs through dynamin related protein 1 (Drp1), which forms a ring structure to encircle and constrict at a site on the OMM upon its interaction with fission protein 1 (Fis1). It is known that mitochondrial fission

proteins are regulated by a range of protein modifications, including phosphorylation. Phosphorylation of Serine at amino acid 616 (Ser616) is likely to activate fission, since it promotes binding to other fission proteins, whereas phosphorylation of Ser637 could be an inactivating step [122,133].

Mitophagy consists in the process of elimination of irreversibly damaged mitochondria through an autophagic process. When mitochondria become damaged, sustained depolarization of their inner membrane occurs, stabilizing the protein PTEN-induced kinase 1 (PINK1) at the OMM. There, PINK1 phosphorylates Mfn2 which, in turn, recruits Parkin to the OMM. Parkin ubiquitylates several proteins that are then recognized by the ubiquitin-binding proteins optineurin (OPTN), p62, NDP52, and NBR1, which recruit the mitochondria to the autophagy pathway. Once in the autophagic pathway, there is the formation of an autophagosome (mitophagosome), which will fuse to a lysosome and lead to the degradation of mitochondria [134] **(Figure 1.12)**.

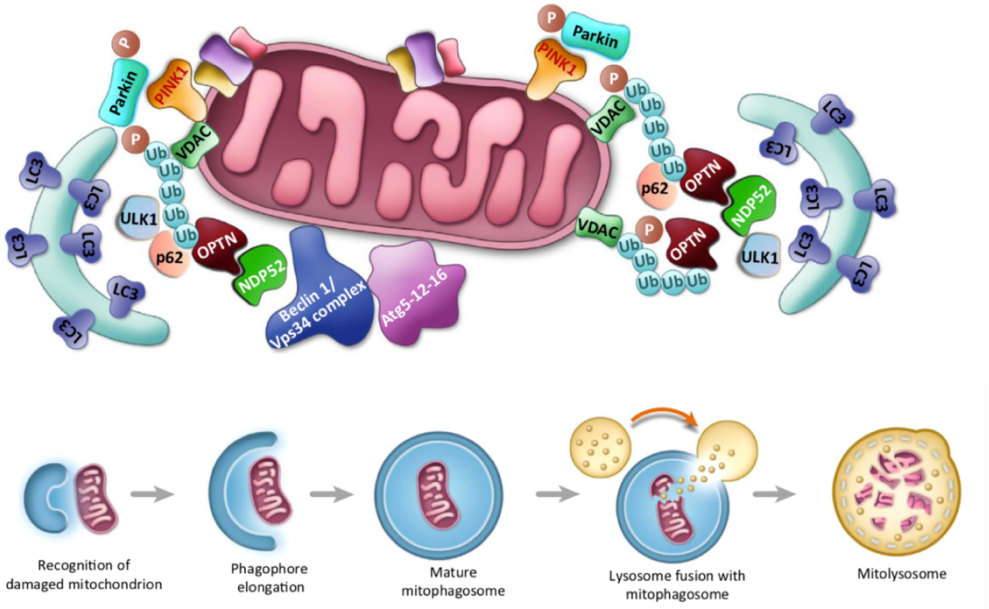


Figure 1.1.2 – Molecular mechanism of mitophagy. When mitochondria become damaged, sustained depolarization of their inner membrane occurs, and this stabilizes the protein PTEN-induced kinase 1 (PINK1) at the outer mitochondrial membrane (OMM). There, PINK1 phosphorylates mitofusin 2 (Mfn2) and ubiquitin, which, in turn, recruits Parkin to the OMM. Parkin ubiquitylates several proteins that are then recognized by the ubiquitin-binding proteins optineurin (OPTN), p62, NDP52, and NBR1, which recruit the mitochondria to the autophagy pathway. Adapted from Kerr *et al.* [134]

There is also a need to produce new mitochondria through a process named mitochondrial biogenesis, which consists in the replication of mtDNA and the synthesis and assembly of mitochondrial components. This process is regulated through the transcription coactivator: peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM) [122]. Mitochondria homeostasis is especially important in neurons due to its complex architecture, energetic demands that fluctuate in time and space, and long lifespan. During neuronal lifetime, the mitochondrial network is continually remodelled and rebuilt in order to maintain a healthy mitochondrial pool [135,136].

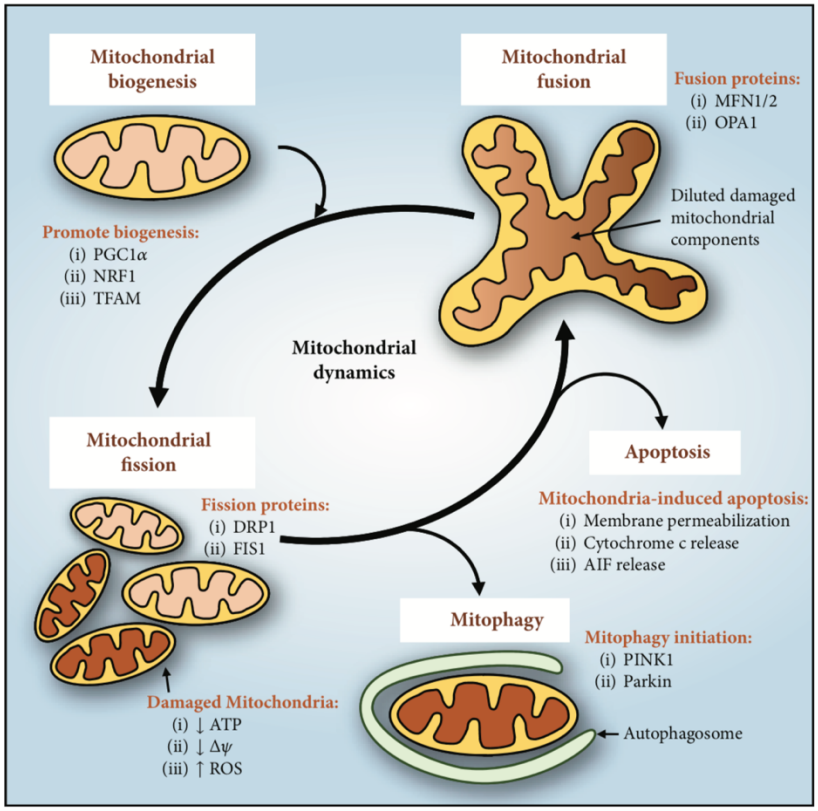


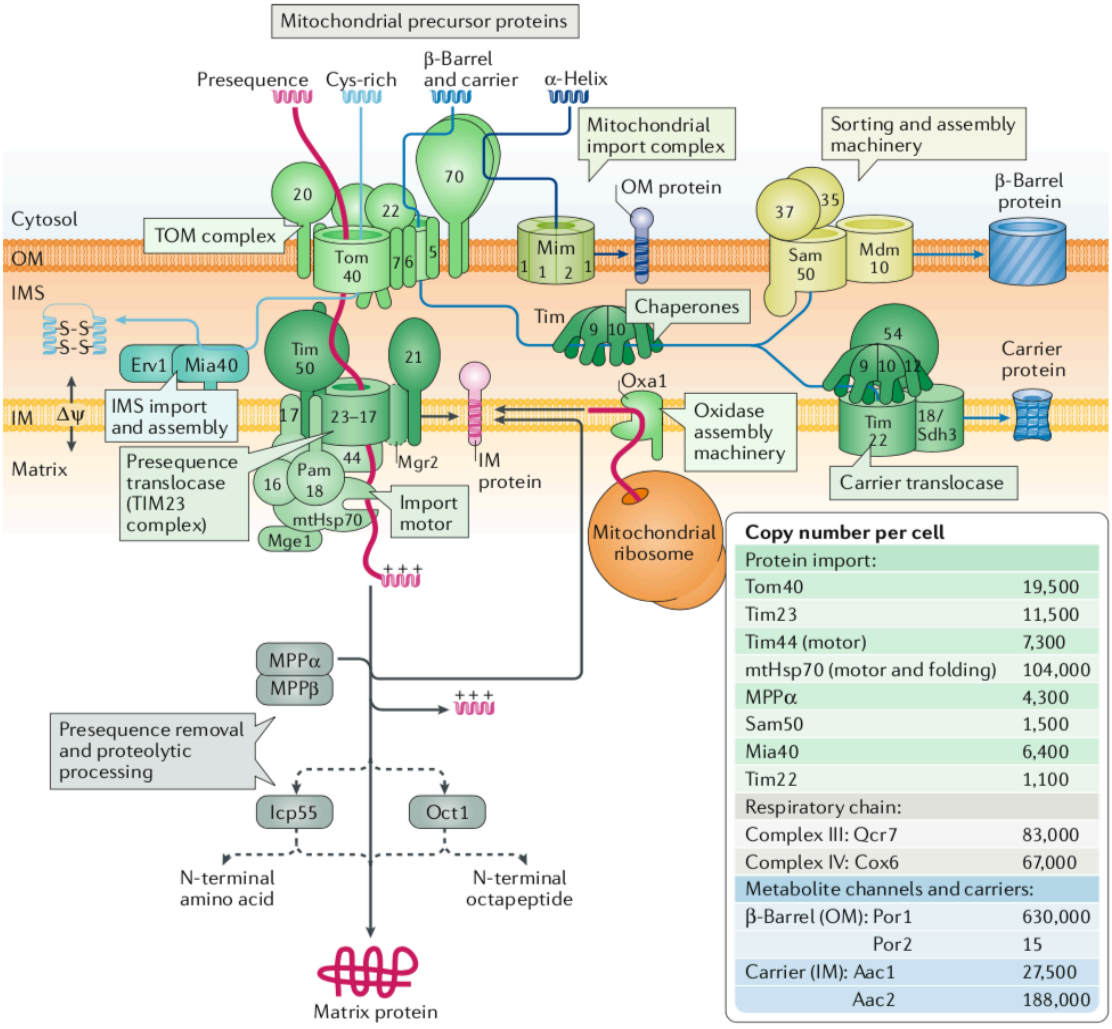
Figure 1.13 – **Mitochondria homeostasis.** Mitochondria pool is dynamically maintained by the processes of mitochondrial (1) biogenesis, (2) mitochondrial fusion/fission, (3) mitophagy, and (4) apoptosis.**(1)** Biogenesis is regulated by peroxisome proliferator-activated receptor γ coactivator-1α (PGC1α), nuclear respiratory factor 1 (NRF1), and mitochondrial transcription factor A (TFAM) **(2)** Mitochondrial fusion is facilitated by mitofusin (MFN) 1 and 2 and OPA1 for the fusion of the outer and inner mitochondrial membranes, respectively, whereas mitochondrial fission involves dynamin-related protein 1 (Drp1) that interacts with fission protein 1 (FIS1), which compartmentalizes damaged mitochondrial components into daughter mitochondria for elimination via mitophagy **(3)**. Dysfunctional mitochondria are detected by -PTEN-induced kinase 1 (PINK1) which recruits Parkin, initiating mitophagy and the subsequent formation of the autophagosome to degrade targeted mitochondria. Damaged mitochondria can also induce apoptosis **(4)** Apoptosis occurs through the permeabilization of the mitochondrial membrane, leading to the release of cytochrome c that can activate caspase-mediated apoptosis, as well as the release of proapoptotic proteins such as apoptosis-inducing factor (AIF) From: Huang *et al*. [122]

1.3.3 – Importance of TOM40 in mitochondria

Mitochondria is the only intracellular organelle containing its own genome. However, only a small set of proteins (37 proteins) are encoded by the mtDNA. The proteins encoded by the mtDNA include 13 essential subunits of the OXPHOS system, as well as the ribosomal RNA (rRNA) and transfer RNA (tRNA) molecules needed for their expression. The remaining mitochondrial proteins (~99%) are encoded by nuclear genes and synthetized in the cytosol [68,118]. These proteins (preproteins) must be imported into mitochondria and have targeting signals that direct them to mitochondrial receptors and then into the proper mitochondrial sub-compartments. Different targeting signals revealed that mitochondria use at least five major pathways. Four of these pathways use the TOM40 for preprotein translocation across the outer membrane and all use the translocase of the outer membrane (TOM) complex (reviewed in Pfanner *et al.* [68]) **(Figure 1.14)**.

The complete TOM complex contains seven subunits: TOM40, TOM22, TOM20, and TOM70, and three smaller proteins, TOM5, TOM6 and TOM7 [118]. TOM40 is the pore-forming protein of the TOM complex encoded by the *TOMM40* gene [67]. TOM20, TOM22 and TOM70 act as receptors recognizing and binding preproteins. TOM6 is involved in the assembly and maintenance of the TOM complex, and TOM7 is involved in its disassembly. Finally, TOM22 and TOM5 assist the passage into the TOM40 channel (reviewed in Gottschalk *et al*. [118]) **(Figure 1.14)**.

As described above, the majority of nuclear-encoded proteins enter mitochondria via TOM40. As the import of mitochondrial proteins into mitochondria is essential for biogenesis and functioning of mitochondria, it is not surprising that TOM40 is essential for life in eukaryotic organisms. The importance of TOM40 was demonstrated in different eukaryotic models such as *S. cerevisiae* [137], *N. crassa* [138], human culture cells [139], *C. elegans* [140] and mice [141]. In *S. cerevisae*, Baker *et al.* demonstrated that yeast cells depleted of TOM40 (then named ISP42) accumulated uncleaved mitochondrial precursor protein and then died, thus concluding that TOM40 is essential for the import of precursor proteins into mitochondria and consequently cell viability



Copy number per cell	
Protein import:	
Tom40	19,500
Tim23	11,500
Tim44 (motor)	7,300
mtHsp70 (motor and folding)	104,000
MPPα	4,300
Sam50	1,500
Mia40	6,400
Tim22	1,100
Respiratory chain:	
Complex III: Qcr7	83,000
Complex IV: Cox6	67,000
Metabolite channels and carriers:	
β-Barrel (OM): Por1	630,000
Por2	15
Carrier (IM): Aac1	27,500
Aac2	188,000

Figure 1.14 – Preproteins import pathways. Different targeting signals revealed that mitochondria use at least five major pathways which have been well conserved from fungi (shown in this figure) to mammals. **(1)** In the presequence pathway, preproteins (~60% of all mitochondrial proteins) are transported through TOM40 pore and TIM23 toward the matrix, where presequences are removed by the mitochondrial processing peptidase (MPP). **(2)** Cysteine-rich proteins are imported into the intermembrane space (IMM) through TOM40 pore and the mitochondrial intermembrane assembly system, which consist in two main components: Mia40 and Erv1. Mia40 recognizes the cysteine-rich proteins and insert disulfide bonds into the imported proteins which lead to its stabilization. **(3)** Precursors of non- cleavable inner membrane proteins such as the carrier proteins are imported by the TOM complex followed by transfer to the small TIM chaperones in the IMS and insertion into the IMM by the TIM22 carrier translocase. **(4)** The precursors of OMM β- barrel proteins use the TOM complex and small TIM chaperones and are inserted into the OMM by the sorting and assembly machinery (SAM). **(5)** OMM proteins with α-helical transmembrane segments are inserted into the membrane by the mitochondrial import (MIM) complex. These proteins do not use the Tom40 channel, but Tom70 can be involved in their recognition. Adapted from: Pfanner *et al*. [68].

[137]. By creating a TOM40 mutant of *N. crassa,* Taylor *et al*. also demonstrated that this gene is essential for the viability of the organism [138]. These authors demonstrated that mitochondria with reduced levels of Tom40 were deficient in the import of mitochondrial preproteins and contained reduced levels of Tom22 and Tom6, suggesting that the import and/or stability of these proteins is dependent on presence of Tom40 [138]. In *C. elegans*, an RNAi screen demonstrated that reduced TOM40 levels arrested growth between the 1st and 3rd larval stages and *TOMM40* knock-out animals exhibited a phenotype similar to that of knock-downs. Moreover, *TOMM40* knock-down collapsed the ΔΨm, blocked the uptake of mitochondrial targeted proteins and elicited the mitochondrial stress response, but markers of cytoplasmic and ER stress were not affected. Unexpectedly, in *C. elegans* the *TOMM40* knock-down also suppressed DAF-28/insulin secretion, which represents the major metabolic insulin pathway in *C. elegans* [140]. Zeh *et al.* studied the influence of a mutation in the *TOMM40* gene on mice and demonstrated that homozygous $Tom40^{-/-}$ mice were not viable, while heterozygous $Tom40^{+/-}$ mice showed normal development but a reduced life span with a 30% higher mortality after two years. Heterozygous $Tom40^{+/-}$ mice had mild cardiac dysfunction and slowly progressing neurological impairments [141]. Altogether, these studies demonstrate the importance of Tom40 for cell and organism survival.

In addition to its well-known role as a mitochondrial protein import translocase, ectopic TOM40 was identified on the cell surface of natural killer (NK) cells, where it was named as p38.5 (Haymaker) protein [142,143].

1.3.4 – Role of reactive oxygen species and oxidative damage in mitochondrial dysfunction

An increasing body of evidence suggests that oxidative stress plays an important role in the pathogenesis and progression of AD. Oxidative stress results from the imbalance between the production of ROS and/or reactive nitrogen species (RNS) **(Table 1.8)** and anti-oxidant defences [144,145] **(reviewed in 1.3.1)**. Under carefully controlled conditions, ROS act as signalling molecules [146]. However, high ROS levels are capable of oxidizing all major biomolecules, including DNA, RNA, protein and lipids causing oxidative damage and were thus described as a “double-edged sword” [147]. Different

ROS/RNS react with biomolecules causing oxidative or nitrosative damage. Since ROS and RNS are usually highly reactive, unstable and have a very short half-life, they are difficult to measure directly and thus specific products that result from oxidative or nitrosative damage are used as biomarkers instead, as reviewed in Butterfield *et al*. [144].

Table 1.8 – Biologically important ROS and RNS

	ROS	RNS
Radical	Superoxide, $O_2^{\bullet-}$ Hydroxyl, $OH^{\bullet}$ Peroxyl, $RO_2^{\bullet}$ Alkoxyl, $RO^{\bullet}$	Nitric oxide, $NO^{\bullet}$ Nitrogen dioxide, $NO_2^{\bullet}$ Nitrate, $NO_3^{\bullet}$
Non Radical	Hydrogen peroxide, H_2O_2 Hypochlorous acid, HOCl Organic peroxides, ROOH Peroxynitrite, $ONOO^-$ Peroxynitrous acid, ONOOH	Nitrous acid, HNO_2 Nitrosyl cation, NO^+ Nitrosyl anion, NO^- Peroxynitrite, $ONOO^-$ Peroxynitrous acid, ONOOH

Adapted from Butterfield *et al.* [144]

Due to electron leakage during electron transfer in the ETC, mitochondria are the main source of ROS (90% of the endogenous ROS). On the other hand, mitochondria are also a major target of oxidative damage. Because of this, mitochondria play a central role on AD, where the relationship between oxidative stress and mitochondria dysfunction forms a downward spiral that amplifies AD-associated deficits. The brain is specifically highly susceptible to oxidative stress due to its high energy demand, high oxygen consumption, rich abundance of easily oxidized polyunsaturated fatty acids, high level of potent ROS catalyst iron and reduced antioxidant capacity [128,145]. However, oxidative stress in AD is not limited to the brain [148,149] and is also detect in the periphery [150,151]

In AD, lipid peroxidation, protein oxidation and DNA/RNA oxidation are the major hallmarks of oxidative damage, as reviewed in Wang *et al*. [145]. Oxidative stress is an early event that can occur during MCI even before deposition of Aβ and p-tau in senile plaques and NFTs, respectively [128,144]. Regarding lipid peroxidation, it was demonstrated that the levels of the isoprostane 8,12-iso-iPF2α-VI were significantly

increased in CSF, plasma, and urine of MCI subjects compared with age-matched control subjects, whereas the levels of AD cerebrospinal fluid markers such as Aβ or tau remained unchanged [152]. Enhanced overall protein peroxidation, as well as oxidative modification of specific proteins (protein carbonyls) were also found in the hippocampi , superior and middle temporal gyri and CSF from MCI subjects [153]. ROS also cause mtDNA damage, which leads to subsequent defects in mtDNA-encoded subunits, namely of the respiratory complex I and III [122]. One important tool to study defects in mtDNA is the cybrid cellular model. Cybrids are prepared through the fusion of mtDNA-depleted cells (p^0 cells) with cells without nuclear DNA, such as platelets, containing the mtDNA of interest [128]. In this way, the nuclear genetic complement is held constant so that the observed effects on OXPHOS can be linked to the introduced mtDNA. Data from our laboratory showed that cybrids expressing mtDNA from AD subjects display reduced cytochrome oxidase (COX; also called complex IV) activity, elevated ROS, and reduced ATP levels, compared with the cells expressing mtDNA from age-matched control subjects [154]

On the other hand, a significant decrease of antioxidant levels such as albumin, bilburin, uric acid vitamins A, C and E was found in AD patients [155,156] and MCI patients [7,157]. A significant decrease in the activity of antioxidant enzymes, such as MnSOD, Cat, GSH and heme-oxygenase was also reported in brain areas associated with AD even that in some cases the levels of this enzymes were increased [158–160]. The same was also observed in MCI patients [161,162].

As referred previously, mitochondria is the major target of oxidative damage. In AD, brain oxidative modification of mitochondrial proteins, such as α-ketoglutarate dehydrogenase, pyruvate dehydrogenase complexes and COX, has been demonstrated by elevated levels of protein carbonyl nitration of tyrosine residues and impaired metabolic activity [163]. Curiously, impaired COX activity also exacerbated mitochondrial-derived ROS production [164], supporting the idea that defective mitochondrial bioenergetics potentiates oxidative stress and vice-versa [165]. There is also a line of evidence suggesting that an inefficient glucose utilization (and thus impaired ATP production) and oxidative damage are intimately related [144]. A major contributor to inefficient glucose utilization may well be oxidative modification of

biomolecules, which often leads to decreased activity of the enzymes involved in glucose metabolism [165].

1.3.5 – Impaired bioenergetics in Alzheimer's Disease

One important process that becomes dysfunctional in AD and MCI is glucose metabolism. In fact, reduced energy metabolism in AD brains is one of the best documented abnormalities in the disease. The first evidences were obtained from PET imaging with the tracer 2-[18F] fluoro-2-deoxy-D-glucose, in which reductions in cerebral glucose transport and utilization were detected in brain areas affected by AD pathology [166,167]. At the moment, low glucose metabolism at baseline and longitudinal glucose metabolism decline in the brain are viewed as sensitive measures useful for monitoring change in cognition and functionality in AD and MCI, and are being increasingly adopted to assist diagnosis and used to predict future cognitive decline [9,168]. It is also known that decreased cerebral metabolism precedes the development of clinical (neuropsychological) or neuroanatomic (imaging) evidence of the disease [169].

Alterations in mitochondrial enzymes likely underlie the reduced energy metabolism in the AD brain through reduced expression or reduced activity. Chandrasekaran *et al*. demonstrated a decrease in COX I and III subunits mRNA in affected brain regions [170]. On the other hand, different studies, including from our laboratory demonstrated decrease activity of COX on AD brain [171,172] and platelets [173,174]. Bubber *et al*. demonstrated a decrease in the activities of the pyruvate dehydrogenase complex, isocitrate dehydrogenase, and the a-ketoglutarate dehydrogenase [175]. As referred previously, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase complexes and COX are also targets of oxidative damage. AD brain is also characterized by a reduction in the expression of glucose transporters-1 and -3 [176]. Another important aspect is the brain insulin resistance present in AD and MCI brain. Indeed, type 2 diabetes, a disease characterized by insulin resistance, is a substantial risk factor for developing AD [165,177].

1.3.6 – Calcium dyshomeostasis

There is a growing body of evidence indicating that perturbed neuronal Ca^{2+} homeostasis is implicated in age-related cognitive impairment and AD [131,178,179]. Based on this, the "calcium hypothesis of brain aging" was formulated in 1982 by Dr. Kachaturian, in which he postulated that an imbalance of cellular Ca^{2+} disrupts neuronal functions and leads to neurodegenerative diseases, including AD [180]. This hypothesis was further revised in 1989 and 2017 [181,182]. The deregulation of Ca^{2+} homeostasis has been reported in brain and peripheral cells of AD patients and also in AD animal models [183–185]

Mitochondria has an important role in this process, since this organelle is a major short-term reservoir of Ca^{2+}, as referred earlier. Ca^{2+} is a second messenger that regulates important facets of neuronal physiology [131,179,186], including ATP production, transmitter release, excitability, dynamics and traffic. However, under excessive Ca^{2+} levels, the following mitochondria-related events occur: uncoupling of OXPHOS, increase ROS production and opening of the mitochondrial permeability transition pore (mPTP) [187]. The mPTP is a voltage- and Ca^{2+}- dependent high-conductance channel breaching the IMM that allows free passage of Ca^{2+} [188], as well as cytochrome c [189] and AIF, from the mitochondrial intermembrane space into the cytoplasm [128] . Release of cytochrome c initiates the apoptosis cascade that ultimately leads to neuronal death [190,191]. On the other hand, AIF can travel further into the nucleus where it causes DNA and chromatin fragmentation in a caspase-independent mechanism **(reviewed in 1.3.1)**. Moreover, enhanced ROS production activates redox-sensitive RyR channels to augment the release of Ca^{2+} from the ER, which then feed-forwards into the mitochondria to further increase ROS production [192]. The function of other Ca^{2+} pumps and channels are altered in AD leading to Ca^{2+} dyshomeostasis (reviewed in Wang *et al*. [129]).

Aß aggregates are also responsible for the upregulation of Ca^{2+} concentration in the cytosol of neurons by forming Ca^{2+} permeable channels in the plasma membrane [193]. Another pathway by which Aß can increase Ca^{2+} concentration in the cytosol of neurons is through excessive NMDA receptor activation and consequent cell Ca^{2+} overload [194,195].

Altered MAM also seems to have a role in calcium dyshomeostasis. Indeed, it was demonstrated that the function MAM and ER–mitochondrial communication proteins are increased significantly in fibroblasts from patients with both familial and sporadic forms of AD [196]. On the other hand, increased expression of several ER-mitochondria interface proteins was also observed in AD [197], which led to the formulation of the MAM hypothesis.

1.3.7 – Mitochondrial-associated ER membrane hypothesis

Besides Ca^{2+} dyshomeostasis, other processes related to MAM, such as aberrant cholesterol and phospholipid metabolism have been described to occur in AD. Based on this information, the MAM hypothesis states that the pathogenesis of AD is mediated by increased ER-mitochondrial communication, which in turn alters the function of proteins that reside at the interface of these two organelles, both in degree and kind [130].

The interest of the MAM role on AD pathogenesis started after finding that presenilins and γ-secretase activity are enriched in MAM [130]. Furthermore, the same authors found that C99 or CTFβ, product of cleavage of full APP by BACE-1 **(see 1.1.5)**, correlated with MAM structure and function [198]. C99 promotes the various features of the disease, including the Ca^{2+} and lipid dyshomeostasis, mitochondrial perturbations, and ultimately plaque and tangle formation in the brain. These authors also observed that accumulation of C99 at MAM resulted in the upregulation of sphingomyelin hydrolysis by sphingomyelinases (SMases). The increase in SMase activity resulted in a notable elevation of the sphingomyelin hydrolysis product, ceramide, which is a proapoptotic molecule and inhibitor of respiration [198].

The authors thus concluded that mitochondrial dysfunction is an early and significant defect in AD, and not a primary insult in the pathogenesis of the disease, but rather a consequence of MAM dysfunction driven by an increased presence of C99 at MAM [199].

1.3.8 – Alterations in mitochondrial homeostasis

Due to its complex architecture and high energetic demands that rely on time and space, neurons need a highly regulated mitochondrial pool. The mechanisms that control the homeostasis of this mitochondrial pool comprise the processes of fusion and fission, biogenesis, and mitophagy (reviewed by Cardoso *et al*. [135]) **(Figure 1.13)**.

Recent studies demonstrated significant changes in the expression of almost all mitochondrial fission and fusion proteins, including Drp1, OPA1, Mfn1/2, and Fis1 in both the brain [200,201] and peripheral tissues of AD patients [202,203]. It is known that levels of Drp1 phosphorylation at Ser616 are increased in AD [200], which leads to an increased recruitment to mitochondria and thus increased fission. Increased levels of Drp1 S-nitrosylation [204] are also observed in AD. By modulating the GTPase activity of Drp1, this post-translation modification also favours the occurrence of mitochondrial fission. On the other hand, reduced levels of mitochondrial fusion proteins OPA1 and mitofusins were documented in hippocampal tissue of AD patients [200]. Excessive fission plays a critical role in ROS overproduction and consequently in oxidative imbalance [205]. Moreover, it was demonstrated that ROS overproduction in AD models could be efficiently prevented or rescued by the inhibition of mitochondrial fission or the promotion of mitochondrial fusion, which demonstrates the contribution of abnormal mitochondrial dynamics to oxidative imbalance in AD [200,202,206]. Defects in mitochondrial fission and fusion can also increase ROS indirectly through negative impact on bioenergetics, Ca^{2+} handling, and mtDNA integrity. Excessive fission leads to rapid accumulation of mtDNA mutations and decreased Ca^{2+} buffering capacity [207,208]. On the other hand, the balance of mitochondria fission and fusion is also sensitive to oxidative imbalance. Studies demonstrated that, through regulation of mitochondrial fission and fusion proteins, such as Drp1 and Mfn2, both endogenous [209] and exogenous [210] applications of ROS might directly impair mitochondrial fission and fusion balance, induce mitochondrial fragmentation and further cause subsequent mitochondrial dysfunction including ROS overproduction and thus form a vicious cycle that amplifies oxidative stress.

Regarding mitophagy, emerging findings suggest that this quality control mechanism is also compromised in AD (reviewed by Kerr *et al.* [134]). The first clue that mitophagy plays a role in AD derived from a study performed in post-mortem AD brain tissue, which revealed the presence of mtDNA and COX in the neuronal cytoplasm, with mtDNA being presented in lipofuscin-containing vacuoles [211]. Consistently, elevated levels of mitochondrial components, namely COX and lipoic acid, within autophagosomes were detected in human post mortem brain tissue, suggesting an increase in the rate of mitochondrial degradation by autophagy [212]. Lipofuscin (biological garbage) is known to accumulate in damaged organelles [128]. Neurons exhibiting abnormal accumulation of autophagosomal vacuoles are a prominent feature in AD and their accumulation may result from lysosomal dysfunction (elevated pH), perhaps secondary to dysregulation of neuronal Ca^{2+} homeostasis [213]. The undegraded dysfunctional mitochondria accumulate in the soma, which may result from the combination of local lysosome dysfunction and impaired mitochondrial transport [214]. These findings suggest that autophagy/mitophagy is stimulated (perhaps secondary to mitochondrial dysfunction and fission) while lysosome function is impaired thereby contributing to the prominent accumulation of autophagosomes in neurons in AD [134].

On the other hand, recent findings suggest that mitochondrial biogenesis is also impaired in AD, as indicated by reduced levels of the transcriptional regulator of mitochondrial biogenesis PGC1α [215]. Analyses of post mortem brain tissue from AD patients and age-matched control subjects have revealed reduced expression of genes related to mitochondrial biogenesis, including PGC1α, TFAM, and NRF1 [216], indicating that the refurbishment of an “old” mitochondrial pool via mitochondrial biogenesis is compromised [217].

1.3.9 – Role of TOM40 on mitochondrial dysfunction

Mitochondrial dysfunction is also, at least in part, due Aβ internalization via TOM40 pore and blockage by APP of this mitochondrial import channel. Anandatheerthavarada *et al.* demonstrated for the first time that APP is incompletely translocated and

accumulated in the TOM40 channel. Incomplete translocation and progressive accumulation of APP lead to the impairment of mitochondrial functions and decreased cellular energy levels [218]. In the same study it was also demonstrated that APP interacts efficiently with mitochondrial outer and inner membrane translocase proteins TOM40, TIM23, and TIM44 [218]. Devi *et al*. reported that full-length and C-terminal truncated APP accumulates exclusively in the protein import channels of mitochondria of brains from AD patients, but not in age-matched controls [219]. Devi *et al.* also demonstrated that this accumulation varied with the severity of AD and levels of arrested mitochondrial APP directly correlated with mitochondrial dysfunction. It was also demonstrated that accumulation of APP inhibited the entry of nuclear encoded COX subunits IV and Vb, which was associated with decreased COX activity and increased levels of H_2O_2. Similar to Anandatheerthavarada *et al.,* Devi et al. also demonstrated that in AD brains, mitochondrial associated APP formed stable 480 kilodalton (kDa) complexes with TOM40 and a super complex of 620 kDa with both mitochondrial TOM40 and TIM23 [219].

In rats, Hansson Petersen *et al*. demonstrated that Aβ is transported into mitochondria via the TOM machinery and that the import was insensitive to valinomycin, indicating that it is independent of the ΔΨm. Immunoelectron microscopy (immuno-EM) after import showed localization of Aβ to mitochondrial cristae. A similar distribution pattern of Aβ in mitochondria was shown by immuno-EM in human cortical brain biopsies obtained from living subjects with normal pressure hydrocephalus [220]. Curiously, trapped APP in TOM40 can be cleaved by the mitochondrial γ-secretase, generating Aβ inside the mitochondria [221,222]. Once inside the mitochondria, Aβ can bind or interact with important mitochondrial proteins, leading to mitochondrial dysfunction. One of the main targets of Aβ is the Aβ-related alcohol dehydrogenase (ABAD). ABAD is responsible for the oxidation of short fatty acids and protection against metabolic damage. ABAD inhibition via Aβ leads to oxidative stress and consequently mitochondrial and cellular damage, as referred earlier [223]. Another target of Aβ inside the mitochondria is the mitochondrial peptide processing enzyme (PreP), which is responsible for the cleavage of presequences from mitochondrial preproteins **(Figure 1.14)**. Aβ-mediated inhibition of this enzyme prevents maturation of mitochondrial

proteins, causing its accumulation in the mitochondrial matrix [224]. Aβ also has the ability to bind heme groups, which constitute critical redox centers of COX, thus interfering with COX activity [225,226]. Inhibition of these proteins by Aβ causes mitochondrial dysfunction at multiple levels, including: impaired respiration, OXPHOS, ATP production, ROS overproduction and increased oxidative damage, defects in maintaining the inner membrane potential and ion homeostasis, abnormal dynamics and increased apoptosis [118].

APOE ε4 (1-272) fragment, a product from *APOE* ε4 cleavage was also associated with mitochondria, causing mitochondrial dysfunction [227,228]. Since mitochondrial localization of ApoE4 (1-272) was dependent on three positive charges in the receptor binding domain (aa 1-170) [227], it was proposed that its mitochondrial localization is also mediated by the TOM complex [118].

1.3.10 – Role of Aβ, Tau and *APOE* ε4 in mitochondrial dysfunction

The two main pathological hallmarks of AD are intracellular NFT and extracellular senile plaques. NFT are formed from paired helical filaments composed of neurofilaments and hyperphosphorylated Tau protein, whereas plaques are mainly composed of Aβ protein **(see 1.1.5)**. The ε4 allele of *APOE* gene is the most highly replicated genetic risk factor for LOAD [42,43] **(see 1.1.9)**. Aβ, Tau and *APOE* ε4 are known to have an impact on mitochondria dysfunction.

As referred above, Aβ can enter mitochondria via TOM40, inhibit ABAD, Prep and bind heme groups, thus causing mitochondrial dysfunction. The negative effects of Aβ were observed in different models (reviewed in Wlikins *et al*. [19]). In neuroblastoma cells, APP overexpression leads to elevated $A\beta_{40}$ with consequent reduction in cellular respiration, ATP levels, and COX activity [229]. In PC12 cells, exogenous Aβ was shown to depolarize the ΔΨm and decrease activities of the mitochondrial ETC complexes I, III, and IV, while also reducing oxygen consumption [230]. Shorter Aβ fragments, such as $A\beta_{25–35}$, also have been shown to impact mitochondria, reducing cellular ATP production, levels of antioxidants, ΔΨm, and ETC activities (CI-IV) in primary cortical

neurons [231], also impairing anterograde transport of mitochondria to synapses in mouse hippocampal neurons [232]. In N2a cell line, $A\beta_{25-35}$ increased caspase activation, ROS production, and cytochrome c release. However, in ρ0 cells that lack a functional respiratory chain caused by an absence of mtDNA, $A\beta_{25-35}$ failed to induce ROS production, caspase activation, or cytochrome c release, demonstrating that Aβ requires functional mitochondria to induce toxicity [233]. Aβ may also alter mitochondrial dynamics by interacting with a mitochondrial fission protein, Drp1 [201]

Hyperphosphorylated Tau is known to impair mitochondrial axonal transportation, mitochondrial dynamics and function (reviewed in *Cheng et al.* [21]). To meet high energy demands and regulate Ca^{2+} buffering of neuronal cells, efficient delivery of mitochondria in neurons is essential. Tau is a microtubule associated protein, which plays an important role in delivering cargoes across axons into synapses, including mitochondria [21]. It has been described that overexpression and hyperphosphorylation of Tau impairs localization and distribution of mitochondria [234–236], which further causes defects in axonal function and synapse loss [237]. The effects of Tau aggregates and tau accumulation on mitochondria distribution have been observed both in animal models and human AD brains [236]. Evidence also suggests that pathological forms of Tau play a significant role in the impairment of mitochondrial fission/fusion dynamics in AD, mainly through a molecular mechanism of increasing mitochondrial fission protein such as Drp1 and decreasing fusion protein including OPA-1 and Mfn1/2. Manczak *et al.* demonstrated that hyperphosphorylated tau interacts with Drp1 causing excessive mitochondrial fission, leading to the degeneration of mitochondria and synapses in brain tissues of APP, APP/PS1, and 3xTg-AD mice. Similar findings were made in brains from AD patients [238]. On the other hand, it was found that truncated tau was combined with reduced levels of OPA-1 [239]. Tau-induced abnormal mitochondrial dynamics and impaired mitochondrial distribution may further lead to mitochondrial dysfunction, as addressed above. Tau dysfunction also leads to impaired mitochondrial function. In both cell culture and transgenic mice, overexpression of tau inhibits mitochondrial function by decreasing the activity of mitochondrial respiratory chain complexes, antioxidant enzymes, ATP synthesis and synaptic function (reviewed in Cheng *et al.* [21]). It was also described that N-terminal-truncated tau localized in mitochondrial membrane impaired ATP synthesis and $\Delta\Psi m$ [240]. Abnormal interaction of phosphorylated tau with voltage-

dependent anion channel 1 protein (VDAC1) was observed in AD patients, as well as in mice, which broke the balance of the opening and closure of mitochondrial pores and led to mitochondrial dysfunction [241]. Mitochondrial dysfunction was also observed in the P301L tau transgenic mice, which presented notable mitochondrial dysfunction. Brains of these mice showed that the activity of mitochondrial complexes was significantly reduced, especially complex I and V. Moreover, impaired ATP synthesis together with decreased mitochondrial respiration and increased ROS levels were also noticed [242]. Similar findings were observed in other Tau animal models (reviewed in Cheng *et al.* [21]).

APOE genotype has also been shown to impact on mitochondrial function (reviewed in Mahoney-Sanchez *et al.* [243]). In a post mortem cohort, cognitive history was shown to be more closely associated with a reporter of mitochondrial function (α-ketoglutarate dehydrogenase complex) than either plaque or tangle pathology in ε4 carriers, but the opposite was observed in non-ε4 carriers [244]. ApoE also decreases mitochondrial mobility in an isoform- specific manner (ApoE ε4 fragments > ApoE ε4 > ApoE ε3 > ApoE ε2) in cultured neuronal cells and transgenic mice expressing ApoE ε4 in neurons [245,246]. ApoE ε4 could also reduce the expression of mitochondrial respiratory enzymes, in particular ETC genes from complexes I–V (NADH dehydrogenase, succinate dehydrogenases, ubiquinol-cytochrome c reductase, amongst others) and translocases of the inner and outer mitochondrial membranes (TIMMs and TOMMs, respectively), which could alter mitochondria function and brain metabolism [247,248]. This effect of ApoE ε4 was observed in post mortem cortical tissue of young ε4 carriers, which suggests that mitochondrial dysfunction could be occurring early on in the development of the disease, before amyloid and tau pathology and cognitive impairment start, and could be linked to the onset and progression of AD [249]. As stated above, ApoE ε4 fragments (1– 272) are also associated with mitochondria dysfunction. Indeed, it has been demonstrated in N2a cells that ApoE ε4 fragments (1– 272) escaped the secretory pathway, co-localised with mitochondria and impaired their function and integrity [227], namely by disrupting ΔΨm and by binding directly to complexes of the ETC enzymes, reducing the respiratory activity of the mitochondria [228].

Chapter 2
Objectives

Nearly a decade ago, the association between the *TOMM40* poly-T and LOAD was discovered, and since the original discovery multiple studies found associations between *TOMM40* poly-T and LOAD-related features, such as AOO and risk of AD, brain structure and cognition, while other studies could not replicate these associations. Thus, investigating the genetic associations between LOAD-related features and *TOMM40* poly T, using additional cohorts, are required and highly valuable. On the other hand, the identity of the *TOMM40* poly-T risk allele has been controversial and needs further exploration. Furthermore, the association of *TOMM40'* 523 polymorphism and MCI has been poorly addressed. To gain a better insight about the risk associated with *TOMM40* poly-T, there is an unmet need to translate these findings to the understanding of the biological mechanism leading to neurodegeneration. As *TOMM40* encodes a critical mitochondrial membrane protein, our hypothesis is that different '523 poly-T lengths can ultimately affect mitochondrial function. In this context, and taking into consideration the state-of-the-art (Chapter 1), the major goals of this work were:

1) Determine the *TOMM40* poly-T profile in a Portuguese (Caucasian) cohort of controls, MCI and AD patients.
2) Study the association of *TOMM40*'523 polymorphism with the risk and time of conversion from MCI to AD
3) Replicate the independent association of *TOMM40*'523 polymorphism with AD risk and AOO.
4) Study the association of *TOMM40*'523 polymorphism with AD CSF biomarkers, namely $A\beta_{42}$, t-tau and p-tau in MCI and AD patients.
5) Study the impact of the *TOMM40*'523 polymorphism in mitochondrial function of AD patients.

Chapter 3

TOMM40'523 and the risk of Mild Cognitive Impairment conversion to Alzheimer's Disease

The scientific content of the present chapter is based on the following international scientific publication:

Cardoso R, Lemos C, Oliveiros B, Rosário Almeida M, Baldeiras I, Fragão Pereira C, Santos A, Duro D, Vieira D, Santana I, Resende Oliveira C. ***APOE*ε4-*TOMM40*L Haplotype Increases the Risk of Mild Cognitive Impairment Conversion to Alzheimer's Disease**. J Alzheimers Dis. 2020 Sep 25. doi: 10.3233/JAD-200556. Epub ahead of print.

Abstract

MCI has been considered as a pre-dementia stage, although the factors leading to AD conversion remain controversial. The *TOMM40* poly-T polymorphism (*TOMM40*' 523) discovered by Roses *et al.* has been so far associated with different AD related features such as AOO and risk of AD, brain structure and cognition of AD patients and cognitively normal individuals. However, this polymorphism has been poorly addressed in MCI cohorts and as far as we know, none of these studies fully addressed the connection between *TOMM40'* 523 polymorphism and the risk and time of conversion from MCI to AD. Thus, in this study we intended to evaluate whether *TOMM40* poly-T polymorphism was associated with the risk and conversion time from MCI to AD and secondly with AD CSF biomarkers, disentangling the *APOE* genotype. For this purpose, 147 AD patients, 102 MCI patients and 105 cognitively normal controls were genotyped for poly-T polymorphism. MCI patients were subdivided into 2 groups, the group of patients that converted to AD (MCI-AD) and the group of those that remained stable (MCI-S). We first observed that T*OMM40*' 523 L allele was significantly more frequent in the MCI-AD group and having at least one L allele significantly increased the risk of conversion from MCI to AD (OR = 8.346, $p < 0.001$, 95%CI: 2.830 to 24.617). However, when adjusted for the presence of *APOE* ε4 allele, both the L allele and ε4 allele lost significance in the model ($p>0.05$). We then analysed the *APOE* ε4-*TOMM40*' 523 L haplotype and observed that patients carrying this haplotype had significantly higher risk (OR=5.83; 95% CI=2.30-14.83) and mean lower times of conversion to AD ($p=0.003$). This haplotype was also significantly associated with a biomarker profile compatible with AD ($p = 0.007$). This study shows that the *APOE* ε4-*TOMM40*' 523 L haplotype is associated with a higher risk and shorter times of conversion from MCI to AD, possibly driven by CSF biomarkers and mitochondrial dysfunction.

3.1 – Introduction

The term MCI describes an intermediate state of cognitive function between the changes seen in aging and those fulfilling the criteria for dementia, namely AD [10]. The rate of conversion from MCI to AD is estimated to reach 10-15% per year in contrast with a rate of 1–2% per year among healthy elderly individuals [250]. When considering MCI as a prodromal state of AD (MCI due to AD) [251] it is of major importance to study the risk factors that predict which MCI patients will convert to AD, namely risk genes, as this group of patients represents a target for future disease modifying therapies [30].

The *APOE* ε4 allele , the most highly replicated genetic risk factor for LOAD [42,43], has been shown to increase the susceptibility for MCI to AD progression, as demonstrated in previous meta-analysis [252,253]. However, the presence of an allele ε4 is neither necessary nor sufficient for MCI to AD progression, showing low sensitivity. Moreover, its use for MCI to AD prediction remains controversial. For this reason, recent models of MCI to AD progression incorporate other predictors besides allele ε4, such as: CSF biomarkers, neuroimaging biomarkers ((e.g. MRI and fluorodeoxyglucose positron emission tomography (FDG-PET)) and neuropsychological tests, in order to increase the accuracy when evaluating the conversion from MCI to AD [254–256]. Apart from *APOE* there is little information about genes that increase the susceptibility for MCI to AD progression. Interestingly, the most relevant discoveries in fine-mapping and genome-wide association studies for AD are within a LD region in Chr:19q13.32 that incorporates *APOE*, *TOMM40* and *APOC1* genes [64–66]. By deep sequencing of this specific region, Roses' group discovered a poly-T polymorphism, rs10524523, in *TOMM40* gene (hereafter, *TOMM40*' 523), reported to be associated with risk and AOO of AD [73]. Meanwhile, based on the distribution behaviour of the number of thymine (T) residues, three categories (alleles) of repeat length were established: short (S, ≤ 19), long (L, 20–29) and very long (VL, ≥ 30) [75]. In the Caucasian population, it has been confirmed that the *APOE* ε4 allele is almost exclusively linked to a L poly-T repeat allele [73,75]. This strong LD (r2 = 0.941) [257] between *TOMM40' 523* L and *APOE* ε4 alleles complicates analytical approaches to disentangle independent effects between these two variants as fleshed out in previous studies [79,82,85]. Nevertheless, these effects could be due

to a combination of both *APOE* and *TOMM40* [257]. As the *APOE* ε3 allele is almost always linked to either a S or a VL poly-T allele, in the Caucasian population, several studies attempted to find instead differences between S and VL alleles in ε3/ε4 or ε3/ε3 cohorts in order to establish an effect of *TOMM40*' 523 independent of *APOE*. However, differences between these two alleles (S and VL) are still controversial (reviewed in Chiba-Falek *et al.*) [114].

Unlike the *APOE* ε4 allele, the role of *TOMM40' 523* has been poorly addressed in MCI cohorts. One study presented a stratification by *TOMM40' 523* and *APOE* genotype of the AOO of cognitive impairment for cognitively normal subjects followed in a prospective cohort, in order to create a risk algorithm for clinical trial enrichment [86], which was further evaluated in other studies [87,88]. A subsequent study evaluated, the utility of *TOMM40* poly-T variable-length polymorphism alleles among other 247 variables, for modelling progression from MCI to AD [258]. Lastly, an additional study reported the effect of *TOMM40*' 523 on spatial navigation in amnestic MCI individuals [103]. However, as far as we know, none of these studies fully addressed the connection between *TOMM40'* 523 polymorphism and the risk and time of conversion from MCI to AD.

The main aim of this part of the study was to investigate the relationship between *TOMM40*' 523 polymorphism with the risk and conversion time from MCI to AD, and replicate the association of *TOMM40*'523 polymorphism with AOO and risk of AD disentangling the effect of *APOE* genotype in that relationship. For this purpose, the poly-T profile was first characterized in three different groups: cognitively normal controls, AD and MCI patients subdivided into patients that converted from MCI to AD (MCI-AD), and patients that remained stable (MCI-S) within a minimum period of two years. Secondly, the association between *TOMM40*' 523/*APOE* genotype and AD CSF biomarkers, particularly $A\beta_{42}$, t-Tau and p-Tau, was also explored.

3.2 – Materials and Methods

3.2.1 – Study subjects

This study included 354 Caucasian subjects: 147 AD patients, 102 MCI patients and 105 cognitively normal controls. CSF and DNA from AD and MCI individuals were available, DNA samples were also available from controls.

AD and MCI patients were recruited and diagnosed at the Dementia Clinic, Neurology Department of Coimbra University Hospital (CHUC), Coimbra, Portugal. The baseline study, as well as the follow-up protocol, have already been published [7]. In summary, patients were enrolled in a systematic way and were subject to biannual clinical observation and annual neuropsychological and functional evaluations. All patients undertook a thorough biochemical, neurological and imaging ((computed tomography (CT) or MRI)) evaluation. PET and genetic studies were more restricted, although considered in younger patients. Essentially, a neurologist completed a medical history with the patient and caregiver and conducted a general physical, neurological, and psychiatric examination in addition to a comprehensive battery-protocol diagnostic, including cognitive instruments. Cognitive instruments included: the Portuguese versions, validated for the Portuguese population, of the Mini Mental State Examination (MMSE) [259,260], the Montreal Cognitive Assessment (MoCA) [261,262], and the Alzheimer's Disease Assessment Scale-Cognitive (ADAS-Cog) [263–265]. A comprehensive neuropsychological battery with normative data for the Portuguese population (Lisbon Battery for Dementia Assessment (BLAD)) [266] exploring memory (Wechsler Memory Scale subtests) and other cognitive domains (including language, praxis, executive functions and visuoconstructive tests) were also used. The evaluation was completed applying standard staging scales which provided objective information about subject performance in various domains, including the Clinical Dementia Rating scale (CDR) [267] for global staging, the Disability Assessment for Dementia (DAD) [268,269] to evaluate the functional status, and, the Neuropsychiatric Inventory (NPI) [270,271] to characterize the psychopathological profile, including the presence of depression. All the available information (baseline cognitive test, staging scales, clinical laboratory and imaging studies) was used to reach a consensus research diagnosis. A

similar approach was used to follow-up annual evaluations.

MCI patients were amnestic type (aMCI) and the diagnosis was completed in agreement with the criteria defined by Petersen *et al.* [12] and, more recently, with the framework for MCI due to AD proposed by NIA-AA criteria [251]. Petersen *et al.*'s criteria were implemented as follows: 1) A subjective complaint of memory decline (reported by the subject or informant); 2) An objective memory impairment (considered when scores on standard Wechsler memory tests were > 1.5 SDs below age/education adjusted norms) with or without deficits in other cognitive domains; 3) Normal general cognition suggested by normal scores in the MMSE and MoCA using the Portuguese cut-off scores [260,262]; 4) Fairly normal daily life activities, evaluated with a functional scale – (DAD) 5) Absence of dementia, indicated by a CDR rating of 0.5 [267]. All patients were in stable condition without acute comorbidities. As exclusion criteria for enrolment, we considered a significant underlying medical or neurological illness revealed by laboratory tests or imaging; a relevant psychiatric disease, including major depression, suggested in the medical interview and confirmed by the NPI; CT or MRI demonstration of significant vascular burden [272].

MCI cases were followed, at least for 2 years, under this comprehensive protocol and they were further dichotomized in those that were cognitively stable (MCI-S) and those that developed dementia due to AD (MCI-AD). Conversion to AD required fulfilling the criteria of the clinical diagnostic for probable AD (see below) and was confirmed by the coordinator of the clinical study (IS). As these criteria are not fully operational and the conversion status decision has some uncertainty and subjectivity, patients in this study were classified as having undergone conversion based on: 1) Objective evidence by cognitive testing of deterioration to dementia using the MMSE, the MoCA and the ADAS-COG scores and qualitative evaluation (i.e., impairment of memory with the addition of other domains); 2) Changes in global CDR rating from 0.5 to 1 or more, confirming the cognitive profile of dementia and autonomy loss. Dementia was diagnosed according to the *Diagnostic and Statistical Manual of Mental Disorders* – fourth edition text review (DSM-IV-TR) criteria [273] , and AD, according to the National Institute of Neurological and Communicative Disorders and Stroke - Alzheimer's Disease and Related Disorders (NINCDS- ADRDA) [274] and, more recently, the 2011 NIA-AA criteria [8]

Cognitively normal controls (≥65 years) were recruited from a group of volunteers

from a Portuguese population study on aging [275], who were subjected to cognitive evaluation and showed no signs of cognitive impairment. These procedures have been approved by the Ethics Board of CHUC, Faculty of Medicine of Coimbra and NOVA Medical School of Universidade NOVA de Lisboa. Informed consent was required from all subjects or responsible caregivers, whatever appropriate.

3.2.2 – *APOE* and *TOMM40* Genotyping

DNA was isolated from whole EDTA-blood using a commercial kit (Roche Diagnostics GmbH, Manheim, Germany). *APOE* genotype was determined by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) assay, as previously described [276]

TOMM40' 523 polymorphism was genotyped as described formerly [74], with some modifications. Briefly, each genomic DNA sample was amplified by the polymerase chain reaction (PCR) using fluorescently labelled forward 5'D4-TGCTGACCTCAAGCTGTCCTC-3' and reverse 5'-GAGGCTGAGAAGGGAGGATT-3' primers. PCR amplification was performed in a 12 µL volume containing 20 ng of genomic DNA, 2,5 µM of each primer and 6µl of Supreme NZYTaq 2x Green MasterMix (Nzytech, Lisbon, Portugal). Amplification was performed, with the following conditions: initial denaturation for 5 min at 96◦ C; 29 cycles of denaturing for 45s at 96◦C, annealing for 45 s at 69◦C and elongation for 45s at 72º; concluded with a single 10 min final elongation step at 72◦C. PCR products with an expected length of 150 + n(T) pb were confirmed in a 2% agarose gel electrophoresis. Afterwards, 1µl of each PCR product was mixed up with 20 µl of formamide (Beckam Coulter, USA) and 0.5µl of DNA Size Standard 400 (Beckam Coulter, USA) and loaded on a capillary automated sequencer CEQ 8000 (Beckman Coulter, USA).

3.2.3 – Genotype Calling

Since we were analysing a homopolymer, "slippage" during each DNA amplification was observed leading to a final product of amplicons with variable lengths around the

real poli-T length. However, as previously described [74,81], the PCR amplicons have a normal distribution with maximum intensity/area of signal near the true count of T (N[T]), but distributed continuously with error around the true value **(Figure 3.1)**. Genotypes were determined using the tool Fragments of the Genome Lab^{TM} software version 10.2 (Beckman Coulter, USA) under the presumption that the highest area peak corresponded to the true count of "Ts". This analysis was always performed by two independent observers who categorized the poly –T fragments according to the poly-T lengths into: short (S, ≤ 19), long (L, 20–29) or very long (VL, ≥ 30) repeats as as previously described [75]. In order to validate and compare the length of poly-T repeats, we sequenced 6 samples by Sanger sequencing and compared the poly-T size with the one determined from the fragment analysis. The results obtained by both techniques were similar, with an estimated error of +/- 1bp.

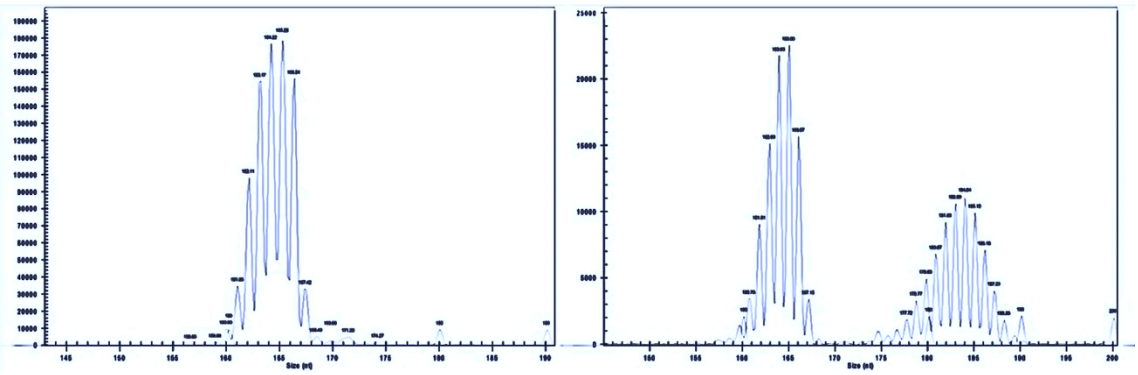


Figure 3.1 – Electropherograms of fragment analysis. Count of polymerase chain reaction (PCR) fragments (intensity) versus size of fragment of a patient homozygous for *TOMM40*' 523 allele (S/S) **(left)** and patient heterozygous for *TOMM40*' 523 allele (S/VL) **(right)**. The PCR amplicons have a normal distribution with maximum intensity of signal near the true count of T (N[T]).

3.2.4 – Cerebrospinal Fluid Biomarkers Analysis

CSF samples were collected from patients as part of their clinical diagnosis investigation routine. Pre-analytical and analytical procedures were done in accordance with previously published recommendations [277]. Briefly, CSF samples were collected into sterile polypropylene tubes, immediately centrifuged at 1800 g for 10 min at 4°C, aliquoted into polypropylene tubes and stored at – 80°C until analysed. CSF Aβ_{42}, t-Tau

and p-Tau-181 levels were measured separately, via commercially available sandwich ELISA kits (Innotest; Innogenetics/Fujirebio, Ghent, Belgium), as aforesaid [278]. All samples were tested in duplicate and assays were performed sequentially in a clinical routine setting. External quality control of the assays was completed under the scope of the Alzheimer's Association Quality Control Program for CSF Biomarkers [279]. The CSF biomarkers profile was classified using the Innotest Amyloid-Tau Index (IATI), with the following formula: $A\beta_{42}/[240+(1.18xTau)]$, and a biomarker profile compatible with AD was defined with a score below 1 [280].

3.2.5 – Statistical Analysis

Categorical data was presented in observed counts and percentages, whereas the mean, standard deviation and range were used for quantitative data. Group differences concerning qualitative data were analysed through the Chi-square test, adjusted for pairwise comparisons using a Bonferroni correction whenever justified, while non-parametric Mann-Whitney and Kruskal Wallis were applied for comparisons between groups in quantitative data. Also, the association between the haplotype and risk for conversion was assessed by a Chi-Square test. Z test adjusted with the Bonferroni correction was used to compare the relative frequencies of poly-T lengths (S, L or VL) among different diagnosis groups. Logistic regression was applied to assess the risk of conversion in MCI patients and the risk of AD. Kaplan-Meyer survival analysis was used to search for predictors in time to conversion in MCI patients and in AOO of AD patients. Both analyses were adjusted for confounding factors such as gender, age and *APOE* genotype, specifically the ε4 allele. All tests were analysed at a significance level of 5 %. Statistical analysis was conducted in SPSS, version 25.

3.3 – Results

3.3.1 – Sample Characteristics

Demographic and clinical characteristics of the 354 participants involved in this study, as well as comparisons by diagnostic group, are summarized in **Table 3.1**. No differences

were found, between groups, in gender distribution (p=0.097). Regarding age, it was observed that controls and MCI-AD patients were significantly older than the remaining groups ($p < 0.001$). Similarly, we observed that MCI-AD patients were significantly older than AD and MCI-S groups regarding AOO ($p < 0.001$). AD and MCI-AD patients had a significant higher percentage of *APOE* ε4 allele carriers (45.6% and 59.7%, respectively) than MCI-S and controls (20% and 18.1%, respectively) ($p < 0.001$). Concerning CSF-AD biomarkers, AD and MCI-AD had significantly lower $A\beta_{42}$ levels and significantly higher t-tau and p-tau levels than MCI-S ($p < 0.001$).

Table 3.1 – Demographics and clinical characteristics of the studied cohort.

	AD (n=147)	MCI-AD (n=62)	MCI-S (n=40)	Controls (n=105)
Females (%)	97 (66%)	38 (61.3%)	25 (62.5%)	53 (50.5%)
Age (years) [Range]	67.1 ± 8.7 [48-84]	73.6 ± 7.6 ###, §§ [49-87]	67.2 ± 7.9 [47-79]	73.2 ± 5.7 ###, §§ [65-89]
Age at onset (years) * [Range]	63.9 ± 9.2 [46-79]	69.8 ± 7.5 ###;§§§ [47-84]	62.5 ± 8.0 [47-79]	-
ApoE-ε4 (%)	67 (45.6%)	37 (59.7%)	8 (20%) ##; ‡‡‡	19 (18.1%) ###, ‡‡‡
$A\beta_{1-42}$ (pg/mL)	436.3 ± 201.9	517.5 ± 192	796.5 ± 334.9 ###;‡‡‡	-
t-tau (pg/mL)	556.8 ± 376.1	525.7± 323.8	282.6 ± 166.5 ###;‡‡‡	-
p-tau-181 (pg/mL)	62.6 ± 37.8	63.9 ± 32.8	38.7 ± 19.4 ###;‡‡‡	-

Data is presented as mean ± SD, except when indicated otherwise. ### P < 0.001 vs. AD; ## P<0.01 vs AD; §§ P<0.01 vs. MCI-S; §§§ P < 0.001 vs. MCI-S; ‡‡‡ P < 0.001 vs MCI-AD

* AD n=(114); MCI-AD n=62; MCI-S n=40 (subjects with available age at onset data)

3.3.2 – *TOMM40* poly-T distribution

In all clinical groups, we observed that the distribution frequency of *TOMM40* poly-T lengths had several peaks in the fragment analysis, consistent with other published studies with European cohorts [79,80], confirming our *TOMM40*' 523 genotyping methodology. Particularly, 4 clusters with peaks around 15, 22, 28 and 33/34 "Ts" with diminished count frequencies on either side of the peak, could be distinguished **(Figure 3.2)**. When comparing MCI-S and MCI-AD distribution of poly-T lengths, it was observed that despite an overall similar pattern, differences between poly-T relative frequencies

could be seen in these groups **(Figure 3.2A).** The same was observed when comparing controls and AD poly-T lengths distribution **(Figure 3.2B).** In order to evaluate if these differences were statistically significant, we compared the relative frequencies of poly-T lengths classified as S, L or VL among different diagnosis groups using a Z test adjusted with the Bonferroni correction **(Table 3.2)**. By comparing MCI-AD with MCI-S group we observed that MCI-AD group had significantly higher frequencies of L alleles ($p < 0.001$) than MCI-S group. Regarding S and VL alleles, MCI-AD group had lower relative frequencies of S and VL alleles than MCI-S group. However, these differences did not reach statistical significance (p>0.05) after Bonferroni correction **(Table 3.2)**. Similar results were observed when comparing AD vs controls. Therefore, this data suggests that, in general MCI-AD is similar to AD and MCI-S to controls regarding *TOMM40*' 523 allele distribution **(Table 3.2)**.

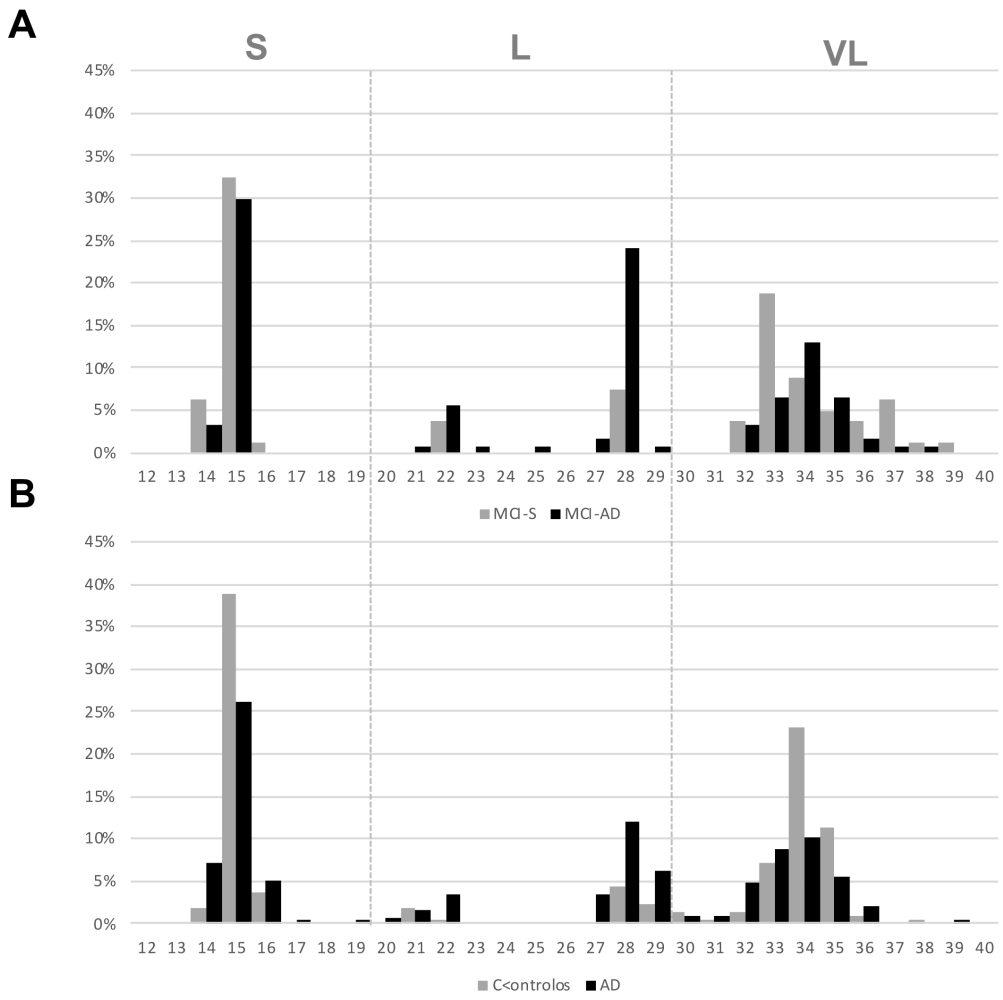


Figure 3.2 – Distribution of *TOMM40*' 523 poly-T lengths. (relative frequencies) in MCI-S vs MCI-AD group **(a)** and controls vs AD group **(b).** *TOMM40*' 523 alleles are classified into: short (S, ≤ 19), long (L, 20–29) or very long (VL, ≥ 30).

The different *TOMM40*' 523 genotypes, for MCI population and its correlation with *APOE* genotype are shown in **Table 3.3**. Here, we observed that in both MCI-S and MCI-AD population, the *TOMM40'* 523 genotype was significantly associated to *APOE* (p < 0.001)**.** *APOE* ε3 allele was found to be associated to *TOMM40'* 523 S and VL alleles, whereas the ε4 allele was almost exclusively associated to L *TOMM40'* 523, as previously described [73,75]. Despite the low frequency of *APOE* ε2 allele carriers, we found that similar to the ε3 allele, the ε2 alleles were also associated with the S and VL alleles. However, some discrepancies were found and are underlined and highlighted in bold in **Table 3.3**. The same association was found in the control and AD group (data not shown).

Table 3.2 – Distribution of *TOMM40* poly-T alleles in all clinical groups.

TOMM40' 523 alleles	AD	MCI-AD	MCI-S	Controls
S	115 (39.12%)	41 (33.06%)	32(40%)	94 (44.76%)
L	80 (27.21%)	43 (34.68%)	9 (11.25%) ##, ‡‡‡	19 (9.05%) ###, ‡‡‡
VL	99 (33.67%)	40(32.26%)	39 (48.75%)	97 (46.19%) #

Data is presented as relative frequencies of *TOMM40* poly-T alleles (S, L and VL) in each clinical group. Statistical analysis was performed using Z test adjusted for pairwise comparisons using Bonferroni adjustment.; ### P < 0.001 vs. AD; ## P<0.01 vs AD; # P<0.05 vs AD; ‡‡‡ P < 0.001 vs MCI-AD

Table 3.3 – Distribution of *TOMM40*' 523 polymorphism according to *APOE* genotype for MCI-S and MCI-AD population.

			TOMM40' 523 genotypes						
			S/S	S/L	S/VL	L/L	L/VL	VL/VL	Total
APOE Genotype	ε2/ε3	MCI-S	1	0	1	0	0	1	3
		MCI-AD	1	0	1	0	0	0	2
	ε3/ε3	MCI-S	6	0	15	0	0	8	29
		MCI-AD	6	0	12	0	**1**	4	23
	ε3/ε4	MCI-S	0	2	0	0	5	0	7
		MCI-AD	**1**	13	0	**1**	16	**1**	32
	ε4/ε4	MCI-S	0	0	0	1	0	0	1
		MCI-AD	0	0	0	5	0	0	5
	Total	MCI-S	7	2	16	1	5	9	40
		MCI-AD	8	13	13	6	17	5	62

Significantly statistic associations (Fisher test) between *APOE* genotypes and *TOMM40*' 523 genotypes are highlighted in dark grey. Discrepancies are underlined and highlighted in bold.

3.3.3 – *TOMM40*' 523 Genotypes and Risk of Mild Cognitive Impairment Conversion to Alzheimer's Disease

We performed a logistic regression analysis in order to assess the risk conferred by the different *TOMM40*' 523 genotypes for MCI to AD conversion. In a preliminary analysis, we observed that *TOMM40* '523 genotype was significantly associated with the risk of MCI to AD conversion and more specifically that *TOMM40*' 523 genotypes S/L and L/L were associated with that risk. Curiously, the L allele, when combined with the VL allele (L/VL) did not reach statistical significance (data not shown). However, when we stratified by *TOMM40*' 523 genotypes, the sample size was very small within each stratum, therefore we considered this analysis only as preliminary, due to low statistical power.

We then analysed the impact of *TOMM40*' 523 L allele on MCI to AD risk of conversion and observed that having at least one L allele significantly increased the risk of conversion (OR = 8.346, $p < 0.001$, 95%CI: 2.830 to 24.617). Age also showed to modestly impact the risk of conversion (OR = 1.140, $p < 0.001$, 95%CI: 1.064 to 1.221) but gender was not a significant factor ($p > 0.05$). However, as demonstrated previously, the L allele is almost exclusively linked to *APOE* ε4 allele, which also demonstrated to increase the risk of conversion (OR = 9.033, $p < 0.001$, 95%CI: 3.055 to 26.709). When we adjusted for ε4 allele, as expected, the L allele and ε4 allele were not independent significant factors for the risk of conversion **(Table 3.4)**.

Table 3.4 - Association of *TOMM40*'523 L and *APOE ε4* with risk of MCI conversion to AD

Variables	Without APOE ε4		Without TOMM40'523 L genotype		With APOE ε4	
	p-Value	OR (95% CI)	p-Value	OR (95% CI)	p-Value	OR (95% CI)
Age (years)	< 0.001	1.14 (1.06-1.22)	< 0.001	1.14 (1.07-1.22)	< 0.001	1.14 (1.07-1.22)
TOMM40' 523 L genotype	< 0.001	8.35 (2.83 – 24.62)	-	-	0.99	1.03 (0.23-46.11)
APOE ε4 genotype	-	-	< 0.001	9.03 (3.06-26.71)	0.27	8.77 (0.19-406.11)

3.3.4 – *APOE* ε4-*TOMM40*'523 L Haplotype on Risk and Time of Conversion

As it was not possible to disentangle if the effect would be due to ε4 allele or due to *TOMM40*' 523 L allele, we analysed instead the *APOE*ε4-*TOMM40*' 523 L haplotype (hereafter ε4-L haplotype) since L allele in our sample occurs in 95.5% of ε4 carriers. We then performed a haplotypic analysis, where we compared the carriers of the ε4-L haplotype with those not carrying that haplotype, and observed a significantly higher risk of conversion for the haplotype carriers (O.R.=5.83; 95% CI=2.30-14.83), $p<0.001$. Similarly we also observed that ε4-L haplotype was associated with a significantly higher risk of AD (O.R.=3.8; 95% CI=1.96-7.39) , $p<0.001$.

When evaluating the impact of the ε4-L haplotype on the time of conversion of MCI patients to AD, we also observed that carriers of this haplotype had significantly lower mean times of conversion: 4.8 years (95%CI: 3.7 to 5.9) than patients without the ε4-L haplotype: 9.1 years (95%CI: 7 to 11.1) ($p=0.003$) **(Figure 3.3A).** ε4-L haplotype was also associated with lower AOO of AD patients: 68,4 years (95%CI: 66,4 to 70.5) when compared to AOO of AD patients without this haplotype: 76,2 years. (95%CI: 73,8 to 78.6) ($p<0.001$) **(Figure 3.3B).** Interestingly when stratified in the different *TOMM40*'523 genotypes we observed that L/VL had significant higher AOO, 74,7 years (95%CI: 72,4 to 77) than S/L, 66,6 years (CI: 63,5 to 69,6) (p=0,003) (data not shown).

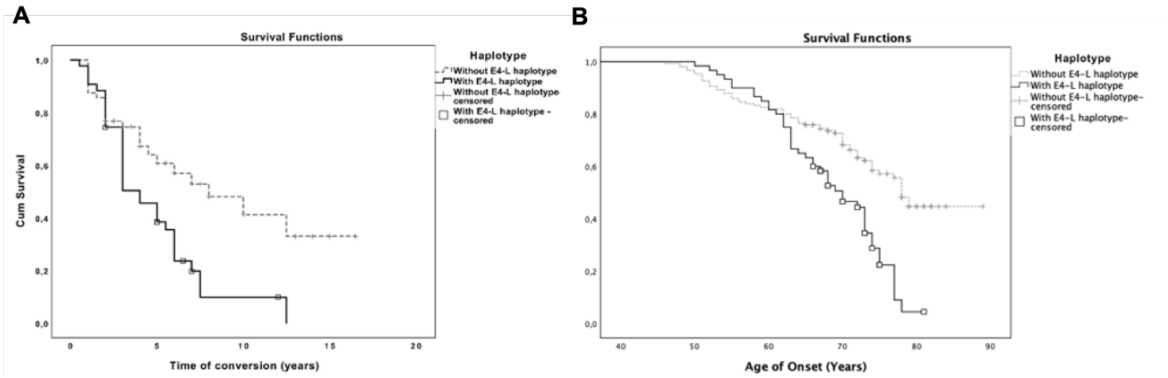


Figure 3.3 – Impact of *APOE* ε4-*TOMM40*' 523 L on time of conversion from MCI to AD and AOO of AD patients. Survival analysis curves (Kaplan-Meier) for mean time of conversion from MCI to AD (years) **(a)** and for AOO of AD patients **(b)** with and without ε4-L haplotype.

3.3.5 – *APOE ε4-TOMM40*' 523 L Haplotype Impact on AD Biomarkers

We further observed that AD biomarkers significantly increased the risk of MCI conversion to AD. Indeed, when classified as having a profile compatible with AD, using the dichotomic variable IATI index [280], AD biomarkers significantly increased this risk of conversion from MCI to AD (O.R =15.6, $p < 0.001$, 95%CI: 5.2 to 46.3). We also evaluated the association of ε4-L haplotype with biomarkers and verified that this haplotype is significantly associated with biomarkers compatible with AD ($p = 0.007$) in whole MCI population **(Table 3.5).** Considering the values of the 3 biomarkers: $A\beta_{42}$, t-Tau and p-Tau we further observed that the ε4-L is significantly associated with lower levels of $A\beta_{42}$ ($p = 0.015$) and higher levels of t-Tau ($p = 0.006$) and p-Tau ($p<0.001$) **(Figure 3.4).** On the other hand, in the AD population the ε4-L haplotype was only significantly associated with higher levels of p-Tau ($p=0.039$). The same association regarding $A\beta_{42}$ ($p = 0.861$) and t-Tau levels ($p = 0.132$) did not reach significance (data not shown).

Table 3.5 – Cross tabulation of ε4-L haplotype and biomarkers classified as having a biomarker profile compatible or not compatible with AD (according to IATI index)

			Biomarkers		
			Non Compatible with AD	Compatible with AD	Total
Haplotype	Without ε4-L haplotype	Count	29	27	56
		% within *TOMM40* genotype	51.8%	48.2%	100%
	With ε4-L haplotype	Count	10	33	43
		% within *TOMM40* genotype	23.3%	76.7%	100%
Total		Count	39	60	99
		% within *TOMM40* genotype	39.4%	60.6%	100%

More cases than expected are highlighted in grey. ε4-L haplotype is associated with a biomarker profile compatible with AD whereas patients without this haplotype are more associated with biomarker profile not compatible with AD

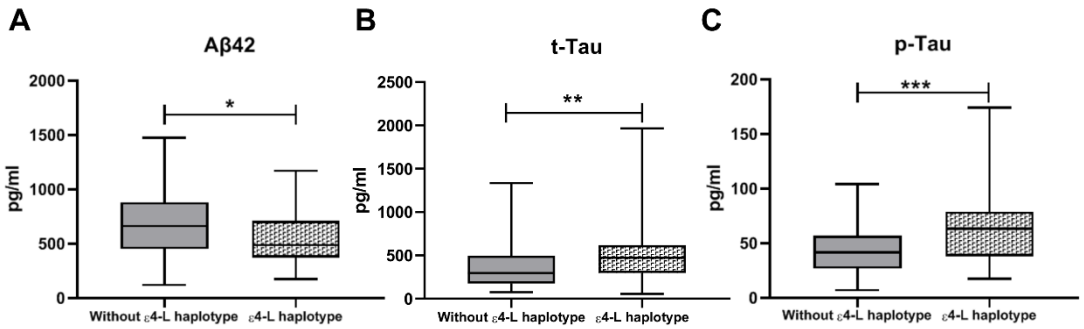


Figure 3.4 – Impact of *APOE ε4-TOMM40*' 523 L Haplotype on AD CSF biomarkers in MCI population. Box plots for CSF concentrations (pg/ml) of **(a)** $A\beta_{42}$, **(b)** t-Tau and **(c)** p-Tau (means ± SD). Statistical significance between groups was determined using Mann Whitney test * p<0,05; ** p<0,01; *** p<0,001

3.4 – Discussion

In this study, we uncover the role of the *APOE-TOMM40* haplotype on the risk and time of conversion from MCI to AD. We first demonstrated that MCI non-converters (MCI-S) and converters (MCI-AD) had a different poli-T distribution, where the L allele was significantly more frequent in the MCI-AD group. We further evaluated how this difference impacted the risk of conversion and found that, having at least one L allele significantly increased the risk of conversion from MCI to AD. Due to the strong LD between *APOE* and *TOMM40* [64–66] we were unable to statistically distinguish the independent effects of *TOMM40*-L and *APOE*-ε4. As it was not possible to disentangle if the effect would be due to *APOE* ε4 allele or due to *TOMM4*0' 523 L allele, we analysed instead the *APOE* ε4-*TOMM40*'523 L haplotype performing a haplotype analysis and verified a significantly higher risk of conversion for the ε4-L haplotype carriers and that patients carrying this haplotype had significantly lower mean times of conversion. Lastly, we observed that the ε4-L haplotype was significantly associated with a CSF biomarker profile compatible with AD, namely, significantly lower levels of $A\beta_{42}$ and higher levels of Tau and p-Tau. Similar results were observed for AD where ε4-L haplotype carriers where associated with a significantly higher risk of AD and lower AOO of AD patients. Regarding the relation of ε4-L haplotype with AD biomarkers it was only significant for p-Tau levels.

In contrast with AD, very few studies have focused on the impact of *TOMM40*' 523 polymorphism on MCI and, to our knowledge, none of them studied the impact of *APOE-TOMM40*' 523 haplotype on risk and conversion time, as we did in this study. Roses´ group, who identified this polymorphism, presented a stratification by *TOMM40* '523 and *APOE* genotype of ages of onset of cognitive impairment, in order to create a risk algorithm for clinical trial enrichment [86,87] . The major difference between these two studies and our study is that the other authors evaluated the risk of conversion from normal cognition to MCI and/or AD, whereas in our work the event studied was the risk of conversion from MCI to AD. Other authors evaluated the utility of *TOMM40* poly-T variable-length polymorphism among other 247 variables, for modelling the progression from MCI to AD but the *TOMM40* '523 genotype variable was not present in the final model. On the other hand, it was not clear how the *TOMM40* '523 poly-T variable was classified [258]. Lastly, Laczo *et al*. studied the impact of *TOMM40* '523 genotype on cognition and brain structure among amnestic MCI individuals [103]. In contrast, regarding the *APOE* ε4 allele, dozens of studies reviewed in previous metanalysis [252,253] showed that the presence of the ε4 allele is a risk factor for the progression from MCI to AD, despite its low sensitivity [255] . Regarding the association of *TOOM40*'523 with AD AOO we observed that L/VL allele was associated with significantly higher AOO of AD than S/L in opposition to what was described by Roses *et al.*[73] . Similar results were described by Cruchaga *et al.* [78].

CSF biomarkers are also used as predictors for the progression from MCI to AD [256]. Li *et al.* showed in a metanalysis that abnormal levels of t-Tau, p-Tau and the ratio t-Tau /$A\beta_{42}$ are associated with high risk of progression from MCI to AD [253]. Here, we also demonstrated that MCI patients with a CSF biomarker profile compatible with AD, using the dichotomic variable IATI index [280], were significantly at higher risk to convert from MCI to AD, and that ε4-L haplotype was significantly associated with a biomarker profile compatible with AD, namely higher levels of $A\beta_{42}$ and lower levels of t-Tau and p-Tau. Previous studies also demonstrated a similar influence of *APOE* ε4 on CSF AD biomarkers [281–283]. Regarding *TOMM40'523* L, the associations observed with this allele and CSF biomarkers were attributed to ε4 allele [78,79,89]. Taking all this data into consideration, we hypothesize that the higher risk of conversion and lower mean times of conversion observed in ε4-L haplotype carriers, can be driven by CSF biomarkers.

In the present study we analysed the role of *APOE* ε4 *TOMM40*'523L haplotype on the risk of MCI to AD conversion instead of *APOE* ε4 and *TOMM40*' 523 L allele separately as explained earlier. A recent study also addressed the impact of the *APOE-TOMM40* haplotype on susceptibility to dementia with Lewis Bodies (DLB), Parkinson's Disease Dementia (PDD) and Parkinson's Disease (PD) in clinically and neuropathologically well-characterized individuals and found that the ε4-L haplotype increased the susceptibility and risk of earlier DLB onset. This association was explained by the co-occurrence of AD pathology [257]. Although in the Caucasian population the ε4 allele is almost exclusively associated with L allele, as demonstrated in this study, the same does not occur in other populations such as in Ghanaian, African Americans and Japanese population where, ε4 is commonly linked to '523-S in addition to the '523-L allele [74,76,284]. Considering this, Yu *et al*. compared the ε4-L haplotypes between older Caucasians and African Americans and demonstrated that the effect size and effect pattern on AD dementia incidence were similar between ε4 and '523-L on the Caucasian, but different in the African carriers. In the same study it was also demonstrated that the risk conferred by the *APOE* ε4 haplotype depends on the *TOMM40*'523 allele in LD, showing that African Americans with ε4-'523-L haplotype show stronger effect on the increased risk for AD dementia than those with either ε4-'523-S or ε4-'523-VL haplotypes [77]. In line with these results, Prokopenko also suggested the possibility that part of the liability of LOAD, commonly ascribed to ε4, might have been caused by *TOMM40* on the basis of its strong LD [257] . Thus, we propose the ε4-L haplotype as a risk factor for MCI to AD conversion as well for AD and that its effect may be due to the combination of both *APOE* and *TOMM40* genes. *APOE* and *TOMM40* encode two very distinct proteins. *APOE* encodes the Apolipoprotein E (ApoE) protein, which is a glycoprotein that regulates lipid homeostasis by mediating lipid transport from one tissue or cell type to another [56,57]. However, other important roles have been described for this protein, such as Aβ metabolism and clearance, Tau phosphorylation, brain activity and atrophy, brain cholesterol transport, synaptic plasticity, inflammation and brain neurogenesis (reviewed in [57]). On the other hand, *TOMM40* encodes the channel subunit of the outer mitochondrial membrane protein complex TOM40, through which the majority of nuclear-encoded proteins enter mitochondria [67,68], including Aβ and APP, which further lead to mitochondrial

dysfunction [218,220]. Mitochondria dysfunction has been shown to be an early and well characterized event in AD [285,286]. *APOE* ε4 contributes to mitochondrial dysfunction, decreasing mitochondrial mobility, expression of mitochondrial respiratory chain enzymes and translocases of the inner and outer mitochondrial membranes (TIMs and TOMs, respectively). It was also demonstrated that apoE4 (1-272), a bioactive carboxyl-terminal-truncated product from apoE4 proteolytic cleavage [287] , is internalized into mitochondria, causing mitochondrial dysfunction [227,288], by reducing mitochondrial membrane potential [227] and by bounding several mitochondrial proteins such as ubiquinol cytochrome c reductase core protein 2 and subunit 4 of cytochrome oxidase [288]. ApoE4 (1-272) internalization probably occurs via TOM40 [118] which shows an interaction between the two genes of the *APOE-TOMM40* haplotype **(Figure 3.5)**.To our knowledge only two studies focused on the relationship of *TOMM40*' 523 and mitochondrial function whose results are contradictory [289,290]. Therefore, further studies addressing the cellular and mitochondrial role of *APOE-TOMM4*0 haplotypes are needed, namely studying how *TOMM40*' 523 alleles can affect apoE internalization into mitochondria and subsequent mitochondria function.

In our study, two main limitations can be found: the low dimension of the sample of MCI subjects and the lack of familial data. The low number of MCI subjects and conversion events decreased the statistical power when we stratified by *TOMM40*' 523 genotypes. The same happened when analyzing the ε3/ε3 and ε3/ε4 stratum, thus making it impossible to find any *TOMM40 '523* independent effect. We tried to overcome this limitation with specific statistical analysis focusing on *APOE-TOMM40'523* haplotype. Furthermore, in the logistic regression analysis, we tried to adjust for several confounding factors and applied multiple testing corrections. Regarding the lack of familial data, that made it impossible for us to establish the phase of the haplotype with the parent-offspring transmission, because we could not assess if the *APOE* ε4 and the L allele were in *cis* or in *trans*.

One of the main strengths of our study is the fact of being performed in a well characterized cohort of patients both clinically and regarding CSF biomarkers of disease. Furthermore, the data presented here reinforce the hypothesis of mitochondrial dysfunction as an early event in the pathophysiology of AD.

In summary, our study shows that the *APOE* ε4 - *TOMM40* '523 L haplotype is associated with a higher risk and shorter times of MCI to AD conversion possibly driven by CSF biomarkers and mitochondrial dysfunction.

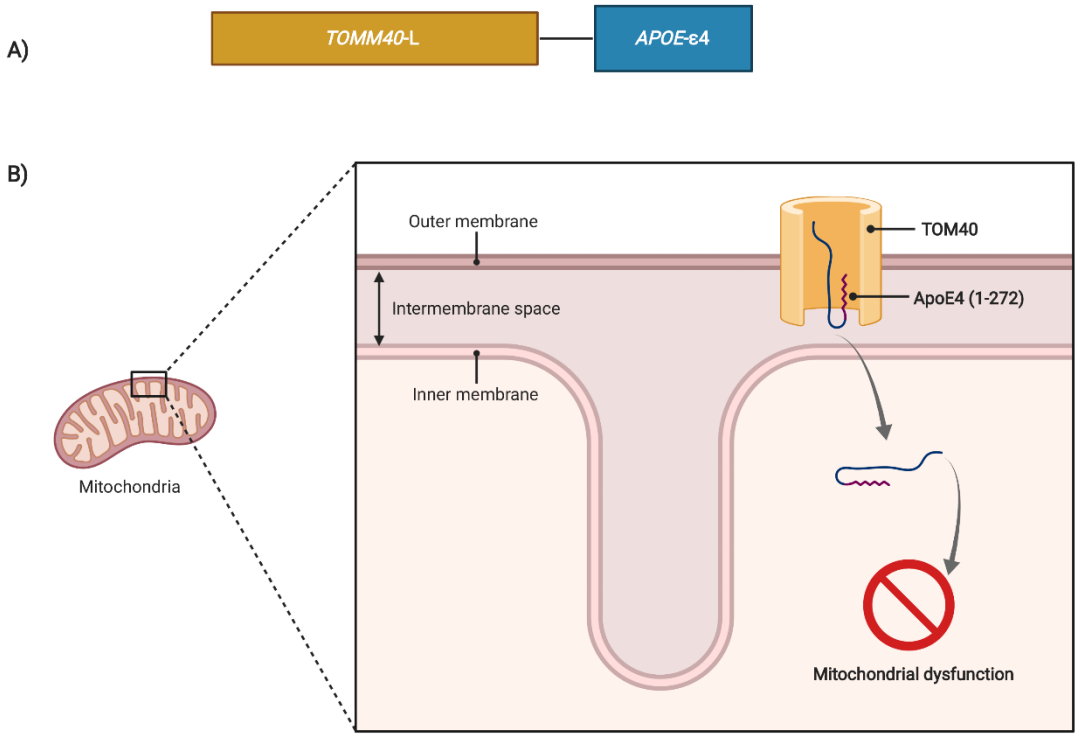


Figure 3.5 – Possible biological interaction between *APOE* and *TOMM40* genes. A) The *TOMM40* gene encodes the channel subunit of the outer mitochondrial membrane protein complex TOM40, through which the majority of nuclear-encoded proteins enter mitochondria. On the other hand, *APOE* codes the Apolipoprotein E (ApoE) protein, which is a glycoprotein that regulates lipid homeostasis by mediating lipid transport from one tissue or cell type to another, nevertheless other important roles in AD pathophysiology have been described for *APOE*. **B)** ApoE4 (1-272) a fragment from full ApoE4, was demonstrated to be internalized into mitochondria, causing mitochondrial dysfunction. ApoE4 (1-272) internalization probably occurs via TOM40 channel which shows an interaction between the two genes, which can have a possible modulatory effect, turning the *APOE-TOMM40* haplotype analysis important to address this possible joint effect. Therefore, studies addressing how *TOMM40*'523 alleles can affect apoE internalization into mitochondria and subsequent mitochondrial dysfunction are also needed.

Chapter 4

TOMM40'523 and mitochondrial dysfunction

Abstract

TOMM40' *523* polymorphism has been associated with different AD related features such as AOO and risk of AD, brain structure and cognition. *TOMM40* gene encodes the channel subunit of the outer mitochondrial membrane protein complex TOM40, through which most of nuclear-encoded proteins enter mitochondria. Mitochondrial dysfunction is one of the earliest events in AD. Considering this, it was hypothesized that *TOMM40*' 523 could have a role on AD through mitochondrial dysfunction. However, the few studies performed so far did not reach clear conclusions. Using Peripheral Blood Mononuclear Cells (PBMCs) from AD patients, we addressed the impact of *TOMM40*' 523 on different mitochondrial features, such as function, structure and apoptosis. We obtained PBMCs from patients homozygous for S, L and VL *TOMM40*' 523 polymorphism, which are thereafter called S, L and VL groups. In this study, we observed that VL group had significant higher levels of TOM40 than S group, similar mitochondrial membrane potential, $\Delta\psi$m, higher mitochondrial fission and lower caspase activation. Although these two last parameters did not reach statistical significance, our data reinforce the hypothesis that. increased levels of TOM40, as have been observed by Zeitlow *et al.* [290] seem to be protective to mitochondria.

4.1 – Introduction

Over 40 years ago, electron microscopy (EM) studies of AD brains revealed altered mitochondria morphology [291]. Nowadays, mitochondria dysfunction is a well characterized event in AD. Mitochondrial dysfunction is an early event in AD and precedes detectable amyloid pathology both in humans [169,292,293] and animal models [294,295]. Several mitochondrial changes have been shown to be involved in AD**,** such as increased levels of ROS and oxidative damage [128,144,145], impaired bioenergetics [165], calcium dyshomeostasis [131,179], alterations in mitochondrial-associated ER membrane (MAM) [130,199] and mitochondrial fitness [134,165]. Alterations in mitochondrial fitness include alterations in fission and fusion, mitophagy and mitochondrial biogenesis. Protein AD hallmarks, such as Aβ [19], Tau [21] and *APOE* ε4 [243] are also known to play a role in mitochondria dysfunction. As different features are altered in mitochondria, several hypotheses have been put forward in order to explain the mitochondrial abnormalities seen in AD, including the AD mitochondrial cascade hypothesis [296,297], the calcium hypothesis of brain aging [180,182], the MAM hypothesis [130,199], and the amyloid cascade hypothesis [33,37]. However, it is difficult to demonstrate if these mitochondrial abnormalities are a trigger or just consequences of AD, and to draw a timeline among them. Moreover, it is known that these mitochondrial abnormalities are linked and ultimately lead to apoptosis and neuronal death.

As addressed previously, several polymorphisms on *TOMM40* have been associated with LOAD risk **(chapter 1.2)**, namely *TOMM40*'523 whose effect on AD risk, AOO, risk of MCI conversion to AD and relationship with CSF AD biomarkers was described in **chapter 3**. *TOMM40* gene encodes the channel subunit of the outer mitochondrial membrane protein complex TOM40, through which most of nuclear-encoded proteins enter mitochondria [67,68]. The majority of mitochondrial proteins are encoded by nuclear genes, synthetized in the cytosol [68,118] and then imported into mitochondria. It is known that preproteins use at least five major import pathways, four of which involve TOM40 for preprotein translocation across the outer membrane and all use the TOM complex [68]. As the import of mitochondrial proteins into mitochondria is essential for biogenesis and functioning of mitochondria, it is not surprising that TOM40

was shown to be essential for life in eukaryotic organisms. The importance of TOM40 was demonstrated in different eukaryotic models, including *S. cerevisae* [137], *N. crassa* [138], human cell lines [139], *C. elegans* [140] and mice [141]. TOM40 channel plays also a role in mitochondrial dysfunction, via Aβ internalization and APP blockade of this mitochondrial import channel. It has been demonstrated that accumulation of APP inhibits the entry of nuclear encoded COX subunits IV and Vb, which was associated with decreased COX activity and increased levels of H_2O_2. [218] On the other hand, Aβ internalization via TOM40 leads to ABAD and Prep inhibition, causing mitochondrial dysfunction [223,224]. *APOE* ε4 (1-272) fragment, a product of *APOE* ε4 cleavage, was also associated with mitochondria and mitochondrial dysfunction [227,228], and it was proposed that its mitochondrial localization is also mediated by the TOM complex. [118].

In the previous chapter, we demonstrated that *APOE* ε4-*TOMM40*' 523 L haplotype is a risk factor for MCI to AD conversion, and proposed that its effect may be due to the combination of both *APOE* and *TOMM40* genes and respective protein products via mitochondrial dysfunction. However, in contrast with *APOE*, very few studies focused on the relation of *TOMM40*' 523 and mitochondrial function and the results are contradictory [289,290]. Using human fibroblasts obtained from cognitively healthy *APOE* ε3/ε4 carriers harbouring VL or S poly-T variants coupled to their APOE ε3 allele, Hedskog *et al.* studied the relation of *TOMM40*'523 polymorphism with expression levels of TOM40 protein and mRNA, TOM40 mRNA splicing, mitochondrial function and morphology [289]. However, these authors did not find significant differences regarding VL or S poly-T variant . Zeitlow *et al.* used an overexpression model (HeLa cells) of TOM40 in order to model the VL effect on *TOMM40* protein expression, according to Linnertz *et al.* [117], and studied how the increased levels of TOM40 protein affect mitochondrial function. Analyzing a variety of parameters, such as mitochondrial number, bioenergetics and cellular response to β-amyloid, suggested that a high expression of TOM40 may play a protective role in mitochondrial function [290].

Accordingly, the main aim of this study was to investigate the impact of *TOMM40*'523 polymorphism in mitochondrial function of AD patients. To address this, PBMCs from AD patients were used and the role of *TOMM40'523* in mitochondrial function, morphology, dynamics and apoptosis was analyzed.

4.2 – Material and Methods

4.2.1 – Materials

Ethylenediaminetetraacetic acid (EDTA) tubes (VACUETTE)® were from Greiner Bio-One. Histopaque®-1077 (10771), RPMI-1640 medium (R7388), tetramethylrhodamine methyl ester perchlorate (TMRM; T5428), carbonyl cyanide 3-chlorophenylhydrazone (CCCP; C2759), oligomycin (O4876), protease inhibitor cocktail (P2714), phenylmethanesulfonyl fluoride (PMSF; P7626), glycerol (G5516), bromophenol blue (B0126), TWEEN® 20 (P9416) , 3[(3-cholamidopropyl) dimethylammonio]-propanesulfonic acid (CHAPS; C5070), epoxy resin (45359-1EA-F) and osmium (75632) were from Sigma-Aldrich. Inactivated fetal bovine serum (FBS) (10270-106) and penicillin-streptomycin (Pen/Strep; 15140-122) were from Gibco. Sodium fluoride (NaF; 201154) was from Fluka. Dithiothreitol (DTT; AMRE-0281-5G) and sodium dodecyl sulfate (SDS; M107) were from Amresco. Tris Base (BP152) was from Fisher Scientific. 30% Acrylamide/Bis gel (#1610156) was from Bio-Rad. Pierce™ bicinchoninic acid (BCA) Protein Assay Kit (23225) was from Thermo Scientific™. Bovine serum albumin (BSA ;MB04602) was from Nzytech. Enhanced chemifluorescence (ECF) substrate for Western Blotting (RPN5785) was from GE Healthcare. Colorimetric substrate for caspase-3 (235400) and polyvinylidene fluoride (PVDF) membrane (IPVH00010) were from Merck Millipore. Sodium cacodylate (R1103) and uranyl acetate (P1260A) were from Agar Scientific . All other chemicals were from Sigma- Aldrich or Fisher.

Table 4.1 – Primary Antibodies.

Primary Antibody	Isotype	Dilution	Target MW (kDa)	Supplier	Reference
Actin	Mouse	1:10.000	42	Sigma	A5316
Fis1 (TTC11)	Rabbit	1:500	~20	NovusBio	NB100-5646
Mfn2 (M03) clone 4H8	Mouse	1:500	~75	Abnova	H00009227-M03
p- Drp1(Ser616)	Rabbit	1:1.000	78 – 82	Cell Signalling	3455
TOM40 (D-2)	Mouse	1:500	40	Santa Cruz	Sc-365467

Table 4.2 – Secondary antibodies.

Secondary Antibody	Host	Dilution	Supplier	Reference
Anti-mouse	Sheep	1:10.000 (1:2.000 for TOM40)	GE Healthcare	NIF1316
Anti-rabbit	Goat	1:20.000	GE Healthcare	NIF1317

4.2.2 – Study subjects

AD patients (n=16) were recruited and diagnosed at the Dementia Clinic, Neurology Department of Coimbra University Hospital (CHUC), Coimbra, Portugal**.** These patients were genotyped for *APOE* and *TOMM40*' 523 as described previously **(see 3.2.2)**. In order to study the biological effect of *TOMM40*' 523 polymorphism on mitochondria, patients homozygous for S, L and VL *TOMM40*'523 alleles were selected (hereafter, denominated as S, L and VL groups). S/S (n=5) and VL/VL (n=6) patients were both homozygous for *APOE* ε3 allele (ε3/ ε3) whereas L/L (n=5) patients were homozygous for *APOE* ε4 allele (ε4/ε4) **(Table 4.3)**. Collection of peripheral blood from each patient was performed by an experienced nurse in the day of the medical appointment upon agreement to participate in the study (signed informed consent by the patient or the legal representative). These procedures have been approved by the Ethics Board of Faculty of Medicine, University of Coimbra and NOVA Medical School of Universidade NOVA de Lisboa.

4.2.3 – Isolation of Peripheral Blood Mononuclear Cells

For each participant, a total of 18 mL of blood was collected by vein puncture in two sterile 9 mL EDTA-coated tubes. After collection the tubes were gently mixed and stored at 4 °C to be processed in the next day. PBMCs were isolated by density gradient centrifugation. Briefly, the collected blood (~9 mL per tube) was carefully layered into 8 mL of Histopaque in a 50 mL falcon tube without disturbing the Histopaque surface. The blood was always checked for possible erythrocyte's lysis. Tubes were centrifuged at 1048xg for 20 min at 18 °C in a swing out rotor centrifuge (Eppendorf 5810R) without brake. After centrifugation, the ring containing mononuclear cells was carefully

removed from the interface using a Pasteur pipette. The harvested fraction was diluted with phosphate saline buffer 1x (PBS 1x) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4 (total volume of 30 mL). Hereafter, cells were pelleted by centrifugation for 10 min at 377xg, 18 °C. The pellet was resuspended in lysis buffer to perform total protein extracts for Western blotting (WB) analysis and measurement of caspase-3 activity or, alternatively, resuspended in RPMI 1640 medium containing 10% (v/v) heat inactivated FBS, 100 U/mL penicillin and 100 mg/mL streptomycin and cultured in T25 culture flasks, at a density of 1×10^6 cells/mL, in a humidified incubator chamber with 95% air and 5% CO_2 at 37 °C, for analysis of mitochondrial membrane potential (TMRM assay) and morphology [(transmission electron microscopy (TEM)].

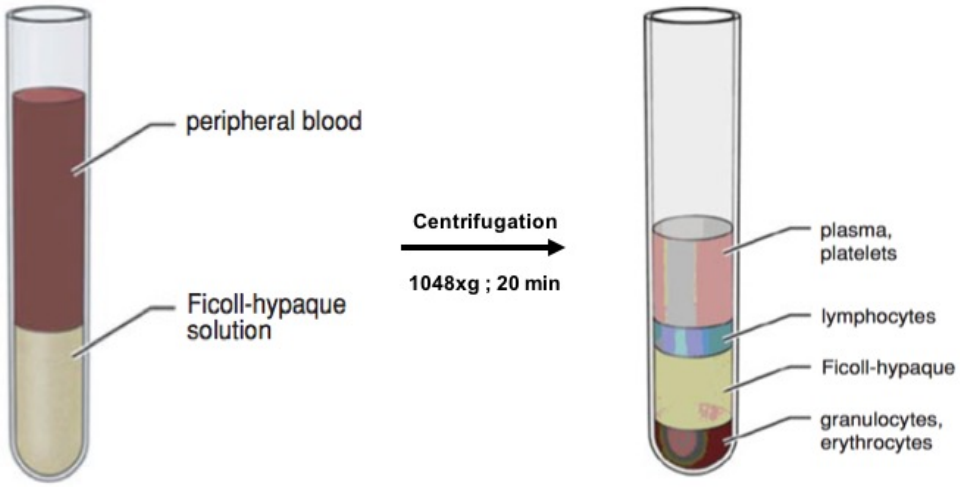


Figure 4.1 – Schematics of Peripheral Blood Mononuclear Cells isolation. Adapted from Fuss *et al.* [298].

4.2.4 – Analysis of mitochondrial membrane potential

To monitor changes in Δψm, the TMRM dye was used. TMRM is a lipophilic cationic compound with positive charge that accumulates in the highly negatively charged interior of mitochondria. Thereby, a decrease in TMRM cellular retention is associated with a decrease in Δψm. In this study TMRM was used in quenching mode [299], which

uses high dye concentrations such that it accumulates in mitochondria and form aggregates, quenching the fluorescence. Therefore, a subsequent mitochondrial depolarization will remove the quenching of the loaded probe and (transiently) increase the fluorescence signal. In a 96-well plate, $0.5x10^6$ cells/well were loaded in the dark with 300 nM TMRM in Krebs buffer (pH 7.4) composed of 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, for 30 min at 37 °C. Basal fluorescence was recorded for 2 min (readings every 30 seconds) at 37 °C (λex=540 nm and λem=590 nm). Afterwards, 40 µM CCCP (protonophore) and 40 µg/ml oligomycin (inhibitor of ATP synthase and Na^+/K^+-ATPase), were added to each well to achieve maximal mitochondrial depolarization and prevent ATP synthase reversal, respectively, and fluorescence was recorded for another 3 min (lectures every 10 seconds) at 37 °C. TMRM retention ability was calculated by the difference between the total fluorescence (after depolarization) and the initial value of fluorescence (basal fluorescence). Measurements were performed using a Spectramax Gemini EM spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

4.2.5 – Preparation of total protein extracts

For WB analysis and measurement of caspase-3 activity, total protein extracts were prepared from PBMCs **(see 4.3.3)**. Briefly, pelleted cells were resuspended in 80 µL of iced lysis buffer [25 mM HEPES, 2 mM $MgCl_2$, 1 mM EDTA and 1 mM EGTA (Ethyleneglycol- bis(β-aminoethyl)-N,N,N',N'-tetraacetic Acid), pH 7.5, supplemented with 1% (v/v) protease inhibitor cocktail, 50 mM NaF, 2 mM sodium ortovanadate, 100 µM PMSF and 2 mM DTT]. Cellular suspensions were frozen three times in liquid nitrogen and centrifuged for 10 min at 20800xg at 4 °C. The resulting supernatants were collected and stored at −20 °C. Protein concentration was determined using the Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions. Measurements were performed using a Spectramax Plus 384 spectrofluorometer (Molecular Devices, Sunnyvale, CA, USA).

4.2.6 – Western blotting analysis

Forty µg of total protein were denatured at 95 °C for 5 min in a 6x concentrated sample buffer [0.5 M Tris, 30% (w/v) glycerol, 10% (w/v) SDS, 0.6 M DTT, 0.012% bromophenol blue] and loaded into a 12% (w/v) polyacrylamide gel. After electrophoresis, the proteins were blotted onto a PVDF membrane and blocked in 5% (w/v) BSA during 60 min, before being incubated with the appropriate primary antibody overnight at 4 °C and with the appropriate secondary antibody for 2 h at room temperature (RT) **(Table 4.1 and 4.2)**. Then, membranes were washed five times during 5 min in Tris-buffer (150 mM NaCl, 25 mM Tris-HCl, pH 7.6) supplemented with 0.1% (v/v) Tween 20 (TBS-T). Finally, membranes were incubated with ECF for a maximum of 5 min at RT. ECF detection was performed using a Molecular Imager Versa Doc MP 4000 System (Bio-Rad) and, for each membrane, the analysis of band intensity was performed using the Image Lab software (Bio-Rad). Equal protein loading was controlled by membrane re-probing with an anti-actin (1:20,000) antibody and followed by incubation with an appropriated secondary antibody **(Table 4.1 and 4.2)**. Stripping was performed to reprobe membranes with another antibody(ies). For ECF removal, membranes were washed overnight with TBS-T at 4 °C or for 30 min with 40% (v/v) Methanol at RT. To remove antibodies, membranes were washed, with continuous agitation, with water for 5 min, NaOH (0.2 M) during 5 min and, finally, with water for 5 min. After stripping, membranes were blocked again with 5% (w/v) BSA for 60 min at RT.

4.2.7 – Determination of caspase-3 activity

To evaluate caspase-3 activity, cell extracts containing 25 µg protein were reacted for 3 h at 37 °C with 100 µM acetyl-Asp-Glu-Val-Asp p-nitroaniline (Ac-DEVD-pNA), the colorimetric substrate for caspase-3, in reaction buffer: 25 mM HEPES, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, 10 mM DTT, pH 7.4. The enzymatic cleavage of the chromophore p-nitroaniline (pNA) from the substrate was detected at 405 nm using a Spectramax Plus 384 spectrophotometer (Molecular Devices, Sunnvale, CA, USA).

4.2.8 – Transmission electron microscopy

PBMCs in culture were collected and centrifuged at 775xg for 5 min. The supernatant was discarded and pelleted cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h. After rinsing in the same buffer, post-fixation was performed using 1% (w/v) osmium tetroxide for 1 h. Cells were washed subsequently in buffer, buffer and distilled water, and finally in distilled water. To enhance contrast, 1% (v/v) aqueous uranyl acetate was added to cells for 1 h. Samples were washed in distilled water and dehydrated in a graded ethanol series (70–100%). Following embedding in 2% (w/v) molten agar, pellets were re-dehydrated in ethanol (30-100%), impregnated and included in Epoxy resin (Fluka Analytical). Ultrathin sections were mounted on copper grids and stained with 0.2% (w/v) lead citrate for 7 min. Observations were carried out on a FEI-Tecnai G2 Spirit BioTwin (Spain).

4.2.9 – Statistical analysis

Statistical analysis was performed using the non-parametric Kruskal-Wallis test, followed by the Dunn's multiple comparison. Values are expressed as mean ± SEM. P values of <0.05 were considered to be significant. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

4.3 – Results

4.3.1 – Sample characteristics

To study the impact of *TOMM40*' 523 polymorphism on mitochondrial function, structure and apoptotic cell death, PBMCs derived from AD patients genotyped for *APOE* and *TOMM40*' 523 were used. Three different study groups were established: S, L and VL groups, which consisted of PBMCs derived from patients homozygous for S, L and VL alleles, respectively (**Table 4.3)**. No significant differences were found neither in gender distribution, age or MMSE scores between study groups **(Table 4.4)**.

Table 4.3 – Demographic, genetic and clinical characteristics of patients.

#Patient	Gender	Age	MMSE	*APOE* genotype	*TOMM40*' 523 genotype
S1	F	69	0	ε3/ ε3	S/S
S2	F	69	29	ε3/ ε3	S/S
S6	M	56	25	ε3/ ε3	S/S
S7	F	87	23	ε3/ ε3	S/S
S8	M	75	7	ε3/ ε3	S/S
L1	F	71	0	ε4/ε4	L/L
L5	F	71	13	ε4/ε4	L/L
L6	F	64	5	ε4/ε4	L/L
L7	M	79	21	ε4/ε4	L/L
L8	F	69	14	ε4/ε4	L/L
VL1	F	82	4	ε3/ ε3	VL/VL
VL2	M	78	ns	ε3/ ε3	VL/VL
VL3	M	80	ns	ε3/ ε3	VL/VL
VL4	F	63	0	ε3/ ε3	VL/VL
VL5	M	66	10	ε3/ ε3	VL/VL
VL5	F	66	10	ε3/ ε3	VL/VL
VL6	F	68	6	ε3/ ε3	VL/VL

Table 4.4 – Comparison between *TOMM40*'523 groups.

	S group	L group	VL group
Females %	60%	80%	66,66%
Age (years) [range]	71,2 ± 11,2 [56-87]	70,8 ± 5,4 [64-79]	71,9 ± 7,8 [63-89]
MMSE	16,8 ± 12,6	10,6 ± 8,2	6 ± 4,2

Data are presented as mean ± SD, except when indicated otherwise.

4.3.2 – *TOMM40*' 523 genotypes and mitochondrial function

In order to study the effect of *TOMM40*' 523 polymorphism in mitochondrial function, we evaluated the effect of each polymorphism (S, L and VL) in mitochondrial potential using the TMRM assay. The L group showed a minor TMRM retention ability in comparison with the other groups, suggesting decreased mitochondrial membrane potential, whereas a similar retention of the fluorescent dye was observed between S and VL genotypes **(Figure 4.1 A)**. A statistically significant difference was seen when comparing L and VL genotypes (p=0.016), but not for the S allele **(Figure 4.1B).**

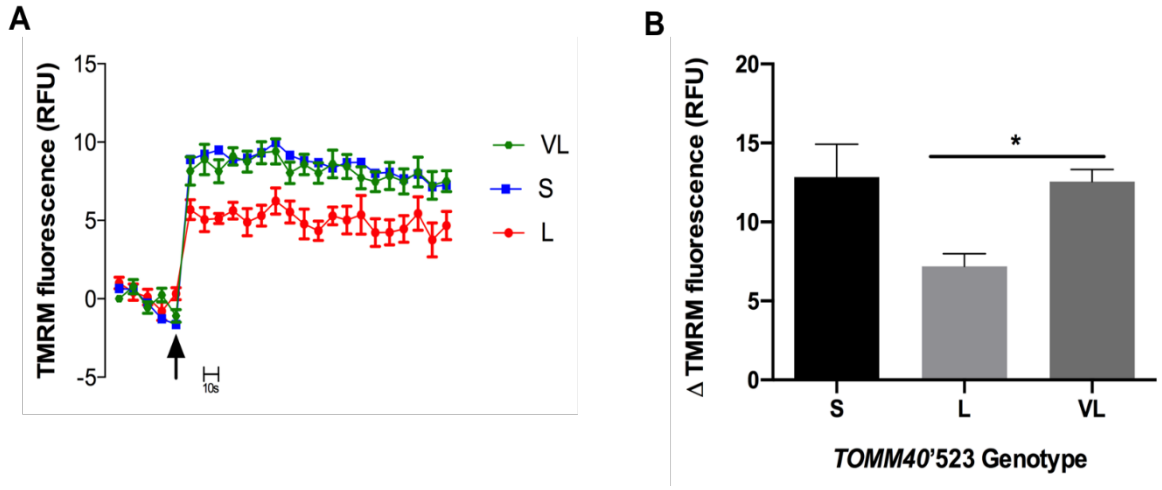


Figure 4.2 – Analysis of mitochondrial membrane potential between different *TOMM40'* 523 genotypes. Differences in mitochondrial membrane potential between different *TOMM40'* 523 genotypes were assessed using the TMRM fluorescent probe (quenching mode) in PBMC samples of S, L and VL groups. **(a)** Basal fluorescence of TMRM was measured for 2 min (readings every 30 seconds) and after stimulus (black arrow) for 3 min (readings every 10 seconds) and is represented as mean ± SEM of 3 patients per group. **(b)** TMRM retention ability was calculated by the difference between the maximum fluorescence (after depolarization) and the initial value of fluorescence (basal fluorescence) for S , L, and VL groups and represented as mean ± SEM of 3 patients per group. Statistical significance between groups was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test: *P<0.05 VL vs L.

4.3.3 – *TOMM40'* 523 genotypes and mitochondrial structure

To study the effect of *TOMM40'* 523 polymorphisms on mitochondrial structure, different strategies were used: **(i)** determination of TOM40 protein levels; **(ii)** analysis of the levels of proteins involved in mitochondrial dynamics (fission and fusion); **(iii)** study of mitochondrial morphology using TEM. Differences were observed between groups when TOM40 protein levels were measured. An increase was observed in TOM40 levels in L and VL groups, in comparison with the S group, reaching statistical significance when S and VL groups were compared (p=0,0427) **(Figure 4.2)**.

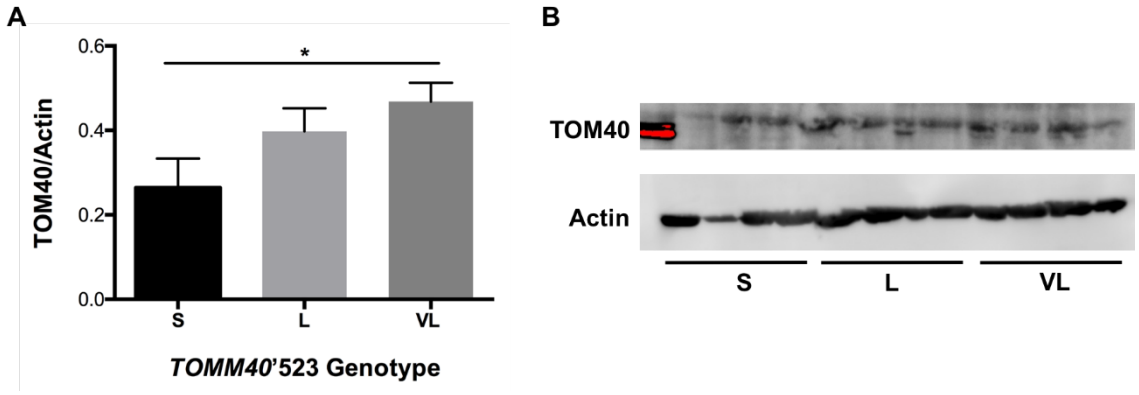


Figure 4.3 – Levels of TOM40 protein between different *TOMM40*' 523 genotypes. The levels of TOM40 protein were evaluated by WB in total protein extracts derived from PBMCs obtained from S, L and VL groups. **(a)** The values of TOM40 protein were normalized to actin and presented as TOM40/actin ratio (mean ± SEM**)** of 4 AD patients per group. **(b)** gel showing the TOM40 and corresponding actin levels for S, L and VL group. Statistical significance between groups was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test: *P<0,05 VL vs L.

In order to evaluate mitochondrial dynamics, the levels of two fission related proteins (p-Drp1 (Ser616) and Fis 1) and one fusion protein (Mfn2) were evaluated **(Figure 4.3)**. Regarding p-Drp1 (Ser616), similar levels were detected in the L and VL groups, while lower levels were observed in the S group, but without statistical significance. For Fis1, the L and VL groups showed higher levels in comparison with the S group. However, differences were not significant. Regarding Mfn2, all groups exhibited similar levels of this protein, which is involved in mitochondrial fusion.

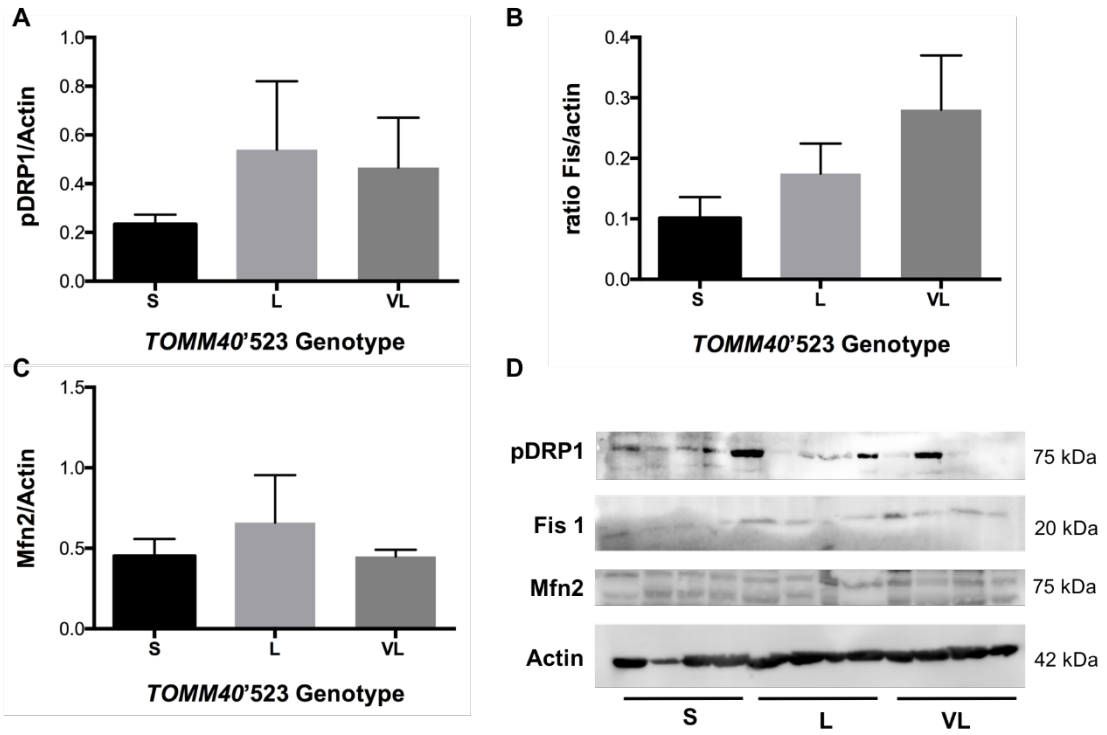


Figure 4.4 – Effect of *TOMM40'* 523 genotypes in mitochondrial dynamics. Protein levels were evaluated by WB in total protein extracts derived from PBMCs isolated from S, L and VL groups: pDrp1 **(a)**, Fis1 **(b)**, and Mfn2 **(c)**. Protein levels were normalized to actin and are presented as ratio of protein/actin (mean ± SEM) of 4 AD patients per group. **(d)** gel showing the p-Drp1 (Ser616), Fis1, Mfn2 and corresponding actin levels for S, L and VL group. Statistical significance between groups was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test.

To study mitochondrial morphology, confocal microscopy analysis of TOM20 or Heat Shock Protein 60 kD (HSP60) mitochondrial proteins immunostaining was first performed in PBMCs isolated from the different groups. However, due to the size of the nucleus in PBMCs, almost all intracellular space is occupied with this organelle, which hinders the investigation of mitochondrial morphology using this technique. Therefore, TEM was used to address the role of the different genotypes on mitochondrial morphology, in comparison with a cognitively healthy control **(Figure 4.4)**. Regarding control cells (PBMCs derived from cognitively healthy controls), uniform round mitochondria were mainly present (**Figure 4.4 a**). In the S group, a shape similar to control was observed, even though with some variability in mitochondria sizes (**Figure 4.4 b**). On the contrary, in the L group, high variability in size and shape of mitochondria

were detected and most of them were swollen (**Figure 4.4 c**). Lastly, it was observed that a great variability in mitochondria shape, with round and elongated mitochondria, occurs in the VL group (**Figure 4.4 d**). Moreover, in this group some fission events could also be observed (data not shown), which is in line with results showing higher levels of fission proteins in this group **(Figure 4.3).**

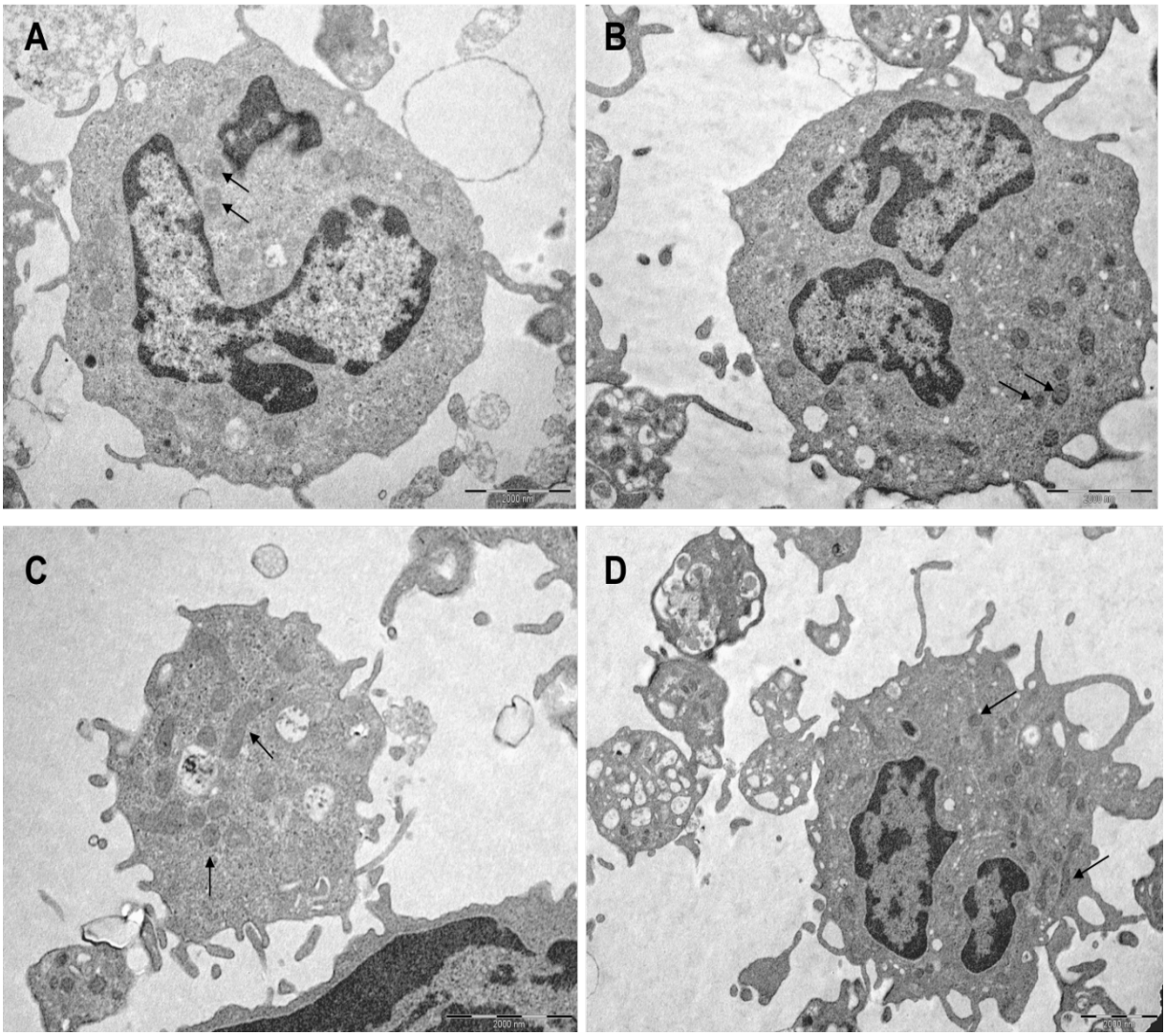


Figure 4.5 – *TOMM40*' 523 genotypes and mitochondrial morphology. Differences in mitochondrial morphology were observed by TEM in PBMCs of cognitively healthy control **(a),** S group **(b),** L group **(c)** and VL group **(d)**. Black arrows point to mitochondria.

4.3.4 – *TOMM40'523* genotypes and cell apoptosis

Cell death provides a defense mechanism by which damaged and potentially dangerous cells can be eliminated. Programmed cell death is known as apoptosis and is controlled by caspases **(see 1.3.1)**. Since mitochondria plays a central role in controlling apoptosis by the release of proapoptotic factors such as cytochrome c, the relation of *TOMM40*' 523 and the activity of caspase 3 in lysates from S, L and VL group PBMC's was studied. Caspase 3-like activity was assessed through the cleavage of a specific colorimetric substrate measured at 405 nm. Using this assay, a lower activity of caspase 3 was observed in the VL group, when compared with the S and L groups. Moreover, the highest activity was found in the S group, despite not reaching statistical significance.

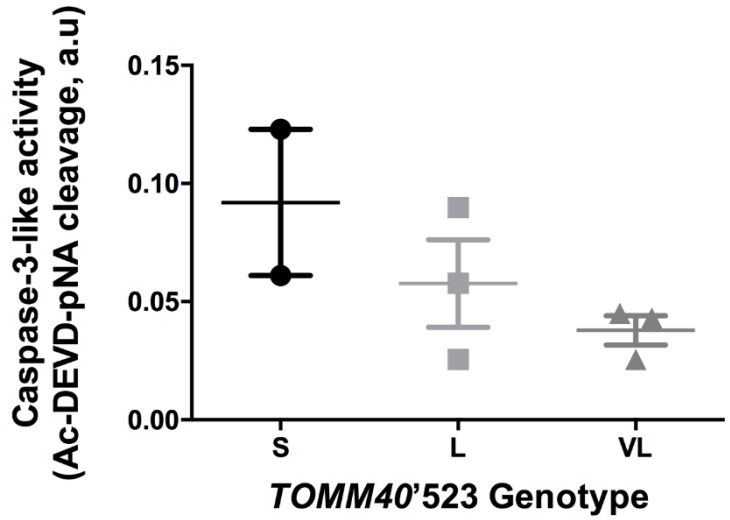


Figure 4.6 – *TOMM40'* 523 genotypes and caspase 3 activity. Values of absorbance corresponding to the generation of the chromophore pNA, an indication of caspase 3 activation, are represented in the scatter plot for S, L and VL groups. Statistical significance between groups was determined using the Kruskal-Wallis test, followed by the Dunn's multiple comparison test: n.s: non-significant

4.5 – Discussion

In this study, we observed significant higher levels of the mitochondrial import protein TOM40 on the VL group, when comparing to the S group. Regarding $\Delta\psi m$, similar results were obtained for the S and VL groups. On the other hand, mitochondrial membrane depolarization was detected in L group. *APOE* ε4, which is in LD with the L allele has been linked to reduced expression of genes encoding for mitochondrial respiratory enzymes, in particular electron transport chain genes from complexes I–V [247,248]. On the other hand, it has been demonstrated that apoE4 (1-272) is internalized into mitochondria, probably via TOM40, causing mitochondrial dysfunction [227,288], by reducing mitochondrial potential [227] and by binding several mitochondrial proteins [288]. Regarding the levels of proteins involved in mitochondrial dynamics (fission and fusion), higher levels of Fis1 and p-Drp1 (Ser616), which are fission-related proteins, were detected in the VL group compared to the S group. However, the differences were not statistically significant. On the other hand, the levels of the fusion protein Mfn2 were similar across all analyzed groups. Differences on mitochondria morphology were observed by TEM across different groups. The S group presented round mitochondria, similar to what was found in healthy control cells, despite some variability in mitochondria sizes. The VL group exhibited round and elongated mitochondria, where fission events were observed. Regarding the L group, despite high variability in size and shape of mitochondria, as observed in the VL group, swollen mitochondria were also detected. These swollen mitochondria can be explained by the mitochondrial dysfunction associated to *APOE* ε4 described above. Finally, caspase 3 activity was lower in the VL group, when compared with that in the S and L groups. However, the differences did not reach statistical significance.

The main objective of this study was to address any independent effect of *TOMM40'523* polymorphism on mitochondrial features and thus on mitochondrial dysfunction seen in AD. However, as the L allele is in LD with ε4 *APOE* allele, the observed effect for this allele can be a combination of both genes in ε4-L haplotype **(chapter 3).** For this reason, in this discussion I will only focus on the differences

between S and VL allele, since these alleles are linked to the same *APOE* allele and thus the different effects can be attributed only to *TOMM40'523.*

Regarding the levels of TOM40, we observed higher levels of TOM40 on VL group (VL/VL) when comparing to S group (S/S), whereas other authors found different results. Zeitlow *et al.* demonstrated a trend towards higher TOM40 protein levels in brain tissue from *APOE* ε3/ε4 AD patients who were S/L carriers, compared with VL/L carriers. However, the statistical significance depended on the protein used for normalization. In the same study, the author found that in the brain tissue from APOE ε3/ε3 subjects, expression of TOM40 did not differ significantly between S/S or VL/VL homozygotic carriers, independently of the protein used for normalization [290]. Furthermore, Hedskog *et al.* did not find differences in the levels of TOM40 protein between S/L and VL/L in fibroblasts from non-demented individuals [289]. The divergence in the results of the studies referred above is probably due to the fact that different tissues, genotypes and normalization methods were used, precluding any conclusion about the levels of TOM40 when comparing S and VL alleles. On the other hand, Linnertz *et al.* found that *TOMM40* mRNA levels were dependent on the number of VL alleles in brain samples from cognitively normal individuals [117], which is in line with higher TOM40 levels found in VL group.

Regarding mitochondrial function, a similar $\Delta\psi m$ was observed in our study when comparing S and VL group. Hedskog *et al.* also did not find differences in $\Delta\psi m$ when comparing fibroblasts from cognitively healthy donors with VL/L and S/L poly-T genotypes [289]. On the other hand, using cells overexpressing TOM40, Zeitlow *et al.* observed that $\Delta\psi m$, basal cellular ATP levels and oxygen utilization rates were higher in these cells, when compared to controls cells [290]. As addressed above, the cells overexpressing TOM40 were intended to model the VL allele according to the study of Linnertz et al [117]. Higher levels of pDrp1 and Fis 1 were also observed in this study in VL group, when compared to S group, despite not reaching statistical significance. Moreover, we observed more fission events by TEM in VL group, when compared to S group. It is known that levels of Drp1 phosphorylation at Ser616 are increased in AD [200] and that excessive fission can lead to mitochondrial dysfunction, including ROS overproduction, accumulation of mtDNA mutations and decreased calcium buffering [205,207,208]. However, despite VL group presenting higher levels of pDrp1 and Fis 1

than S group, the difference did not reach statistical significance. On the other hand, despite the negative effect of fission on AD, we need more data, such as ROS and Ca^{2+} levels, to state that the higher levels of fission in VL group has a negative impact on mitochondria. It is known that excessive Ca^{2+} levels lead to the mitochondrial permeability transition pore (mPTP) opening [71] and release of cytochrome c, which initiates the apoptosis cascade with caspase 3 activation [190]. In fact, when caspase 3 activation was evaluated, we observed lower levels of caspase 3 in VL group, when compared to S group, despite not being statistically significant.

Due to the high variability in the experiments described above, it was difficult to find significant statistical differences between *TOMM40*'523 groups in some of the parameters evaluated, precluding solid conclusions. Thus, the experiments here presented should be replicated with a higher number of samples per group in order to obtain more robust results. On the other hand, mitochondrial dysfunction involves a wide range of abnormal parameters **(see 1.3),** which should be further analyzed. Therefore, using the same model (PBMCs) we intend to extend these studies by evaluating the following parameters: (1) cellular quality control; (2) stress response and (3) mitochondria communication with endoplasmic reticulum (ER). Since its known that Aβ has several negative effects on mitochondria **(see 1.3.10)** and that Aβ is imported to mitochondria via TOM40 channel [220], we intend to evaluate mitochondria-specific accumulation of Aβ among the different *TOMM40*' 523 groups using mitochondrial fractions.

In future studies, we also intend to address the cellular and mitochondrial role of *APOE-TOMM4*0 haplotypes, by studying how *TOMM40*' 523 alleles can affect ApoE internalization into mitochondria and subsequent mitochondria function. These studies should be replicated also in the MCI population in order to address if mitochondrial disfunction leads to MCI to AD conversion, as hypothesized in **Chapter 3**.

Part of the limitations of this study are related with the nature of the study sample: human PBMCs obtained from blood of AD patients. One of the main limitations was related with the amount and availability of the sample. Since we were collecting samples of AD patients with specific *TOMM40'* 523 genotypes, the availability of eligible patients was limited, as most of the patients genotyped in the first part of the work **(Chapter 3)** were no longer accompanied in the clinic. For this reason, the number of patients per

group was low. On the other hand, since we were performing a significant number of assays and the amount of blood collected per patient was limited, the number of patients in each assay was reduced, varying from two to four per group, influencing the intra-group variability in most of the assays and therefore the statistical power of the results. For this reason, we had to reduce the number of assays performed and the number of biological replicates. A critical example was the TEM experience where, due to the cost of the assay and lack of sample, only one patient per group was tested. Finally, another limitation was the lack of healthy controls in the majority of the assays performed, since it was technically impossible to obtain a healthy control for each *TOMM40'* 523 genotype. Accordingly, we designed the experiments only with groups of AD patients relative to each genotype (S, L and VL) and intergroup comparisons were made. Thus, in future experiments healthy controls should also be included.

Despite all the limitations discussed above, this study correlated for the first time *TOMM40'* 523 polymorphism with mitochondria alterations in PBMCs derived from AD patients. Our findings support that this genetic alteration may affect mitochondria and give important clues for future research on the impact of *TOMM40'* 523 in mitochondrial phenotype. On the other hand, there is an unmet need to understand the biological mechanisms of *TOMM40'523* polymorphisms. Therefore, this study provides a useful and practical *in vitro* model to study the effect of these *polymorphisms*, which can be obtained from AD patients with different degrees of cognitive impairment but also from cognitively normal subjects. PBMCs are easy to collect when compared with other types of specimens, such as fibroblasts or brain tissue and can be used to address the *TOMM40*'523 phenotype in large cohorts.

In summary, we observed that VL group had significant higher levels of TOM40 than S group, similar mitochondrial membrane potential ($\Delta\psi$m), higher fission and lower caspase activation, despite not being statistically significant. The small sample size, as well as the technical limitations described above, precluded more robust conclusions and further experiments should be done.

Chapter 5

Conclusions and future perspectives

More than one century after the first description of AD by Alois Alzheimer we still do not have any therapy that stop or at least delay the progression of this disease for which ageing is the major risk factor. Part of the problem is that we do not fully understand the pathophysiology of this disease. Other issue is that we are starting to treat the disease too late in the disease pathogenesis, when the pathophysiologic processes have been underway perhaps a decade or longer [300]. AD risk genes allow a better understanding of the pathophysiology of the disease [41] and can even help in early diagnosis of AD giving us the possibility to treat patients earlier. It has been proposed that intervening earlier in the disease trajectory, before irreparable neuronal injury has accrued, will be a more successful strategy [300] and that the overall frequency of the disease would be decreased by nearly 50% if the onset of the disease could be delayed by 5 year [30]

TOMM40, namely its polymorphism, rs10524523, is a potential AD risk gene. Some studies associated this polymorphism with the risk and AOO of AD, although this aspect remains debatable. However, this gene encodes the channel subunit of the outer mitochondrial membrane protein complex TOM40, through which most of nuclear-encoded proteins enter mitochondria [67,68] and mitochondria dysfunction has been shown to be an early and well characterized event in AD [285] . Therefore, in this work we aimed to study the potential role of *TOMM40'*523 as a risk gene for AD and its involvement in AD pathophysiology through mitochondrial dysfunction, under the perspective "from clinics to mitochondria".

Regarding the potential of *TOMM40'*523 as a risk gene, we tried to replicate some of the previous associations of *TOMM40'*523 with AD risk, AD AOO and CSF core biomarkers of the disease. Accordingly, we also studied the role of *TOMM40'*523 on the MCI to AD risk and time of conversion, a poorly addressed feature under the scope of the disease. We found a poli-T pattern similar to that found in other published studies using European cohorts (which validated our methodology) [79,80] in contrast with what was observed in American Caucasian cohorts. This highlights not only the importance of studying this gene in different cohorts around the world, as stated by Linnertz el al [74], but also the need of using the same genotyping methodology.

However, we confronted some difficulties in this study. The principal difficulty was the ability to separate the effects of *TOMM40*' 523 from those triggered by the *APOE*

gene, and thus find independent associations of *TOMM40' 523* polymorphism, since the two genes are adjacent and reside within a region of LD, a well described problem in literature [78,79,81,82]. As it was not possible to disentangle if the effect would be due to *APOE* ε4 allele or to *TOMM4*0' 523 L allele, we analysed instead the *APOE* ε4-*TOMM40*'523 L (ε4-L) haplotype, by performing a haplotype analysis, and verified a significantly higher risk of MCI to AD conversion in ε4-L haplotype carriers and that patients carrying this haplotype had significantly lower mean times of conversion. Similar results were found for the ε4-L haplotype with the risk, and AOO of AD. Lastly, we observed that the ε4-L haplotype was significantly associated with a CSF biomarker profile compatible with AD, namely, significantly lower levels of $A\beta_{42}$ and higher levels of Tau and p-Tau in MCI population. It has been proposed that the effects observed for the ε4-L haplotype would be explained by the combination of both *APOE* and *TOMM40* genes.

Using PBMCs as study model we further addressed the relation of *TOMM40*' 523 and mitochondrial function. In this part of the study we observed that VL group had significant higher levels of TOM40 than S group, similar mitochondrial membrane potential ($\Delta\psi m$), higher fission and lower caspase activation despite not statistically significant. In contrast with our results, according to Zeitlow *et al.* [301], increased levels of TOM40, as observed in VL group, are suggested to be protective to mitochondria. However, we must point that this is a preliminary and proof concept study which due to the limitation of the sample hinders robust conclusions. So further studies to replicate these results in a larger cohort should be done. On the other hand this study opens doors to tackling the possible effect of *TOMM40' 523* on mitochondria dysfunction in AD , an aspect that to our knowledge, was only addressed in a few studies [289,290]. Beyond the mitochondrial aspects analyzed in this thesis other aspect of mitochondrial dysfunction can be studied such as ROS production and oxidative damage, calcium dyshomeostasis and alterations in mitochondrial homeostasis as reviewed in chapter 1.3.

Interestingly, recent studies showed that TOM40 has other important cellular functions beyond channel subunit of the outer mitochondrial membrane. Besides the indirect impact that TOM40 can have in apoptosis through mitochondrial dysfunction, some studies point a direct role of TOM40 in the apoptosis process. Veresov *et al.* using

computational modelling demonstrated that, under apoptotic conditions, TOM40 can form a complex with Bax, which leads to mitochondrial outer membrane permeabilization [302]. Furthermore, Frank *et al.* found an interaction of BIM with TOM40 [303]. A couple of recent papers also describe a relation of TOM40 with autophagy and the proteins involved in this process. Okatsu *et al.* demonstrated that TOM40 protein is required for PINK1 accumulation at OMM after mitochondria depolarization[304] and Neethling *et al.* showed that LRRK2 also interacts with TOM40 under DMSO and CCCP conditions [305]. In a more recent study it was shown that TOM40/70 directs ATG2 to MAM to mediate phagophores extention [306]. Curiously, other study by Namba *et al.* also demonstrates an important role of TOM40 in MAM, through the interaction with the protein BAP31 forming a ER-mitochondria bridging complex, which regulates mitochondrial function [307] These studies, showing that TOM40 protein plays a direct role in apoptosis, autophagy and MAM, which are known to be altered in AD, reinforces its relevance in the pathogenesis of the disease . Taking this into account, besides mitochondrial dysfunction, we hypothesize that *TOMM40*'523 can also impact apoptosis, autophagy and MAM through the processes above mentioned, this being an issue that we intend to further study in the context of *TOMM40*'523 polymorphism and AD pathophysiology, mechanisms in which our laboratory has experience as demonstrated in previous papers [184,185,308,309]

Since TOM40 interacts with proteins involved in the pathophysiology of Parkinson's disease, such as PINK1 and LRRK2, it will also be interesting to study *TOMM40*'523 polymorphism in Parkinson's disease pathophysiology, although results already published did not demonstrated an association between *TOMM40*'523 and PD risk [310,311] .

In conclusion, the work performed in this thesis demonstrates the multifactorial characteristics and the complexity of Alzheimer's disease pathophysiology and the relevance to identify disease risk factors allowing an early diagnosis and, consequently ,an early therapeutical approach to this devastating neurodegenerative disease.

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