VNIVERSITAT DE VALÈNCIA
Facultat de Medicina i Odontologia
Departamento de Fisiología

PROGRAMA DE DOCTORADO EN MEDICINA

Estudio prospectivo longitudinal en sujetos portadores del alelo 4 de la *APOE*.

Tesis Doctoral

Mariana Pinheiro Nepomuceno

Dirigida por:
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Dr. José Viña Ribes

Valencia, Mayo de 2023

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CERTIFICAN:

Que Dña. Mariana Pinheiro Nepomuceno, Licencia en Medicina por la Universidad Estácio de Sá y Máster en Neurociencias por la Universidad de Valencia, ha realizado bajo su dirección para la obtención del título de Doctor la presente Tesis Doctoral titulada:

"Estudio prospectivo longitudinal en sujetos portadores del alelo 4 de la *APOE*"

Y para que conste a los efectos oportunos, firman la presente certificación en Valencia en el año 2023.

D. José Viña Ribes Dña. Ana Lloret Alcañiz Dña. Paloma Monllor Taltavull

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Abbreviations

Aβ	Amyloid β peptide
AD	Alzheimer's Disease
apoE	Apolipoprotein E protein
ApoE2	Apolipoprotein E isoform 2
ApoE3	Apolipoprotein E isoform 3
ApoE4	Apolipoprotein E isoform 4
APOE	Apolipoprotein gene
APOE2	*APOE* gene ε2 allele
APOE3	*APOE* gene ε3 allele
APOE4	*APOE* gene ε4 allele
APP	Amyloid precursor protein
Arg	Arginine
BBB	Blood brain barrier
BSA	Bovine serum albumin
cDNA	Complementary DNA
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	C-terminal domain of a protein
Cys	Cysteine
DAPI	4', 6-diamidino-2-phenylindole
DCF	Dichlorofluorescein
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithio-bis-2-nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay

EOAD	Early-onset Alzheimer's Disease
ER	Endoplasmic reticulum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCLC	Glutamate-cysteine ligase catalytic subunit
GCLM	Glutamate-cysteine ligase modifier subunit
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSK3β	Glycogen synthase kinase 3 beta
GSSG	Oxidized glutathione disulfide
H_2O_2	Hydrogen peroxide
HDL	High-density lipoprotein
HNE	Hydroxynonenal
HPLC	High performance liquid chromatography
IL-1β	Interleukin-1β
LDL	Low-density lipoprotein
LDLR	LDL receptor
LOAD	Late-onset Alzheimer's Disease
MAPK	Mitogen-activated protein kinases
MAPT	Microtubule-associated protein tau
MCI	Mild cognitive impairment
MDA	Malondialdehyde
MFE	Memory Failures in Everyday questionnaire
MFI AU	Mean fluorescence intensity arbitrary units
mRNA	Messenger RNA
NADP	Nicotinamide adenine dinucleotide phosphate - Oxidized
$NADPH^+$	Nicotinamide adenine dinucleotide phosphate - Reduced

NEM	N-Ethylmaleimide
NFκβ	Nuclear factor kappa-beta
NFT	Neurofibrillary tangles
NRT	No-Reverse Transcription control
NT	N-terminal domain of the protein
NTC	No-Template Control
p38	p38 MAPK
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PET	Positron emission tomography
PI	Propidium iodide
PKR	eukaryotic translation initiation factor 2 alpha kinase 2
p-p38	Phosphorylated p38 MAPK
p-tau	Phosphorylated tau protein
p-tau231	Tau protein phosphorylated at threonine 231
PUFA	Polyunsaturated fatty acids
qPCR	Quantitative PCR (real-time PCR)
RAVLT	Rey Auditory Verbal Learning Test
RCAN1	Regulator of calcineurin 1
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Reverse transcriptase
RT-PCR	Reverse transcription PCR
SCD	Subjective cognitive decline
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SMC	Subjective memory complaints
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1 (Cu-Zn SOD)
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TNF-α	Tumor necrosis factor α
VLDL	Very-low density lipoprotein
WB	Western blotting

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Summary

Apolipoprotein E [apoE] is a multifunctional protein, whose main function is the maintenance of lipid homeostasis, but that also affects many other physiological functions, including inflammation, immunity, antioxidant response, and other. The Apolipoprotein E gene [*APOE*], in chromosome 19q13.3, presents 3 main alleles, ε2, ε3 and ε4, which code for their respective isoforms apoE2, apoE3 and apoE4. These isoforms differ by an amino acid exchange in positions 112 or 158. While apoE3, the most common isoform, presents cysteine in position 112 and arginine in position 158, apoE2 the least common isoform presents cysteine in both positions and apoE4, which has a worldwide frequency of about 14%, presents arginine in both positions.

This amino acid substitution changes the structure and function of the protein. In the case of apoE4, it presents gain of toxic functions and loss of normal functions that cause many pathological alterations. This makes *APOE4* carriers more likely to develop many disorders, but especially Alzheimer's Disease [AD]. AD is the most common age-related neurodegenerative disorder. It causes a progressive decline in cognitive functions that eventually leave the individual completely dependent. Thus, AD causes great socio-sanitary, economic and personal burden. The sporadic form of AD is the most common and presents many risk factors. However, the main genetic risk factor is the presence of *APOE4*, which induces or increases several of the pathological alterations seen in AD, including processing and deposition of tau and amyloid beta [Aβ], oxidative and cellular stress and cell death. However, pathological alterations have been found even in individuals who are cognitively normal. Therefore, knowing the

changes caused by *APOE4* before the beginning of clinical symptoms might help to better understand the disease and to create better tools for prevention.

The aim of this study is to conduct a 11-year prospective longitudinal study with a cohort of subjects carrying at least one *APOE4* allele, and compare them with non-carriers.

For that, we conducted a follow up of 24 *APOE4* carriers (10 homozygous, 14 heterozygous), which had participated in the previous study. We further analyzed other 23 individuals; 15 of which did not have the *APOE4* allele and were considered control group; the other 8 presented at least one allele (3 homozygous, 5 heterozygous) and were considered volunteers and evaluated only in cross-sectional analysis. We conducted individual interviews with the participants, and performed neuropsychological tests: The Rey Auditory Verbal Learning Test [RAVLT]; The Stroop Color Word test; The Memory Failures in Everyday questionnaire and The Hamilton Depression Rating Scale. Furthermore, blood samples were drawn and processed to analyze: whole blood reduced [GSH] and oxidized [GSSG] glutathione by spectrophotometry; plasma malondialdehyde [MDA] by High performance liquid chromatography [HPLC]; plasma Interleukin-1β and TNF-α levels by Enzyme-linked immunosorbent assay [ELISA]; Cell death and reactive oxygen species by flow cytometry; protein expression of calcineurin, regulator of calcineurin 1 [RCAN1], glycogen synthase kinase-3 beta [GSK3β], p38 mitogen-activated protein kinases [p38], phosphorylated-p38 [p-p38] and tau phosphorylated at threonine 231 [p-Tau231] with western blotting [WB]; and gene expression of calcineurin, RCAN1, GSK3β, glutamate-cysteine ligase catalytic subunit [GCLC], glutamate-cysteine ligase modifier subunit

[GCLM], glutathione peroxidase 1 [GPx1], eukaryotic translation initiation factor 2 alpha kinase 2 [PKR], and superoxide dismutase 1 [SOD1] by real-time polymerase chain reaction [qPCR].

We found that the reductive stress previously present in young *APOE4* carriers has now reversed and carriers now present increased oxidative damage and cell death, compared with non-carriers. Currently, *APOE4* carriers present increased plasma levels of IL-1β, in an allele dose-dependent manner. They also present increased MDA and lower GPx1 expression. Environmental risk factors for AD only influenced MDA levels in heterozygous carriers and not in non-carriers. Moreover, homozygous carriers present lower RCAN1 protein expression when compared with heterozygous and with non-carriers. Conversely, heterozygous carriers presented lower GSK3β gene expression and higher PKR gene expression when compared to non-carriers. This was not associated with alterations in subjective or objective cognition.

In conclusion, our results show that inflammation, redox homeostasis and expression of stress-related proteins involved in AD pathology change with age in cognitively healthy *APOE4* carriers. Thus, studying *APOE4* carriers is important to better understand AD pathology before its clinical onset.

INTRODUCTION

1. Introduction

1.1. Apolipoprotein E

1.1.1. Introduction to Apolipoprotein E

Apolipoprotein E [ApoE] is a 34 kda glycoprotein, with 299 amino acids that belongs to the family of exchangeable apolipoproteins; that is, those that can be transferred between different lipoprotein particles during their time in the (Su & Peng, 2020; Zhao, Liu, Qiao, & Bu, 2018). It is a multifunctional protein that has 3 common isoforms, which are coded for by their respective alleles (Mahley, 2016a).

ApoE was discovered in the early 1970's by Havel and Kane (Havel & Kane, 1973) when it was described as an unknown serine-rich protein present in very-low density lipoprotein [VLDL] particles of patients with familial hypercholesterolemia type III. Later, it was further characterized by (Utermann, Jaeschke, & Menzel, 1975), who named it apolipoprotein E. Due to its discovery in a disorder related to lipid metabolism, it was first studied as a therapeutic target in dyslipidemia. However, in 1993, Strittmatter and colleagues (Strittmatter, Weisgraber et al., 1993; Strittmatter, Saunders et al., 1993) discovered the important association of the ε4 allele of the *APOE* gene [*APOE4*] with Alzheimer's Disease [AD]. The group further described an allele dose-dependent increase in AD risk and a decrease in the mean age of onset (Corder et al., 1993). Since then, apoE has been extensively investigated and it has been shown to have multiple physiologic functions and to be implicated in various diseases.

1.1.2. ApoE metabolism

ApoE is mainly produced by hepatocytes and astrocytes (Semenkovich, Goldberg, & Goldberg, 2016), but many other cell types also synthesize the protein, including macrophages and adipocytes (Kockx, Traini, & Kritharides, 2018). It is first translated in the endoplasmic reticulum [ER] as a pre-protein with 317 amino acids, which is subsequently cleaved, producing the mature protein with 299 amino acids. The apoE protein then passes through the Golgi apparatus, where it can be modified by glycosylation and sialylation (Lee et al., 2010). Sites and percentage of glycosylation and sialylation vary between the periphery and the central nervous system [CNS] and between apoE isoforms (Subramanian & Gundry, 2022). Thus, the final conformation of apoE may differ according to the cell type and isoform (Marais, 2019). Figure 1 shows apoE biosynthesis.

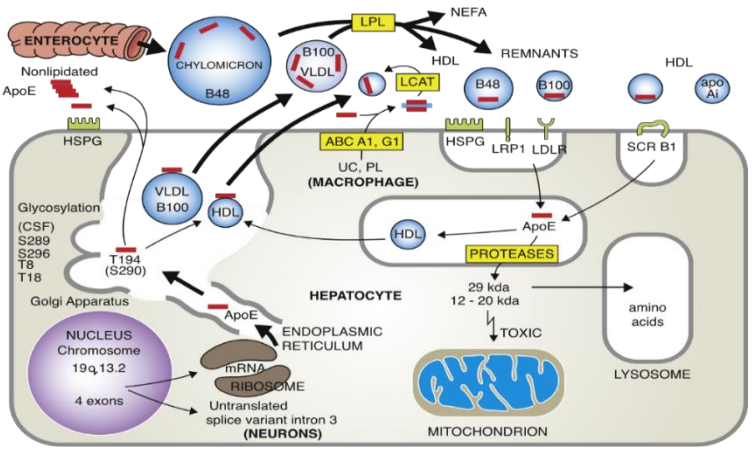


Figure 1: ApoE biosynthesis. Extracted from *(Marais, 2019)*.

The liver can secrete lipid-free apoE that may self-associate into a tetramer, or it may accept cholesterol and phospholipid from other cells such as macrophages. However, the bulk of apoE is associated with VLDL in the Golgi apparatus of hepatocytes, which enhances VLDL synthesis and secretion (Marais, 2019). ApoE can also be incorporated into nascent high-density lipoprotein [HDL] and enter the plasma in the form of discoidal HDL, which will then be lipidated to form spherical, mature HDL (Rye, Bright, Psaltis, & Barter, 2006). Once in the plasma, apoE can be exchanged between lipoprotein particles; consequently, it can circulate as a part of every lipoprotein, although intermediate-density lipoprotein and low-density lipoprotein [LDL] have little to none apoE on their surface (Su & Peng, 2020). After it reaches its destiny, apoE is internalized by receptor-mediated endocytosis (Phillips, 2014). It has broad ligand activity and can binding to heparan proteoglycan, receptors of the LDL receptor [LDLR] family and other receptors, such as scavenger receptors (Schneider, 2016). Internalized apoE can be degraded by intracellular proteases, but the majority will be recycled back to the plasma membrane and be re-secreted to become part of an apoE-containing HDL pool (Phillips, 2014).

There are two different apoE pools, one in the CNS and one in the periphery, because apoE cannot penetrate the blood brain barrier [BBB] (Martínez-Martínez et al., 2020). In the CNS, apoE is the most abundant apolipoprotein and the major protein component of lipoproteins, which are HDL-like particles. It is mainly produced by astrocytes and less in microglial cells and vascular cells in the choroid plexus (Marais, 2019). Neurons also produce apoE protein when injured (Mahley & Huang, 2012).

ApoE lipidation by astrocytes is dependent on transporter proteins in the plasma membrane, which mediate cholesterol efflux to the nascent apoE particle (Liao, Yoon, & Kim, 2017). When it reaches neurons, apoE is taken up by via receptor mediated endocytosis. Once inside the cell, free apoE is either released or degraded by proteolysis. However, it can also be degraded by extracellular proteolysis via a secreted neuronal protease, with hippocampal neurons being more efficient in mediating this apoE cleavage than cortical neurons (Tamboli, Heo, & Rebeck, 2014)

1.1.3. Structure of ApoE

Human apoE is a 299-residue molecule that contains multiple amphipathic α-helices that form two structural domains separated by a flexible hinge region. The N-terminal domain [NT], amino acids 1–198, contains the receptor binding region and forms a four-helix antiparallel bundle. The C-terminal domain [CT], amino acids 238–299, forms 3 helices and contains the major lipid binding region (Mahley, Weisgraber, & Huang, 2009). Figure 2 shows the Lipid-free and Lipid-bound structure of apoE3.

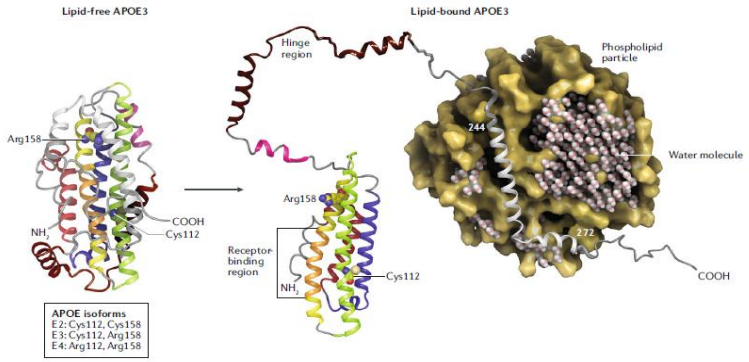


Figure 2: Lipid-free and Lipid-bound apoE3 structure. Extracted from *(Yamazaki, Zhao, Caulfield, Liu, & Bu, 2019)*.

Lipid-free apoE presents an inactive conformation of the NT bundle and a partially exposed LDLR-binding region, which does do not allow receptor-binding. During the folding of lipid-free apoE, the NT domain is the first to adopt helical conformation; the CT domain and hinge region will fold later, using hydrogen-bonds and salt-bridges created when they interact with the NT domain. This creates security mechanisms that ensure optimal apoE-receptor binding, and also inhibits the intracellular interaction between apoE and receptor during their production (Chen, Li, & Wang, 2011). However, in situations of low lipid, apoE can polymerize in a process that involves the CT domain, forming tetramers at low apoE concentration, and aggregates at higher concentrations (Frieden, Wang, & Ho, 2017).

Although the definite mechanism of apoE lipid-binding has not been elucidated, the most common hypothesis suggests that the apoE-lipid interaction causes major conformational changes in the apoE molecule (Nguyen, Dhanasekaran, Phillips, & Lund-Katz, 2009). This would occur in a two-step process: In the first step, lipid-binding in the CT region would break the bonds and bridges that maintained domain interaction, causing the sequential dissociation of the protein's domains; once dissociated, both domains could move away. In the second step, the NT helix-bundle would open, creating the final lipoprotein-associated structure, which presents a completely open conformation with a fully-exposed, active LDLR-binding region (Chen et al., 2011). However, on the surface of lipoproteins, apoE molecules can be present in different lipidation states, depending on the size

and cholesterol content of the lipoprotein and on the number of apoE molecules (Phillips, 2014).

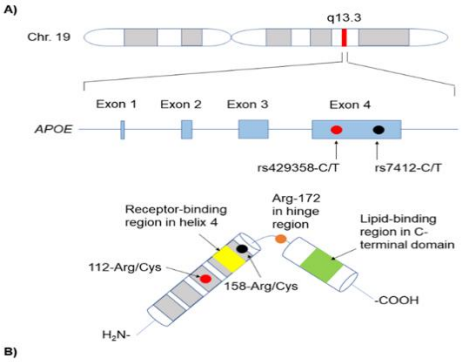
Nevertheless, this lipid-binding mechanism of apoE might only be applicable to plasma apoE, as lipid composition in the brain is different. Recently, Frieden, Wang and Ho (Frieden et al., 2017) proposed a new mechanism for lipid binding in the brain that involves simple changes in domain-domain interactions and does not need important protein rearrangement. These changes cause movement of the NT and CT domains, which allows regions that define apoE function to become accessible. They also suggest that structural differences between apoE isoforms are located in regions where residue exchanges might define binding and specificity. As apoE's functions are dependent on its structure, mutations and modifications of the apoE molecule can harm its function (Marais, 2019).

1.1.4. ApoE polymorphism

The human apolipoprotein gene [*APOE*] is located on chromosome 19q13.3, comprises 4 exons, and presents three main alleles, ε2, ε3 and ε4 (Abondio et al., 2019). An individual inherits an allele from each parent, making 6 possible genotypes, 3 homozygous (ε2/ε2, ε3/ε3 and ε4/ε4) and 3 heterozygous (ε2/ε3, ε2/ε4 and ε3/ε4) (Mahley, 2016a).

Human apoE protein occurs in three main isoforms, apoE2, apoE3, and apoE4, which are coded for by their respective alleles. The three isoforms differ by amino acid substitutions at positions 112 and 158. ApoE3 contains cysteine [Cys] at position 112 and arginine [Arg] at position at 158, whereas

apoE2 contains Cys at both positions and apoE4 contains Arg at both sites (Figure 3) (Abondio et al., 2019; Semenkovich et al., 2016).



Allele	ε2	ε3	ε4
Haplotype	rs429358-T rs7412-T	rs429358-T rs7412-C	rs429358-C rs7412-C
Residue combination	112-Cys 158-Cys	112-Cys 158-Arg	112-Arg 158-Arg

Figure 3: ApoE gene, RNA and protein. A) *APOE* gene on chromosome 19, RNA and apoE protein. B) Human *APOE* polymorphism. Extracted from *(Abondio et al., 2019)*

ApoE3 is the most common isoform, while apoE4 is the second most common and apoE2 the least common of the three isoforms. Other isoforms exist, most of which are related to changes in other amino acid sites, but these are fairly rare (Marais, 2019).

The single amino acid substitution changes the protein's structure and properties (Figure 4). ApoE2 presents a Cys residue at position 158, which indirectly affects the LDLR binding capacity (Marais, 2019; Phillips, 2014) Conversely, the Cys to Arg substitution at position 112 that creates apoE4 is located at the NT domain and does not affect LDLR binding. However, the presence of Arg in apoE4 causes key structural changes in the molecule, which render apoE4 thermodynamically less stable, less structured, and less

rigid compared to apoE3 (Mahley et al., 2009). Furthermore, this substitution eliminates the only Cys residue in the domain, leaving apoE4 without any Cys residue available to create Cys-Cys bonds. Structural models of monomeric apoE4 have suggested that the main change in the molecule is the presence of a salt bridge between Arg 61 side chain and glutamic acid in position 255, which is absent in apoE3 (Belloy, Napolioni, & Greicius, 2019). However, newer computational models suggest that the amino acid exchange creates new salt bridges between different residues (Frieden et al., 2017; Ray, Ahalawat, & Mondal, 2017).

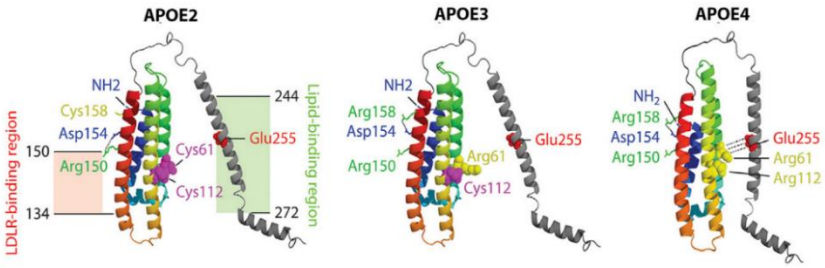


Figure 4: Structures of apoE isoforms. Note that the single amino acid change alters the protein's structure. Extracted from *(Belloy et al., 2019)*.

Independently of the location of new salt bridges, the substitution creates altered interactions between the NT and CT domains. This affects the protein's tertiary structure, leading to a rearrangement and destabilization of the apoE4 molecule, generating partially folded apoE4 (Phillips, 2014). Furthermore, it also affects its preference for lipoprotein binding, which shifts from HDL to LDL/VLDL (Abondio et al., 2019).

1.1.5. *APOE* allele frequency

The *APOE* alleles have a worldwide distribution of approximately 8%, 78% and 14% for ε2, ε3 and ε4, respectively (Eisenberg, Kuzawa, & Hayes, 2010; Farrer et al., 1997). This distribution makes the ε3/ε3 genotype the most frequent (around 60%), followed by the ε3/ε4 genotype (around 20%) (Phillips, 2014).

However, these frequencies are different in different areas and populations. The highest frequencies are found in indigenous populations of Central Africa, Oceania and Mexico (Figure 5). Furthermore, across Europe, *APOE4* presents a distinct latitudinal gradient, with higher frequencies in places with higher latitude (Abondio et al., 2019).

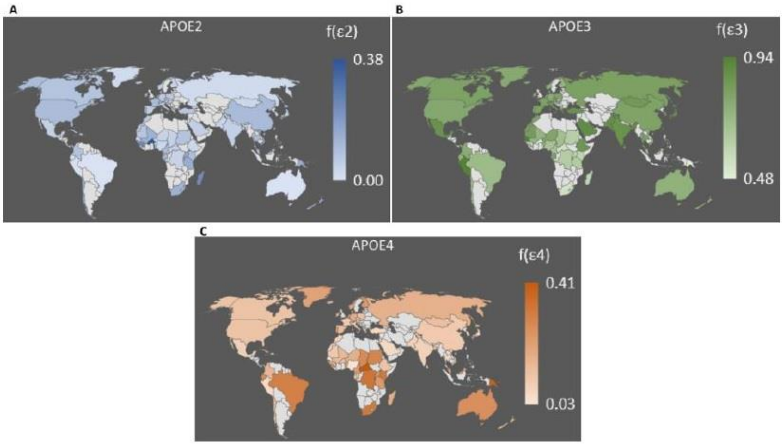


Figure 5: Frequency distribution of APOE alleles. (A) Frequency of the ε2 allele. (B) Frequency of the ε3 allele. (C) Frequency of the ε4 allele. Extracted from *(Abondio et al., 2019)*.

1.1.6. Functions of ApoE

ApoE is a multifunctional protein that presents different functions in several tissues and cells. Its main role is in the maintenance of lipid homeostasis

throughout the body. However, it is also involved in many other physiological functions, including inflammation, cellular repair, vascular function, BBB maintenance and antioxidant response (Kockx et al., 2018). It is also responsible for some key neuroprotective processes, including clearance of neurotoxic debris and amyloid β peptide [Aβ], promotion of dendritic arborization, and regulation of synaptic plasticity, cellular maturation and neuronal signaling (Ben Khedher, Haddad, Laurin, & Ramassamy, 2021; Diaz et al., 2022; Flowers & Rebeck, 2020). Furthermore, apoE modulates multiple cellular pathways including ion homeostasis, glucose metabolism, insulin signaling, mitochondrial function, and tau and Aβ metabolism (Tai et al., 2016). Figure 6 summarizes its main roles in different cell types.

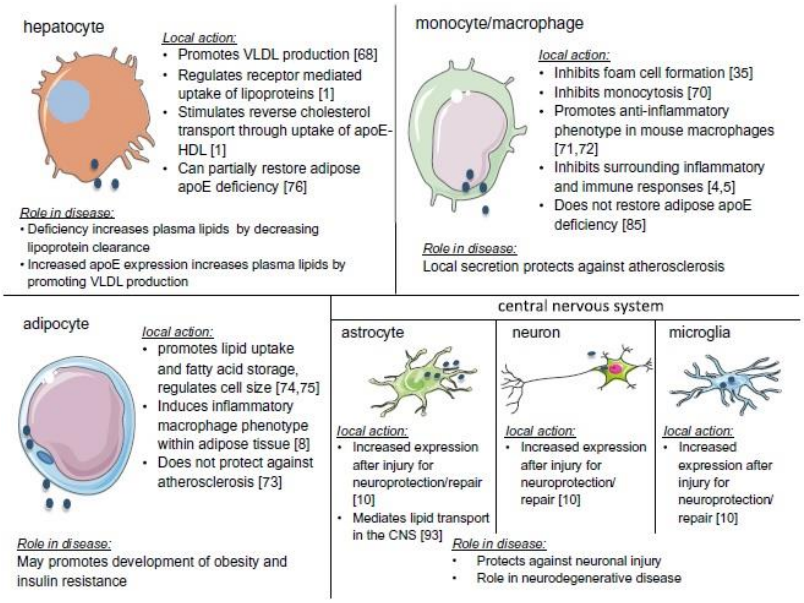


Figure 6: Functions of apoE in diverse cell types. Extracted from *(Kockx et al., 2018)*.

This section will further explain some of the protein's main effects.

Lipid Metabolism

ApoE is an important molecule for lipid homeostasis, as it is responsible for the clearance of VLDL and chylomicron remnants (Marais, 2019)Furthermore, apoE in HDL participates in reverse cholesterol transport, which removes excess cholesterol from tissues and transports it back to the liver (Phillips, 2014). ApoE also influences lipid metabolism in other tissues and cells, like adipocytes, where it is highly expressed in adipogenesis and has a close relationship with adipose tissue and body fat (Su & Peng, 2020).

In the CNS, apoE plays a key role in the lipid metabolism through the formation of apoE-enriched lipoproteins that are similar to HDL in size (HDL-like). This allows apoE to deliver cholesterol and other lipids to cells, which are then used for repair and remodeling (Mahley, 2016b). Furthermore, apoE promotes cholesterol efflux from cells, which prevents intracellular lipid accumulation and lipotoxicity (Ioannou et al., 2019).

Immunomodulatory effects

ApoE controls the immune system and inflammation both at peripheral and central levels (Chernick, Ortiz-Valle, Jeong, Qu, & Li, 2019). In the innate immune system, it modulates the function of macrophages, inducing the conversion of proinflammatory M1 phenotype to the anti-inflammatory M2 phenotype, thus suppressing inflammation (Baitsch et al., 2011). ApoE also affects neutrophils and facilitates lipid antigen presentation to natural killer T cells. Furthermore, it can bind lipopolysaccharide and attenuate the inflammatory response and reduce its lethality (Vitek, Brown, & Colton, 2009). ApoE also affects adaptive immune response: it suppresses T cell activation and proliferation, promotes regulatory and anti-inflammatory

responses, and suppresses proinflammatory immune profiles and cytokines (Dai, Tang, Liu, & Huang, 2021; Zhang, Wu, & Zhu, 2010b). Lastly, apoE controls macrophage infiltration and inflammation through the suppression of endothelial activation and of adhesion molecules on the peripheral vasculature (Martínez-Martínez et al., 2020; Stannard et al., 2001).

Antioxidant effects

ApoE is important for the defense against reactive species. Miyata and Smith (Miyata & Smith, 1996) were the first to described the direct antioxidant activity of apoE, which was seen to protected against hydrogen peroxide- and Aβ-related cytotoxicity. Since then, the antioxidant function of apoE has been described in mice studies (Kitagawa et al., 2002).

ApoE also helps detoxify cells from oxidated species, especially products of lipid peroxidation, and is also involved in deoxyribonucleic acid [DNA] damage recognition and repair after oxidative damage (Roque et al., 2021). Furthermore, apoE regulates oxidative damage by other mechanisms, such as the activation of anti-oxidative signaling cascades (Dai et al., 2021; Yang et al., 2018) and the regulation of immune cells (Laskowitz et al., 1998).

Lastly, apoE sequesters redox active metals ions, which stops them from interacting with other molecules. Thus, its ability to interact with metals maintains both metal and redox homeostasis (Ma et al., 2021), allows the correct functioning of the brain (Xu, Finkelstein, & Adlard, 2014), and protects against oxidative stress caused by metal overload (Marques et al., 2019).

Cellular Function

ApoE is extremely important for cellular function as it binds to its receptors of the LDLR family, causing modulation and integration of several signaling pathways. Furthermore, apoE has direct transcriptional effects; it can directly function as a transcription regulator of multiple genes by undergoing nuclear translocation and binding to double-stranded DNA (Levros, Labrie, Charfi, & Rassart, 2013; Theendakara et al., 2016). Also, apoE influences intracellular expression of proteins related to cellular stress (Osada, Kosuge, Kihara, Ishige, & Ito, 2009) and regulates the expression of genes and proteins related to autophagy and mitophagy, which are necessary for proteostasis (Parcon et al., 2018; Sohn et al., 2021). Lastly, apoE regulates other intracellular pathways, including those related to calcium homeostasis, glucose metabolism and insulin signaling (Misra et al., 2001; Rhea, Raber, & Banks, 2020; Wu, Zhang, & Zhao, 2018), and their defect can lead to cellular stress and dysfunction.

1.2. Alzheimer's Disease

1.2.1. Introduction to Alzheimer's Disease

AD was named after Dr. Alois Alzheimer, who first described the disease's neuropathological hallmarks, i.e., neurofibrillary tangles and amyloid plaques, in a 51-year-old patient with cognitive degeneration (Möller & Graeber, 1998). Since then, AD has been recognized as AD the most common age-related neurodegenerative disorder and has been extensively studied. AD is now described as a neurodegenerative disorder that causes the progressive decline of superior cognitive functions, usually starting with episodic memory, leading to a gradual impairment in the activities of daily

living until the individual becomes completely dependent (American Psychiatric Association, 2013).

AD can be classified by as sporadic or hereditary familial AD. Sporadic AD is a multifactorial disease that presents risk factors, but no determinant genetic alteration; it is responsible for most AD cases and is age-related, usually starting after the age of 65 years. Conversely, hereditary familial AD is a hereditary disorder, typically autosomal dominant, determined by a genetic mutation in a gene related to the Aβ pathway. It is responsible for about 1% of AD cases and it usually starts before the age of 65 years (Moulder et al., 2013; Nussbaum & Ellis, 2003; Piaceri, Nacmias, & Sorbi, 2013).

AD can also be divided into early-onset AD [EOAD] and late-onset AD [LOAD], when it starts before the age of 65 years or in individuals who are 65 years or older, respectively. LOAD is the most common form, while EOAD make up 5–10% of AD cases. However, both forms are mainly sporadic in nature, as only 10–15% of EOAD cases show known genetic mutations (Ayodele, Rogaeva, Kurup, Beecham, & Reitz, 2021).

1.2.2. AD Epidemiology

AD is the most common cause of dementia, being responsible for about 60% to 70% of all dementia cases (World Health Organization, 2017). Worldwide, more than 50 million people suffer from the disease (Li et al., 2022) and about 800.000 of those live in Spain (Sociedad Española de Neurología, 2018). However, as the world's population ages, AD and other age-related dementing disorders become more prevalent; thus, the number of

individuals suffering from AD is expected to triple in a few decades (World Health Organization, 2017).

The increase in prevalence comes with a great cost. Dementia is now the 7th leading cause of mortality globally (Gauthier S, Rosa-Neto P, Morais JA, & Webster C., 2021). People with AD survive an average of four to eight years after the diagnosis, although some live as long as 20 years. However, most of these years are spent in the severe phase, which creates a great burden for the individual, their family and society. Furthermore, AD is associated with many comorbidities, which further increase its economical, physical and mental burden (Alzheimer's Association, 2022).

1.2.3. AD Pathophysiology

AD pathology involves multiple mechanisms and many hypotheses on the pathogenesis of AD have been suggested. The most studied is the amyloid hypothesis, where an accumulation of Aβ results in oxidative stress and inflammation, which in turn leads to energy deficit and synaptic dysfunction (Querfurth & LaFerla, 2010). Aβ deposition as the initial event in AD has been extensively studied and many clinical trials with anti-amyloid medications have been conducted. However, only a few succeeded in reducing amyloid deposition and they have yet to show clinical benefit and real-world validation of their effectiveness (Cummings et al., 2021). Therefore, investigators have suggested that this hypothesis might need to be reconsidered (Frisoni et al., 2022).

Nevertheless, the hallmarks of AD are the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles, which are caused by the

deposition of Aβ and of hyperphosphorylated tau protein [p-tau], respectively (Figure 7). Besides protein aggregation, AD pathogenesis also involves other important pathological alterations that include: inflammation, oxidative stress, mitochondrial dysfunction, autophagy impairments, metal and calcium dyshomeostasis, lipid dysmetabolism and other metabolic alterations, and synaptic and neurotransmitter dysfunction. All of these changes will eventually lead to cell death (Querfurth & LaFerla, 2010). Although AD pathophysiology has been extensively studied, much remains to be learned. We will further explore some of these alterations in this section.

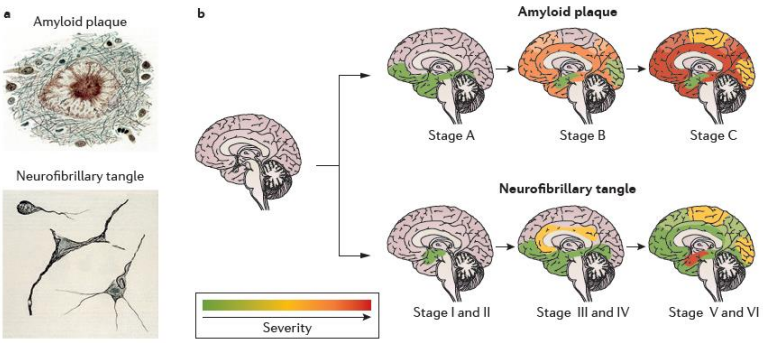


Figure 7: The pathological evolution of Aβ and tau abnormalities. a) Amyloid plaques and neurofibrillary tangles; b) The evolution of Aβ and p-tau deposition through the brain as the disease progresses. Extracted from *(Masters et al., 2015)*.

Amyloid plaques

Amyloid plaques are one of the hallmarks of AD pathology. These extracellular plaques are spherical microscopic lesions formed by a central nucleus, composed mainly of Aβ with surrounding dystrophic neurites (Duyckaerts, Delatour, & Potier, 2009; Kumar, Sidhu, Goyal, & Tsao, 2022).

Many other molecules can also be found in plaques, such as proteoglycans, cytokines, immunoglobulins, metal ions, apoE, proteases, antioxidant enzymes and other proteins (Atwood, Martins, Smith, & Perry, 2002). A subset of amyloid plaques, called neuritic plaques, appear closely associated with neuronal injury and are characterized by the occurrence of dystrophic neurites that frequently have p-tau immunoreactivity (Montine et al., 2012).

Aβ peptides are products that derive from the normal metabolism of a transmembrane protein known as amyloid precursor protein [APP]. It is generated at high levels in neurons, but also by other cell types, throughout an individual's lifetime. Aβ production and release are regulated by synaptic activity and modulated by the sleep–wake cycle, with higher production and release during wakefulness and higher clearance during sleep (Boespflug & Iliff, 2018; Cirrito et al., 2005; Kang et al., 2009).

APP can be cleaved through two main pathways, as shown in figure 8. In the non-amyloidogenic pathway, APP is sequentially cleaved by α–secretase and γ-secretase, forming APPsα, the extracellular peptide p3 and the intracellular fragment AICD. In the amyloidogenic pathway, APP is cleaved by a β-secretase, into APPsβ and βCTF. The latter is then cleaved by γ-secretase to form Aβ and AICD. Aβ is produced as peptides of varying length and slightly different characteristics, with most abundant species having between 38 and 43 amino acids in length. However, monomers of $A\beta_{40}$ are much more prevalent than the damaging $A\beta_{42}$ species (Knopman et al., 2021).

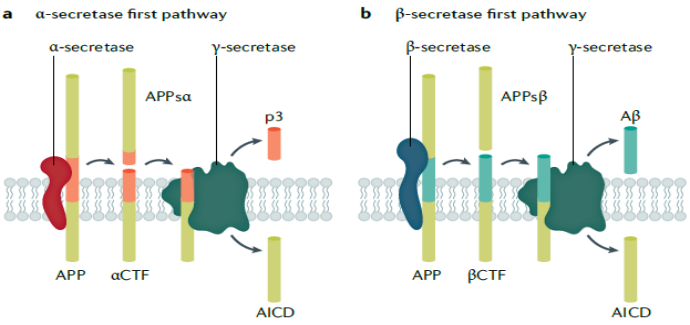


Figure 8: APP processing. APP processing through the non-amyloidogenic pathway (a) and through the amyloidogenic pathway (b). Extracted from *(Knopman et al., 2021)*

After its production, Aβ is released into the extracellular milieu as diffusible monomers. There, Aβ can bind to apoE and be transported into the perivascular space, where it is removed from the CNS by the glymphatic pathway (Hampel et al., 2021). It is important to mention that the reverse pathway also occur, where Aβ can enter from the blood into the CNS using BBB receptors (Wang, D. et al., 2021). Conversely, Aβ can be taken up by astrocytes and glial cells via receptor-mediated endocytosis, which depend on apoE-containing lipoproteins (Hampel et al., 2021).

Aβ can accumulate as a result of increased production, decreased clearance, or both. Aβ peptides will then aggregate in the intercellular space, particularly the $A\beta_{42}$ species, forming fibrils that assemble into plaques. They can also aggregate within the perivascular space, which can damage the vascular wall, leading to microhemorrhages. Aβ fibrils and plaques can be cleared from the brain via degradation by astrocytes and microglia (Knopman et al., 2021).

The strongest evidence for the involvement of Aβ in AD comes from the study of individuals with hereditary familial AD, most of which present an autosomal dominant mutation in one of three different genes: *APP, PSEN1* or *PSEN2* (Bateman et al., 2011). The *APP* gene is responsible for the production of the APP protein, which is the substrate for the production of Aβ. *PSEN1* and *PSEN2* are genes that code for the presenilin 1 and presenilin 2 proteins, respectively, which are part of the γ-secretase. Most mutations result in the overproduction of $A\beta_{42}$ due to alterations in its processing. The fact that, mutations within three different genes cause similar changes in Aβ products lead to the creation of the amyloid hypothesis, which puts Aβ pathology as the common pathway in AD pathogenesis (Knopman et al., 2021; Masters et al., 2015).

In typical cases, amyloid deposition precedes neurofibrillary and neuritic changes and it follows a distinct sequence, in which the regions are hierarchically involved as the disease progresses. Initially, it is found exclusively in the neocortex, with an apparent origin in the frontal and temporal lobes, hippocampus and limbic system (Masters et al., 2015). Then, it progressively affects allocortical brain regions, diencephalic nuclei, the striatum, the cholinergic nuclei of the basal forebrain, brainstem nuclei and the cerebellum (Thal, Rüb, Orantes, & Braak, 2002). It is important to note that Aβ deposition starts in the preclinical phase, decades before cognitive decline and brain atrophy appear (Jack et al., 2013; Villemagne et al., 2013). Furthermore, amyloid plaques can be found in many elderly individuals without clinical symptoms; more specifically, neuropathological studies have found that prevalence of Aβ rises steeply with increasing age, and can be seen in up to 74% of 80-year-olds (Andersen, 2020).

The presence of increased soluble amyloid and amyloid plaques causes many pathological alterations that will eventually lead to neurodegeneration and cell death. Soluble Aβ oligomers can be internalized and cause oxidative stress; as they can affect mitochondrial function, more reactive oxygen species [ROS] are produced and ATP production is reduced (Lloret et al., 2008). Extracellularly, the presence of amyloid plaques can activate microglia and astrocytes, producing neuroinflammation and increasing ROS generation (Simpson & Oliver, 2020). Furthermore, redox active metal ions, such as iron and zinc, are also deposited in plaques, which catalyzes reactive species production and leads to a reduction of antioxidant agents (Cheignon et al., 2018). The disequilibrium between oxidants and antioxidants will then lead to the damage of proteins, DNA and lipids, causing altered function and cell damage. In the case of lipids, it causes lipid peroxidation in the cell membrane, with the production of species such as malondialdehyde and hydroxynonenal [HNE], which will further propagate lipid oxidation (Wang et al., 2014).

Membrane damage leads to further pathology. Membrane transporters, receptors and channels are affected, causing alteration in calcium homeostasis, lipid and glucose metabolism and membrane potential. Furthermore, receptor alterations associated with increased levels of ligands, such as glutamate, lead to cell hyperactivation and excitotoxicity (Fuchsberger et al., 2016). Conversely, Aβ oligomers can directly interact with receptors, altering their function (Mroczko, Groblewska, Litman-Zawadzka, Kornhuber, & Lewczuk, 2018). Aβ also causes endocytic and autophagic dysfunction which affect proteostasis and the expression of membrane receptors, transporters and channels (Knopman et al., 2021).

Soluble Aβ oligomers are also toxic to adjacent synapses. They cause pathological changes in dendritic spines, and decrease synaptic efficiency and content. Furthermore, Aβ causes synaptic dysfunction through increase calcium flux, altered synaptic homeostasis and through deficits in proteostasis (Knopman et al., 2021; Mroczko et al., 2018). Likewise, Aβ species cause elevated calcium release from intracellular stores into the cytoplasm, mitochondrial dysfunction and induce the hyperphosphorylation and aggregation proteins, such as tau and α-synuclein (Lazzari, Kipanyula, Agostini, Pozzan, & Fasolato, 2015; Masters et al., 2015). Lastly, Aβ can also alter its own clearance as it affects brain vasculature, the BBB, proteostasis and membrane composition.

All of these alterations cause an activation of stress responses which, at first, increase the expression and activation of stress proteins, such as regulator of calcineurin 1 [RCAN1], eukaryotic translation initiation factor 2 alpha kinase 2 [PKR] and p38 mitogen-activated protein kinases [p38], in an attempt to restore homeostasis. However, increased chronic stress will eventually lead to cell death.

Neurofibrillary Tangles

Tau deposition is a characteristic of AD and other tauopathies (Zhang, Wu, Yang, Dong, & Yu, 2022). Tau, also called microtubule-associated protein tau [MAPT], is a microtubule-associated protein which binds to tubulin, promoting its polymerization and stabilization into microtubules. It has six isoforms that are generated by alternative splicing of the *MAPT* gene (Kovacs, 2018).

In AD, there is neuronal hyperphosphorylation and intracellular aggregation of tau; thus, it forms an insoluble fibrillary material that appears as neuropil threads in cellular processes and neurofibrillary tangles [NFT] in neuronal somata (Braak & Del Tredici, 2011). Accumulation of NFTs follows a regional progression across brain regions which is distinct from that seen with Aβ (see figure 8 above) (Montine et al., 2012). This progression is uniform and has permitted the classification of tau pathology into 6 different stages by Braak and Braak in 1991 (Braak & Braak, 1991). It starts at the transentorhinal region (stage I), and progresses into the entorhinal region and hippocampal formation (stage II) and the temporal neocortex (stage III). Then, additional neocortical regions are affected: first, parietal and occipital association areas and prefrontal areas (stages IV and V); then, sensory association area and premotor areas (stage V); and, lastly, the primary cortical areas (stage VI) (Braak & Del Tredici, 2018).

Tau pathology spreads along axons from one neuron to the next, suggesting a "prion-like" spread of tau aggregates (Mudher et al., 2017). This occurs due to the presence of pathological tau seeds, that is, tau aggregates that are capable of recruiting and misfolding monomeric tau, which move along synaptically connected neurons (DeVos et al., 2018). Tau seeding occurs before the presence of overt tau pathology and its activity begins in the transentorhinal/entorhinal regions (Kaufman, Del Tredici, Thomas, Braak, & Diamond, 2018) and seems to progress in a cortico-cortical top-down manner (Braak & Del Tredici, 2018).

Tau is a protein whose phosphorylation state depends on the balance of kinase and phosphatase activity. Tau hyperphosphorylation in AD has been linked to an imbalance of many of these proteins. For example, glycogen

synthase kinase 3 beta [GSK3β], PKR and p38, have all been shown to be overexpressed or overly activated in AD patients (D'Mello, 2021; Hugon & Paquet, 2021). Furthermore, other enzymes related to the activation of these kinases, such as RCAN1, are also increased in AD (Wong et al., 2022). These kinases and enzymes are also involved in the interaction of Aβ and tau pathologies (Giraldo, Lloret, Fuchsberger, & Viña, 2014; Llorens-Martín, Jurado, Hernández, & Avila, 2014).

Tau pathology causes many pathophysiological alterations related to AD. Hyperphosphorylated tau is neurotoxic and leads to defective microtubule assembly and functioning, mitochondrial dysfunction, disrupted axonal transport, and induces neuronal cell death. Furthermore, it causes synaptic loss and disfunction, causing impaired long-term potentiation (Fan et al., 2019). Tau pathology also amplifies Aβ deposition, neurotoxicity and pathogenesis. As Aβ induces tauopathy, both proteins are related to each other through a potential feedback loop (Guo et al., 2020).

Once begun, tauopathy progresses for decades without remission (Braak & Del Tredici, 2011; Braak & Del Tredici, 2018). In AD, tau deposition is related to cognitive performance (Dang et al., 2022) and to neuropsychiatric symptoms, but correlations can also be seen in cognitively normal older adults at the preclinical stage (Brier et al., 2016; Gatchel et al., 2017). Furthermore, longitudinal changes in tau are associated with cognitive changes and mild cognitive impairment [MCI] (Hanseeuw et al., 2019; Leuzy et al., 2019). Lastly, tau spreading patterns in AD are influenced by variables such as older age and *APOE4*, and the differential patterns seen in tau positron emission tomography [PET] are associated with different clinical phenotypes (La Joie et al., 2021).

Inflammation

Chronic inflammation is a major driver of AD pathology. Increased inflammatory response in AD results from a disequilibrium between anti- and pro-inflammatory signaling (Kinney et al., 2018). Altered inflammation can be found in the CNS and also in the periphery, especially during early stages of the disease (Holmes et al., 2009; King et al., 2018).

Microglia and astrocytes are initially activated to deal with Aβ accumulation and other products of cellular stress. However, chronic activation leads to increased release of damaging chemokines, cytokines, acute-phase reactants and ROS; this increases tau phosphorylation, causes oxidative stress and contributes to neuronal damage and loss (Nordengen et al., 2019; Querfurth & LaFerla, 2010). Furthermore, inflammation increases Aβ generation and reduces Aβ degradation (Wang, Tan, Yu, & Tan, 2015), and it also facilitates and exacerbates NFT pathology (Kinney et al., 2018). In fact, several pro-inflammatory cytokines, including tumor necrosis factor α [TNF-α] and interleukin-1β [IL-1β], have been shown to alter tau and Aβ metabolism (Domingues, da Cruz E Silva, & Henriques, 2017).

AD also leads to increased expression of pro-inflammatory cytokines by endothelial cells, which causes vascular alterations and BBB disruption (Grammas & Ovase, 2001). BBB breakdown further increases neuroinflammation as it allows infiltration of the brain by blood-derived molecules and immune cells (Mietelska-Porowska & Wojda, 2017). Chronic inflammation also increases the damage to brain cells and extends focal damage to nearby heathy tissue by inducing ER unfolded protein response and increasing oxidative stress (Galasko & Montine, 2010; Rath & Haller,

2011). Conversely, these alterations can also increase inflammatory response.

The correlation between inflammation and AD pathology can also be found in cognitively healthy individuals, where higher levels of proinflammatory cytokines are associated with altered levels of tau and $A\beta_{42}$ in the cerebrospinal fluid [CSF] (Bettcher et al., 2018) and with future cognitive decline (Singh-Manoux et al., 2014). Furthermore, increased chronic inflammation is also associated with many known risk factors for cognitive decline and AD, such as diabetes mellitus, obesity and *APOE* genotype (Kinney et al., 2018).

Redox dysregulation and Oxidative stress

Oxidative stress is a frequent pathological mechanism in the onset and progression of several diseases (Pizzino et al., 2017). It occurs when the level of oxidant species is higher than what the cell's antioxidant defense can cope with, leading to a disruption of redox signaling and molecular damage (Sies, Berndt, & Jones, 2017).

ROS and reactive nitrogen species [RNS] are the most potent oxidants as they present a very high tendency to react with biomolecules (Winterbourn, 2008). They are products of normal cellular metabolism and have important roles in many physiological processes when at low/moderate concentrations (Herb, Gluschko, & Schramm, 2021). However, harmful effects occur when there's an imbalance between pro- and anti-oxidant molecules. When this happens, radicals react with proteins, lipids and DNA, creating other radicals and causing oxidative damage (Figure 9) (Valko et al., 2007). Oxidative damage activates cellular stress responses, including the stimulation

mitogen-activated protein kinases [MAPK] JNK and p38, that eventually lead to cell death (Kannan & Jain, 2000; Son et al., 2011). Furthermore, oxidative stress disrupts of intracellular ion homeostasis by activation of Ca^{2+} channels, which also induces cell death (Malko & Jiang, 2020).

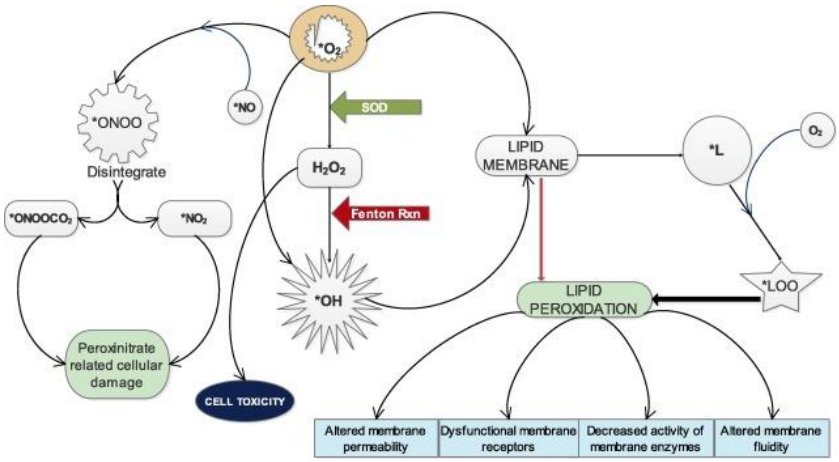


Figure 9: Main reactive species and related cell damage. ROS are initially formed as superoxide anion radical ($O_2^{\bullet -}$) in the mitochondria during the synthesis of ATP. Then, superoxide can be converted to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). H_2O_2, in turn, is scavenged by the enzyme glutathione peroxidase (GPx), which uses reduced glutathione as the electron donor. However, H_2O_2 can also interact with transition metals, particularly Fe^{2+}, to form the highly reactive hydroxyl radical ($^{\bullet}OH$). Furthermore, nitric oxide can react with the superoxide anion to produce the highly cytotoxic peroxynitrite anion. Reactive species then cause lipid peroxidation and cell damage. Extracted from *(Ighodaro & Akinloye, 2018)*

The principal types of oxidative damage are DNA/RNA oxidation, protein oxidation and lipid peroxidation (Halliwell & Gutteridge, 2015). The first two are more limited, but can cause cellular dysfunction, stress and death (Sies et al., 2017). Conversely, lipid peroxidation is a self-propagating process that can continue as long as substrates are available or it's terminated by a

reaction with cellular antioxidants; thus, an initially small concentration of free radicals can be greatly amplified. If not detoxified, reactive lipid species can cause extensive cell damage (Jakaria, Belaidi, Bush, & Ayton, 2021). The peroxidation of polyunsaturated fatty acids [PUFA] can produce end-products which cause cell damage, including malondialdehyde [MDA]. MDA is the most mutagenic end-product of lipid peroxidation due to its high capability of reaction with multiple biomolecules, altering their function. Furthermore, the reaction of MDA with a biomolecule can produce MDA-adducts, which can have further deleterious effects (Ayala, Muñoz, & Argüelles, 2014).

The brain is especially susceptible to oxidative damage, due to the high levels of PUFA content, the high exposure to inspired oxygen, and the presence of redox-active transition metal ions, associated with a modest antioxidant defense (Cobley, Fiorello, & Bailey, 2018). Due to this increased susceptibility, increased levels of lipid peroxidation and protein, DNA, and RNA oxidation can be seen in vulnerable regions of the brain of AD patients (Chen & Zhong, 2014). This occurs even at early stages; in fact, a really important study by Nunomura et al (Nunomura et al., 2001) showed that oxidative damage is an early-stage event in the process of neurodegeneration in AD.

Oxidative stress could be one of the first pathophysiological changes of AD (Sutherland, Chami, Youssef, & Witting, 2013) as it affects the synthesis and function of several proteins, and causes metabolic dysfunction, excitotoxicity, dysregulation of the cell cycle, and Aβ and tau pathology (Sultana & Butterfield, 2010). However, it may also play a role in the progression of disease as oxidative stress markers, both in brain and

periphery, are related to AD pathology and clinical symptoms (Chen & Zhong, 2014; Perrotte et al., 2019; Sultana & Butterfield, 2010). This would occur because oxidative stress can be induced by all other pathological alterations of AD, including dysfunctional mitochondria, Aβ and tau pathology, ion dyshomeostasis, inflammation and cellular activation (Querfurth & LaFerla, 2010).

Defects in the antioxidant defense were also seen in AD, with reports of decreased protein levels and lower activity of enzymatic and non-enzymatic antioxidants (Lovell & Markesbery, 2007; Torres et al., 2011). Antioxidants are molecules responsible for regulating the redox state by preventing or delaying the oxidation of other substrates (Zhang et al., 2020). Enzymatic antioxidants scavenge ROS in a multi-step process by catalyzing reactions that lead to the conversion of superoxide, hydrogen peroxide and lipid hydroperoxides into non-reactive species (Bazinet & Doyen, 2017). Figure 10 shows the main reactions of the enzymatic antioxidant defense systems.

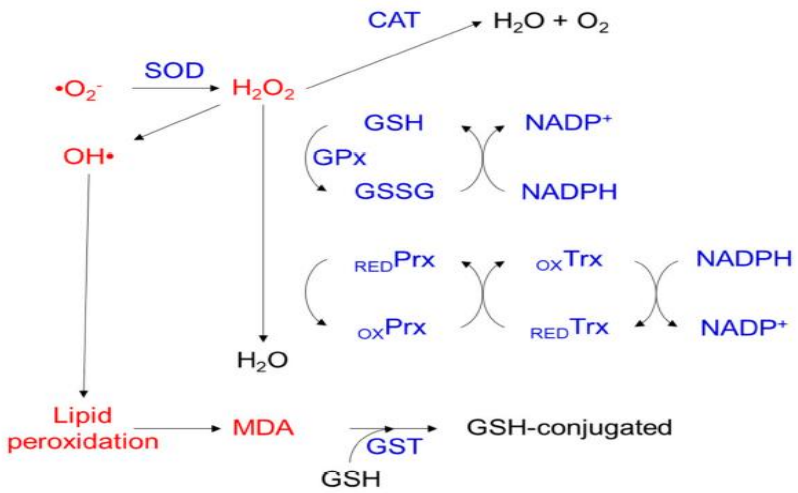


Figure 10: Main reactions of the enzymatic antioxidant defense systems. CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; PRX: peroxiredoxin; TRX: thioredoxin; RED: reduced; OX: oxidized; MDA: malondialdehyde; GST: glutathione S-transferase. Extracted from *(Hsueh et al., 2022)*

Two major enzymatic antioxidants are the family of enzymes superoxide dismutase [SOD] and glutathione peroxidase [GPx] (Valko et al., 2007). SODs catalyze the dismutation of superoxide anions into oxygen and hydrogen peroxide [H_2O_2] (Zelko, Mariani, & Folz, 2002) and they have been implicated in AD pathology, with studies showing alterations in their levels and activity (Ihara et al., 1997; Marcus, Strafaci, & Freedman, 2006). GPxs are selenium-containing enzymes that catalyze the breakdown of H_2O_2 and lipid hydroperoxides to water and lipid alcohols, using reduced glutathione as an electron donor (Zhang et al., 2020). There are several isoforms of GPx that differ in their substrate specificity, but the cytosolic GPx1 is the predominantly expressed enzyme. Its normal levels are essential for cellular detoxification of peroxides and to prevent their toxicity (Liddell, Dringen, Crack, & Robinson, 2006) with altered levels of GPx1 been related to many diseases, including AD (Huang, Zhou, Wu, Ren, & Lei, 2018).

Non-enzymatic antioxidants are molecules characterized by the ability to rapidly inactivate radicals and oxidants (Mirończuk-Chodakowska, Witkowska, & Zujko, 2018). They can be endogenous, such as metal-binding proteins and thiols, or exogenous, such as certain vitamins. Oxidation and reduction of thiols is thought to be the major mechanism by which reactive oxidants integrate into cellular signal transduction pathways and by which redox homeostasis is maintained (Winterbourn, 2008). Glutathione (γ-glutamyl-l-cysteinylglycine) is the most abundant nonprotein thiol in the cell

(Sastre, Pallardó, & Viña, 1996) It can act directly by neutralizing ROS and RNS, and indirectly by supporting enzymatic activity as a reducing agent (Bajic et al., 2019). Upon oxidation, reduced glutathione [GSH] changes into a molecule which dimerizes to create oxidized glutathione disulfide [GSSG]. Both forms are interconvertible, which creates a GSH–GSSG cycle that depends on the presence of two enzymes: GPx, which converts GSH to GSSG, and glutathione reductase [GR], which reduces GSSG back to GSH. GR uses reduced nicotinamide adenine dinucleotide phosphate [$NADPH^+$] as an electron donor in this reaction, changing it into its oxidized form [NADP]. Both the GSSG/GSH and the NADP/$NADPH^+$ couples play crucial roles in the reduction of peroxides, and their balance is crucial for the maintenance of the redox status and cell survival (Xiao & Loscalzo, 2020). Consequently, changes in the concentration of these molecules result in redox imbalance and weakened reducing power, which can be seen in the aging process and in an array of human diseases (Dwivedi, Megha, Mishra, & Mandal, 2020). In AD, studies have found depleted of reduced GSH, associated with elevated oxidized/reduced ratios both in brain and in blood samples (Saharan & Mandal, 2014).

AD is also related to metal dyshomeostasis, which causes oxidative damage. Iron, zinc, and copper are the most abundant physiological transition metals related to AD neuropathology. Although they have essential roles in normal physiology, their dysregulation causes neurotoxicity, neurological damage and cell death (Huat et al., 2019; Lei, Ayton, & Bush, 2021). Ferroptosis is a unique form of iron-dependent, lipid peroxidation-driven programmed cell death, which can be seen in AD (Chen et al., 2021). It is induced by “ferroptotic stress”, a type of oxidative stress that involves iron-induced

peroxidation of plasma membrane phospholipids, especially PUFAs, and alteration of antioxidant enzymes. The main enzyme involved in ferroptosis is GPx4, although other enzymes are also involved. As shown in figure 11, although iron has a crucial role in ferroptotic cell death, ferroptosis may occur at all iron concentrations through multiple other mechanisms, including inhibition of GPx4 and glutathione depletion (Ayton et al., 2021; Jakaria et al., 2021)

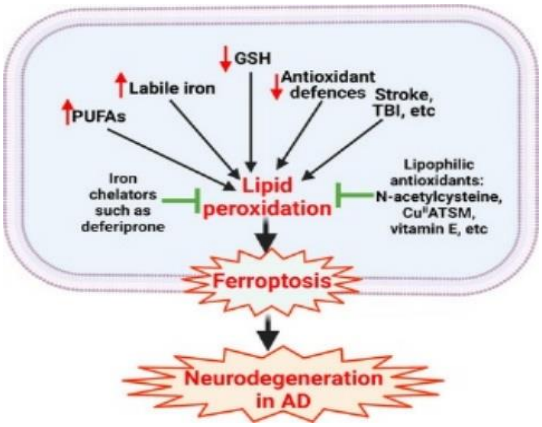


Figure 11: Ferroptosis in AD. Redox dysregulation and oxidative damage leading to ferroptosis in AD. Extracted from *(Jakaria et al., 2021)*.

Cellular Death

Chronic response to oxidative stress, inflammation and accumulation of misfolded proteins, causes intense cellular stress (Iatrou, Clark, & Wang, 2021). It activates intracellular stress response pathways to try and restore homeostasis (Hetz, Zhang, & Kaufman, 2020), causing a transcriptional reprogramming that deeply affects the cell (Oliveira & Lourenco, 2016).

However, in AD, ER stress and the activation of stress responses lead to further accumulation of abnormal proteins and can trigger an inflammatory response (Uddin, Yu, & Lim, 2021). Furthermore, autophagy, which is a normal response to cellular stress, is progressively impaired in AD (Van Acker, Bretou, & Annaert, 2019). This causes synaptic plasticity deficits, tau hyperphosphorylation and neurodegeneration and leads to the progression of AD (Knopman et al., 2021).

Chronic cellular stress eventually leads to neurodegeneration. Cell death is the final result of all the pathological changes caused by AD (Donev, Kolev, Millet, & Thome, 2009), as they activate caspases and the apoptotic cascade, and alter the expression of several pro- and anti-apoptotic factors (Paquet, Dumurgier, & Hugon, 2015). Furthermore, non-apoptotic programmed cell death can also occur (Zhang, G. et al., 2021).

Progressive neuronal death can be seen as brain atrophy in imaging techniques and in autopsy. Atrophy occurs in a stereotypical pattern of cortical neurodegeneration: initially, it affects the medial temporal lobe, i.e., entorhinal cortex and hippocampus, and later it extends through the cortex in a temporal-parietal-frontal trajectory; motor areas are the last to be affected (Pini et al., 2016). This progression correlates with cognitive and behavioral symptoms, disease severity and clinical subtypes of AD (Serra et al., 2010; Whitwell et al., 2012).

1.2.4. Clinical Features and Diagnosis

AD is a disease that presents different stages and begins years before the initial clinical symptoms (figure 12). Therefore, current diagnostic criteria for

AD rely on the association of biomarkers with the absence/presence and severity of clinical symptoms. Current diagnostic criteria for AD are described in Annex 1 (Dubois et al., 2021). Yet, how long individuals spend in each stage varies as it depends on many variables including age, sex, genetics and modifiable factors.

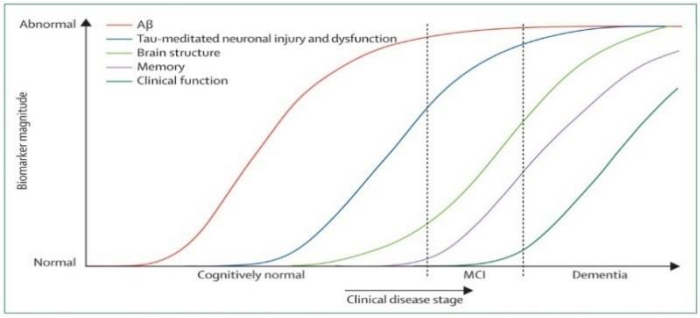


Figure 12: Evolution of biomarkers in relation to disease stage. Biomarkers become altered before the beginning of clinical symptoms. Extracted from *(Jack et al., 2010)*.

The stages of the AD continuum, mainly divided into preclinical/at risk, MCI and AD dementia, are described in the figure below (Figure 13).

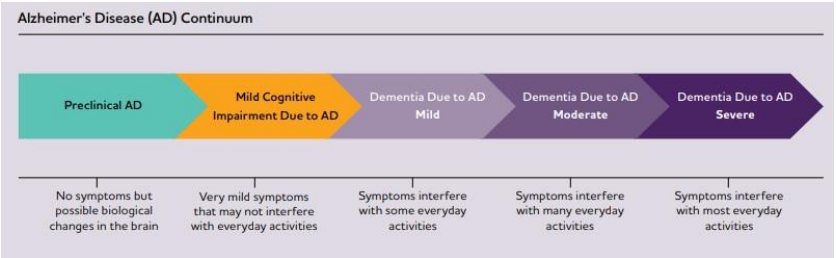


Figure 13: AD continuum. AD stages and symptoms. Extracted from *(Alzheimer's Association, 2022)*.

Preclinical AD / At risk of AD

This phase starts many years before the onset of clinical symptoms. It is characterized by the presence of measurable AD biomarkers, but with absent objective clinical symptoms (Sperling et al., 2011). Even though there are no objective cognitive deficits, individuals may already present subjective cognitive decline [SCD]; that is, a self-perceived decline in cognitive abilities compared to a previously normal cognition, which is unrelated to an acute event. It is important to note, though, that SCD is not a diagnostic category in diagnostic manuals, and that it has variety of potential underlying causes. Nevertheless, its presence increases the risk of future cognitive decline (Jessen et al., 2014).

Although individuals in this phase present AD pathology, many of them will not progress to MCI or dementia due to AD (Bennett et al., 2006), as resistance and resilience to AD is affected by genetic, environmental, and behavioral factors (Andersen, 2020). With this in mind, the International Working Group (IWG) recently recommended that AD diagnosis be restricted to people with positive biomarkers and specific AD phenotypes, while those who are biomarker-positive but cognitively unimpaired should be considered only at-risk for progression to AD (Dubois et al., 2021). However, they also consider that biomarker-positive asymptomatic individuals who are heterozygous *APOE4* carriers have increased risk, while homozygous carriers are considered high risk. Thus, this is an important phase to apply prevention strategies and is the focus of many recent studies (Davis et al., 2018).

Mild Cognitive Impairment Due to Alzheimer's Disease

This stage is characterized by positive biomarker evidence plus new but subtle cognitive symptoms that do not interfere with individuals' ability to carry out normal activities of daily living (Albert et al., 2011).

The annual progression rate to mild AD is variable and not all individuals with MCI progress to dementia, with some individuals even reverting to normal cognition (Davis et al., 2018).

Dementia Due to Alzheimer's Disease

Alzheimer's dementia, is characterized by noticeable cognitive/neuropsychological symptoms that impair activities of daily living, combined with positive biomarkers (McKhann et al., 2011). The most common form of sporadic AD is an amnestic multidomain dementia (Weintraub et al., 2020), which begins with gradual memory loss, specially of the episodic, short-term memory (Murray et al., 2011), and progressively impairs other cognitive domains. However, other less common variants of AD can begin as aphasic, visuospatial, and frontal behavioral-type dementias (Weintraub et al., 2020). Furthermore, neuropsychiatric symptoms, such as apathy, depression, anxiety and sleep disorder are also highly prevalent throughout the disease, even at early disease stages (Leung, Chan, Spector, & Wong, 2021).

The disease progresses, in a variable amount of time, from mild dementia to moderate and, finally, severe dementia. Ultimately, the patient becomes completely dependent and prone to several medical complications (Arvanitakis, Shah, & Bennett, 2019). Medications and non-pharmacological treatments are available to manage cognitive and behavioral symptoms;

however, none of them are proven to be disease modifying (Joe & Ringman, 2019).

1.2.5. AD Risk Factors

1.2.5.1. Environmental risk factors

The greatest risk factors for LOAD are older age, family history of AD and genetics (Hebert et al., 2010; Mayeux, Sano, Chen, Tatemichi, & Stern, 1991; Saunders et al., 1993). However, sporadic AD is a multifactorial disease and many other factors contribute to disease risk, including education, and lifestyle (Alzheimer's Association, 2022).

Even though AD is not necessarily the outcome of aging, age is the principal risk factor for AD (Nelson et al., 2011). Both the prevalence and the incidence of AD increase with age (Querfurth & LaFerla, 2010), with more than 30% of individuals over 85 years presenting the disease (Alzheimer's Association, 2022).

In sporadic AD, a family history of AD is not necessary for an individual to develop the disease; but having a parent or a sibling with the disease increases the risk of the disorder, possibly through shared genetic and non-genetic factors (Loy, Schofield, Turner, & Kwok, 2014; Mayeux et al., 1991).

Sex also plays a role in AD risk, with women showing a higher prevalence. Many factors may contribute for the increased risk, such as life expectancy, education, health behaviors, and hormonal differences. It is also possible that these factors may interact differently with genetic risk factors (Alzheimer's Association, 2022).

The previously mentioned risk factors cannot be modified. However, there are many modifiable risk factors related to AD, which were reported by the Lancet Commission in 2020 (Figure 14). They suggested that addressing these factors might prevent or delay dementia in up to 40% of cases (Livingston et al., 2020).

	Relative risk for dementia (95% CI)	Risk factor prevalence	Communality	Unweighted PAF	Weighted PAF*
Early life (<45 years)					
Less education	1·6 (1·3–2·0)	40·0%	61·2%	19·4%	7·1%
Midlife (age 45–65 years)					
Hearing loss	1·9 (1·4–2·7)	31·7%	45·6%	22·2%	8·2%
TBI	1·8 (1·5–2·2)	12·1%	55·2%	9·2%	3·4%
Hypertension	1·6 (1·2–2·2)	8·9%	68·3%	5·1%	1·9%
Alcohol (>21 units/week)	1·2 (1·1–1·3)	11·8%	73·3%	2·1%	0·8%
Obesity (body-mass index ≥30)	1·6 (1·3–1·9)	3·4%	58·5%	2·0%	0·7%
Later life (age >65 years)					
Smoking	1·6 (1·2–2·2)	27·4%	62·3%	14·1%	5·2%
Depression	1·9 (1·6–2·3)	13·2%	69·8%	10·6%	3·9%
Social isolation	1·6 (1·3–1·9)	11·0%	28·1%	4·2%	3·5%
Physical inactivity	1·4 (1·2–1·7)	17·7%	55·2%	9·6%	1·6%
Diabetes	1·5 (1·3–1·8)	6·4%	71·4%	3·1%	1·1%
Air pollution	1·1 (1·1–1·1)	75·0%	13·3%	6·3%	2·3%

Data are relative risk (95% CI) or %. Overall weighted PAF=39·7%. PAF=population attributable fraction. TBI=traumatic brain injury. *Weighted PAF is the relative contribution of each risk factor to the overall PAF when adjusted for communality.

Table 1: **PAF for 12 dementia risk factors**

Figure 14: Environmental risk factors for dementia. Environmental risk factors for dementia according to age ranges, their relative risk and prevalence in the population. Extracted from *(Livingston et al., 2020)*.

1.2.5.2. Genetic

Several genetic risk factors for sporadic AD have been described, as can be seen in figure 15. Recently, many studies have been analyzing whole genome for polygenic risk scores, which could be used to identify at-risk subjects

before they develop pathology (de Rojas et al., 2021). However, the *APOE* ε4 allele remains the most important genetic risk factor for AD.

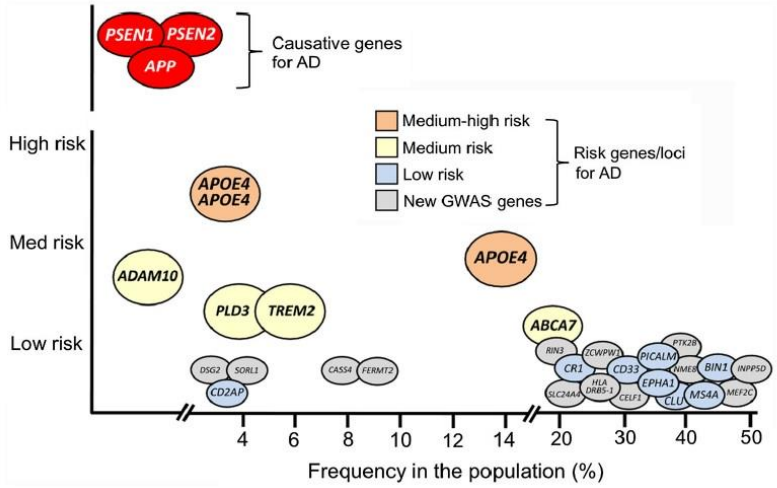


Figure 15: Genetic causative/risk factors for AD. The vertical axis represents the increase in risk, while the horizontal axis represents the frequency of the risk gene in general population. PSEN1, PSEN2 and APP mutations are causative genes for dominantly-inherited familial AD. In sporadic AD, the APOE ε4 allele is the most important risk factor, with a risk that increases in a dose-dependent manner. Extracted from *(Yamazaki, Painter, Bu, & Kanekiyo, 2016)*.

1.2.5.2.1. *APOE4* and Disease

The *ε4* allele has been associated with decreased longevity, increased risk for death and it has been implicated in many other diseases and pathological alterations (Mahley, 2016a; Sebastiani et al., 2019; Steele et al., 2022). However, *APOE4* is mostly related to AD, as it is the main genetic risk factor for the disease. It increases the risk of the disease by 3- to 4-fold in heterozygous carriers and by 15-fold in homozygous carriers, and over 60%

of AD patients carry the allele. Furthermore, *APOE4* also decreases the age of onset by 7 to 9 years per allele. Thus, many cases of EOAD are related to *APOE4* (Michaelson, 2014; Raulin et al., 2019).

However, the effect of *APOE4* on AD risk is distinct between ethnic groups and it is influenced by the ancestry of the *APOE* and surrounding genes (Abondio et al., 2019; Michaelson, 2014). Other factors also modulate *APOE4* effects on AD risk, including sex, with women carriers having much higher risk than their male counterparts (Farrer et al., 1997) and parasitic exposure levels (Trumble et al., 2017). Conversely, *APOE4* can modulate the effects of other risk factors, such as depression, diabetes and lack of physical exercise (Abondio et al., 2019).

1.2.5.2.2. ApoE4 functional alterations

ApoE4 influences the risk of AD through multiple mechanisms, including synaptic dysfunction, alterations in neuronal activity, vascular dysfunction, impaired BBB integrity, altered ion and metal homeostasis, altered glucose metabolism and insulin signaling, and so on (Steele et al., 2022). Figure 16 resumes the main effects of ApoE4 on AD pathology. These alterations derive from apoE4's altered structure, which creates both a loss of normal functions and a gain of toxic functions that alter peripheral and central pathways. Here, we will proceed to further explore some of these alterations as they pertain to this thesis and to its main function, lipid homeostasis.

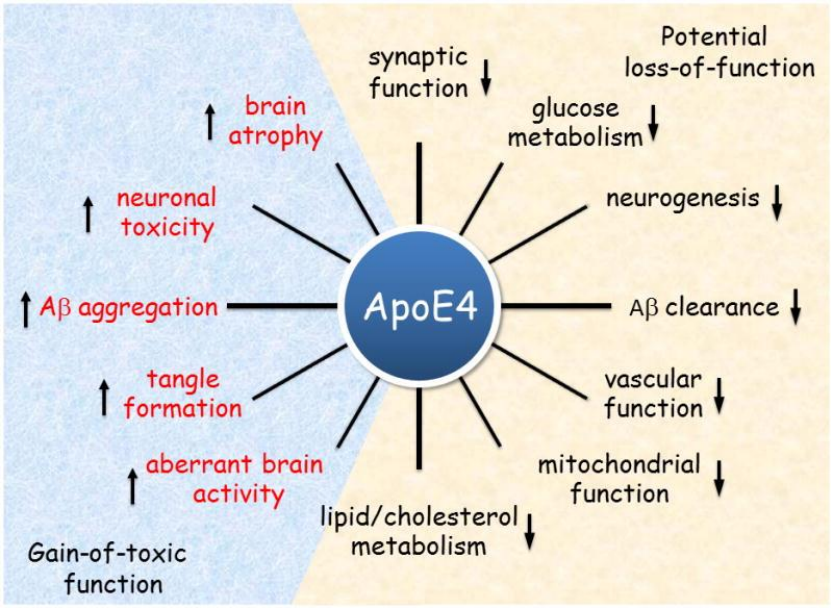


Figure 16: Effects of apoE4 on AD pathology. To the left, gain of toxic functions, to the right, loss of normal function. Extracted from *(Liu, Liu, Kanekiyo, Xu, & Bu, 2013)*.

Aβ and tau metabolism

ApoE4 is abundantly present in amyloid plaques. It promotes the aggregation of Aβ, causing a greater amyloid plaque burden (Wisniewski & Drummond, 2020). Furthermore, it stabilizes the highly toxic Aβ oligomers, increasing their levels in the brain (Hashimoto et al., 2012). ApoE4 impairs processing and clearance of Aβ, but it also increases Aβ production and secretion by neurons (Belloy et al., 2019; Lin et al., 2018). Hence, *APOE4* genotype is associated with increased Aβ burden and an earlier age of onset of Aβ positivity in amyloid PET (Burnham et al., 2020; Lopresti et al., 2020).

APOE4 is also associated with increased tau pathology. Astrocytic apoE4 was found to increase the phosphorylation and misfolding of neuronal tau and to

promote tau aggregation and accumulation (Jablonski et al., 2021). Furthermore, apoE4 exacerbates tau-induced synaptic loss and neurodegeneration (Shi et al., 2017; Wang, C. et al., 2021). Accordingly, *APOE4* carriers show an increased tau deposition in the entorhinal cortex and hippocampus, both in the presence and absence of Aβ plaques (Farfel, Yu, De Jager, Schneider, & Bennett, 2016; Therriault et al., 2020). Furthermore, in AD, *APOE4* promotes a more medial temporal lobe-predominant pattern of tau pathology (La Joie et al., 2021); and higher CSF tau levels are associated with faster AD progression in *APOE4* carriers only (Koch et al., 2017).

ApoE4 fragments are also known to affect Aβ and tau pathology. ApoE4 has an altered degradation in brain, which generates higher levels of low molecular weight fragments compared to apoE3 (Tamboli et al., 2014). These apoE4 fragments are neurotoxic and increase the formation of Aβ plaques, tau hyperphosphorylation and NFT formation, and the initiation of neurodegeneration (Muñoz, Garner, & Ooi, 2019). Moreover, certain apoE4 fragments can promote the cellular uptake of extracellular Aβ species, which promote intracellular Aβ accumulation and increased ROS production (Dafnis et al., 2016).

Lipid Metabolism

The apoE4 isoform is less efficient in accepting cholesterol and phospholipid than apoE3. It also shows an altered lipoprotein affinity, which shifts from HDL to VLDL (Li et al., 2013; Marais, 2019) Furthermore, apoE4 presents increased intracellular concentration due to an impaired recycling, which leads to intracellular cholesterol accumulation and reduced LDLR surface expression (Heeren et al., 2004; Mahley, 2016a). These alterations affect

VLDL metabolism, which alters the plasma lipid profile to one that is more atherogenic, that is, higher VLDL/HDL ratio (Mahley et al., 2009; Whitacre et al., 2022).

Altered lipid metabolism caused by apoE4 is also seen in the CNS and results in reduced neuronal protection or repair and increased inflammation (Rebeck, 2017). ApoE4 promotes less lipid efflux from astrocytes and neurons than the other isoforms, leading to lipid accumulation and lipotoxicity. It also alters fatty acid handling and metabolism, causing further lipid accumulation in astrocytes (Qi et al., 2021). Furthermore, apoE4 in the CNS is less lipidated due to the lower affinity it presents for the CNS HDL-like lipoproteins (Mahley, 2016b). Lipidation status is important for the attachment of lipoproteins to their receptors; thus, CNS apoE4 presents altered receptor-dependent functions (Flowers & Rebeck, 2020). Furthermore, unlipidated apoE4 presents altered self-association in the CSF, which increases the formation of large neurotoxic oligomers (Hatters, Zhong, Rutenber, & Weisgraber, 2006).

ApoE concentration is also important for correct function. *APOE4* carriers have lower levels of apoE protein than non-carriers in CSF and in astrocytes (Flowers & Rebeck, 2020) This could be due to the lower stability of the apoE4 protein which allows cells to recognize it as an unfolded protein, thus enhancing its degradation and the intracellular accumulation of fragments (Mahley, 2016b). Moreover, apoE4 also presents increased extracellular proteolysis (Tamboli et al., 2014).

Immunomodulatory effects

ApoE4 is the least efficient isoform in suppressing inflammation and it can, in fact, facilitate pro-inflammatory cell and cytokine profiles. Mice expressing apoE4 produce more proinflammatory cytokines, have a higher activation of nuclear factor kappa-light-chain-enhancer of activated B cells [NFκβ]-regulated genes and have lower levels of anti-inflammatory cytokines when compared to apoE3 mice (Zhang et al., 2010b). Similarly, human studies found that *APOE4* carriers present lower levels of Interleukin 10, and higher levels of IL-1β and of nitric oxide, both at basal situations and after stimulation (Colton et al., 2004; Olgiati et al., 2010; Tziakas et al., 2006).

ApoE4, when compared to apoE3, also induces higher activation of astrocytes and microglia, which increases the secretion of pro-inflammatory cytokines and causes greater neurotoxicity and postsynaptic neuronal damage (Maezawa, Nivison, Montine, Maeda, & Montine, 2006).

Lastly, apoE4 can also influence neuroinflammation by other mechanisms, such as higher inflammation induced by Aβ deposition, oxidative and cellular stress, increased lipotoxicity and higher levels of neurotoxic apoE fragments in neurons (Rebeck, 2017).

Antioxidant effects and Redox Balance

ApoE is important for the defense against reactive species. However, apoE4's antioxidant activity is the least effective of the three isoforms (Miyata & Smith, 1996), which could be related to the absence of reactive cysteine residues in apoE4 (Martinez-Banaclocha, 2022). Not only apoE4 presents lower antioxidant function, but it also leads to increased production of reactive species; Furthermore, it increases inflammation and activation of

pro-inflammatory pathways which increase ROS and RNS production (Jofre-Monseny, Loboda et al., 2007; Jofre-Monseny et al., 2007).

ApoE4 is also linked to elevated oxidative damage through other toxic mechanisms. Full-length apoE4 and some of its fragments promote oxidative damage by inducing Ca^{2+} accumulation, and mitochondrial dysfunction (Steele et al., 2022). Furthermore, apoE4 protein may also trigger a variety of oxidative intracellular pathways, leading to carbonylation of protein and apolipoprotein dysregulation (Safieh, Korczyn, & Michaelson, 2019). Moreover, apoE4 induces higher accumulation of Aβ and it is less effective in modulating Aβ-induced oxidation (Lauderback et al., 2002). Additionally, apoE4 is less efficient in the detoxification of oxidized molecules, which allows them to oxidize other neuronal proteins and cause cell death (Montine et al., 1996). *APOE4* also reduces the expression of antioxidants (Khan et al., 2022), is less able to suppress metal-induced oxidation than the other isoforms (Mabile et al., 2003), and has been related an increased susceptibility to ferroptosis, an oxidative iron-related cell death (Belaidi et al., 2022).

Human studies have seen the effect of apoE isoforms on oxidative damage. In AD brain samples, the degree of oxidative damage is directly associated with *APOE* allele in the order ε2<ε3<ε4, opposite to the order of antioxidant capacity of the alleles (Dose, Huebbe, Nebel, & Rimbach, 2016; Miyata & Smith, 1996). In post-mortem studies in AD subjects, *APOE4* was associated with increased levels of lipid peroxide, lower levels of GSH, and lower catalase and GPx activities in hippocampus (Ramassamy et al., 1999; Ramassamy et al., 2000). Elevated markers of lipid peroxidation and reduced antioxidant activity can also be seen in the CSF and in the periphery in AD

and MCI *APOE4* carriers (Ben Khedher et al., 2021; Butterfield & Mattson, 2020; Liou, Chen, Lin, Tsai, & Chang, 2021). Oxidative damage and altered antioxidant capacity have also been seen in in healthy *APOE4* carriers (Piccarducci et al., 2019; Smith, Miyata, Poulin, Neveux, & Craig, 1998). However, *APOE4* is also related to reductive stress in young carriers (Badía et al., 2013). In AD mouse models, reductive stress can be found at a young age, which later converts to oxidative stress (Lloret, Fuchsberger, Giraldo, & Vina, 2016). Thus, *APOE4* can lead to both reductive and oxidative stress, depending on age.

Cellular Stress and Cellular Death

ApoE4 causes ER stress, activates stress responses and leads to cellular dysfunction and death (Zhong, Ramaswamy, & Weisgraber, 2009). ApoE4 can lead to ER stress through different mechanisms. It is suggested that the altered structure of apoE4 might affect its trafficking through the secretory pathway and allow it to be recognized as misfolded by the ER (Brodbeck et al., 2011). ApoE4 is also more prone to proteolytic cleavage in neurons, which leads to increased fragments; these are not only directly neurotoxic, but they can also aggregate, increasing cellular stress. Furthermore, ER stress triggered by apoE4 can result in intracellular mislocalization and redistribution of proteins, which increases stress (Theendakara et al., 2016).

ApoE4 also affects cellular function and creates cellular stress by its differential binding to several promoter regions in the DNA, and by altering the expression of several genes and proteins. This affects pathways involved in trophic support, axon guidance, neuronal signaling, synaptic function, glucose and insulin metabolism and energy homeostasis, programmed cell death and inflammation (Theendakara et al., 2013; Theendakara et al.,

2016). Additionally, it induces inflammation and cellular stress through increased NFκβ activity, increased expression of inflammatory cytokines and cell death (Theendakara et al., 2016).

ApoE4 also affects the response to cellular stress and inhibits the expression of autophagy- and mitophagy-related genes, leading to autophagy dysfunction, intracellular accumulation and aggregation of misfolded proteins, destabilization of the cytoskeleton and cell death (Nuriel et al., 2017; Parcon et al., 2018; Sohn et al., 2021). These effects might be facilitated by the nuclear translocation of apoE4, its lower lipidation, its self-aggregation capacity and by the higher intraneuronal accumulation of this isoform (Diaz et al., 2022; Theendakara et al., 2016).

Lastly, alterations of other intracellular pathways caused by apoE4 can also cause increased ER stress and induce apoptosis, such as calcium dysregulation, and impaired insulin signaling (Dose et al., 2016).

1.2.5.2.3. *APOE4* in Healthy individuals

Due to its association with AD risk, *APOE4* has been extensively studied in individuals with diagnosis of AD and MCI. However, it is now known that AD pathology starts decades before the initial clinical symptoms. Thus, the effects of *APOE4* carriage on healthy individuals has recently become subject of many studies.

In the plasma, studies in children and young adults have shown that the concentrations of serum total cholesterol, LDL-cholesterol, apolipoprotein B and triglyceride were different according to *APOE* genotype, with *APOE4* carriers presenting the highest levels (Karjalainen et al., 2019).

In the brain, magnetic resonance imaging studies have found conflicting results regarding the effects of *APOE4* on normal grey matter structure in younger individuals. It is suggested that, at younger ages, the effects of *APOE4* may be limited to specific substructures, or may change substantially with normal development (Flowers & Rebeck, 2020). Studies in middle-aged *APOE4* carriers also found divergent results, with some finding reductions in grey matter volume (Cacciaglia et al., 2018), while others did not find any difference (Jack et al., 2015).

Altered vascular functions with worse cerebrovascular health have also been described in healthy *APOE4* carriers. These individuals present greater white matter intensities (Lyall et al., 2020) and increased microbleeds (Ingala et al., 2020). Furthermore, a very recent study by Montagne et al (Montagne et al., 2020) found that cognitively normal *APOE4* carriers had a breakdown of the BBB in the hippocampus and the parahippocampal gyrus, which was independent of AD pathology and of systemic vascular risk factors. Altered metabolism has also been seen, with cognitively normal *APOE4* carriers presenting hypometabolism in cerebral areas related to AD, decades prior to the onset of clinical symptoms (Langbaum et al., 2010).

Relative to AD biomarkers, *APOE4* was associated with high Aβ plaque levels in the brain, low levels of CSF Aβ42, and lower Aβ42/Aβ40 ratio (Wang, Y., Grydeland, Roe, Pan, Magnussen, Amlien, Watne, Idland, Bertram, Gundersen, Pascual-Leone, Cabello-Toscano, Tormos, Bartres-Faz, Drevon, Fjell, & Walhovd, 2022). Furthermore, in PET scans, *APOE4* is associated with greater amyloid deposition and earlier amyloid PET positivity in an allele-dose-dependent manner, with more than 10% of carriers being positive by age 60 years (Jack et al., 2015).

Young *APOE4*-positive individuals have higher co-activations of the default mode networks, showing higher resting brain activity and altered functional connectivity when compared to young *APOE3*-positive individuals (Koelewijn et al., 2019). Furthermore, young *APOE4* carriers present altered brain activity depending on the cognitive task analyzed, with increased activity during certain cognitive tasks and lower activity during others (Flowers & Rebeck, 2020).

Studies have reported better cognitive performance of young *APOE4* carriers when compared to non-carriers; this lead the hypothesis of antagonistic pleiotropy, that is, a superior cognitive performance in young *APOE4* carriers which contrasts with the increased risk of cognitive decline in older age (Tuminello & Han, 2011). However, other studies found divergent results, with reports of decreased and even no difference between carriers and non-carriers (Flowers & Rebeck, 2020; O'Donoghue, Murphy, Zamboni, Nobre, & Mackay, 2018). Thus, a possible pleiotropic effect of *APOE4* on cognition cannot yet be established.

Beneficial effects associated with *APOE4* have also been reported on certain cognitively healthy carriers. *APOE4* has been associated with increased fertility under adverse environments, such as exposure to high pathogen levels (Oriá, de Almeida, Moreira, Guerrant, & Figueiredo, 2020). *APOE4* is also an advantageous factor on survival in environments with high exposure to infectious diseases (van Exel et al., 2017). Similarly, *APOE4* is associated with lower chronic infection and liver fibrosis due to the hepatitis C virus (Mueller et al., 2016). Furthermore, *APOE4* has been shown to be related to better outcomes in childhood diarrheal infections (Oriá et al., 2010), and also to protect adult cognitive performance in a population exposed to high

parasite burden (Trumble et al., 2017). Thus, the *APOE4* allele might confer benefits in contexts of high-pathogen and energy-limitation associated with an active lifestyle (Garcia et al., 2021).

It is suggested that *APOE4* is associated with increased brain activity and macromolecule turnover in young healthy individuals, with the reverse happening in elderly carriers (Diaz et al., 2022; Smith, Ashford, & Perfetti, 2019). Thus, Smith and Ashford (Smith & Ashford, 2017) hypothesized that *APOE4*-related pathology might be due to increased lifetime exposure to neurotoxic processes. The highest turnover of proteins, lipids, and other macromolecules in neurons of *APOE4* carriers might cause a larger detriment when exposed to certain endogenous or exogenous stressors.

Even though *APOE4* has been extensively studied, the changes it causes have not yet been fully elucidated. Furthermore, as AD pathology starts years or decades before the onset of clinical symptoms, it is extremely important to know and target the alterations that occur in the preclinical phase of the AD continuum. Thus, studying the effects of *APOE4* in cognitively healthy individuals is a vital tool to better understand the disease and its possible prevention.

OBJECTIVES

2. Objectives

2.1. General Objective

To conduct a prospective longitudinal study with a cohort of subjects carrying at least one *APOE4* allele, followed for 11 years and compare them with non-carriers.

2.2. Specific objectives

1) To asses current cognitive, oxidative and inflammatory status along with stress-related protein levels in subjects with at least one *APOE4* allele compared with non-carries.
2) To compare cognitive, oxidative and inflammatory status along with stress-related protein levels in subjects with at least one *APOE4* allele in a 11-year follow-up study.

MATERIALS AND METHODS

3. Materials and Methods

3.1. Materials and Equipment

3.1.1. Reagents, culture media and kits

- Apolipoprotein E4 (human) ELISA Kit; K4699-100; BioVision.
- Human IL-1 beta ELISA Kit; 2ab214025; Abcam.
- Human TNF alpha Simple Step ELISA® Kit; ab181421; Abcam.
- Restore Western Blot STRIPPING BUFFER; 21059; Thermo Scientific.
- Immobilon Classico Western HRP Substrate (Luminol o Luminata); WBLUC0500; Millipore.
- NucleoSpin TriPrep, Mini kit for RNA, DNA, and protein purification; 74096650; Macherey-Nagel.
- Taqman Fast Advanced master mix; 12634225; Thermo Fisher Scientific.
- High capacity cDNa Reverse Transcription Kit; 4368814; Thermo Fisher Scientific.
- Dulbeccos Phosphate Buffered Saline w/o Magnesium w/o Calcium (PBS); L0615-500; Biowest (LabClinics).
- Roswell Park Memorial Institute (RPMI) medium.
- Penicillin/Streptomycin (P/S) 10,000 U/mL Antibiotic; GIBCO.
- Inactivated Fetal Bovine Serum (FBS); Invitrogen.

- Culture medium: consisting of 44mL of RPMI, 5mL of FBS and 1mL of antibiotic.
- Lowry Reagent; L3540-1VL; Sigma-Aldrich.
- Folin & Ciocalteu's phenol reagent; F9252-500mL; Sigma-Aldrich.
- Other reagents were obtained at Sigma-Aldrich, Millipore, Merck (Germany), Molecular Probes (Eugene, Oregon, United States), J.T. Baker, Invitrogen, Thermo Fisher Scientific (United States) and Panreac Quimica SLU (Spain).

3.1.2. Probes, antibodies and primers

- **Annexin V:** Annexin V apoptosis detection, ANXVF-200T, immunostep.
- **Propidium Iodide:** Propidium iodide; 81845; sigma-aldrich.
- **H_2DCFDA:** H2DCFDA, D399; Molecular Probes.
- **DHE:** Dihydroethidium; 37291; Sigma-Aldrich.
- **DAPI:** DAPI for nucleic acid staining; D9542; Sigma-Aldrich.
- **Calcineurin:** Pan-Calcineurin A antibody; 2614; Cell Signaling.
- **RCAN1:** Recombinant Anti-Calcipressin 1/ RCAN1 antibody [EPR 8911]; ab140131; abcam.
- **GSK3β:** Anti-GSK3β; ep 7194; MBL.
- **p38:** p38 MAPK antibody; 9212S; Cell Signaling.

- **p-p38:** Phospho-p38 MAPK (Thr180/Tyr182) Antibody; 9211S; Cell Signaling.
- **p-Tau231:** Tau Antibody (Phospho-Thr231); A00406; GenScript.
- **β-Actin:** Anti-β-Actin antibody; A1978; Sigma-Aldrich.
- **Anti-mouse:** Anti-mouse IgG H&L Chain Specific Peroxidase Conjugate; 401215; Calbiochem- Merk.
- **Anti-rabbit:** Anti-rabbit IgG HRP-linked; 7074S; Cell Signaling.
- **GCLC:** glutamate-cysteine ligase catalytic subunit; Hs00155249_m1; Thermo Fisher Scientific.
- **GCLM:** glutamate-cysteine ligase modifier subunit; Hs00157694_m1; Thermo Fisher Scientific.
- **GPx1**: glutathione peroxidase 1; Hs00829989_gH; Thermo Fisher Scientific.
- **GSK3β:** glycogen synthase kinase 3 beta; Hs00275656_m1; Thermo Fisher Scientific.
- **PKR (EIF2AK2):** eukaryotic translation initiation factor 2 alpha kinase 2 Hs00169345_m1; Thermo Fisher Scientific.
- **PPP3R2 (Calcineurin):** protein phosphatase 3 regulatory subunit B, beta; Hs00330865_s1; Thermo Fisher Scientific.
- **RCAN1:** regulator of calcineurin 1; Hs01120954_m1; Thermo Fisher Scientific.
- **SOD1:** superoxide dismutase 1, soluble; Hs00533490_m1; Thermo Fisher Scientific.

- **GAPDH:** glyceraldehyde-3-phosphate dehydrogenase; Hs02786624_g1; Thermo Fisher Scientific.

3.1.3. Equipment

The laboratory equipment employed in this study are part of the Department of Physiology and of the Unidad Central de Investigación de Medicina (UCIM), both located at the Faculty of Medicine and Odontology of the University of Valencia. The infrastructure used in this study was the one designated to the investigation group directed by Drs. José Viña Ribes and Ana Lloret Alcañiz.

- -80ºC Ultra low temperature freezer Bio Memory 690L; Froilabo.
- -20ºC freezer Comfort Nofrost; Liebherr.
- 4ºC refrigerator; Lynx.
- Milli-Q; Millipore.
- Purelab flex; Elga.
- Block heater SBH130D; Stuart.
- Thermomixer Compact; EPPENDORF®.
- Hot plate stirrer multiposition; SB162-3; Stuart.
- Fume Hood OR-ST 1500; Burdinola.
- Sorvall Legend XTR centrifuge; Thermo Scientific.
- Mikro 220R centrifuge; Hettich.
- Mini Centrifuge; MC7000 series; LBX Instruments.

- SIGMA 1-14 Microcentrifuge; Sigma.
- pH meter GLP21; Crison.
- Spectrophotometer 7315, Jenway.
- Vortex-vib - Vortex shaker; Selecta.
- Reax top vortex mixer; 541-10000-00; Heidolph.
- Duomax 1030 platform shaker; Heidolph.
- Analytical Balance AS 60/220/C/2 (±0,01mg); Radwag .
- Precision weighing balance 6110 (± 0.0001g); Sartorius.
- Sterilizer Autester-G; Selecta.
- Microplate Washer - Hydroflex microplate washer, Tecan.
- BD FACSVerse™ Flow Cytometer with BD FACSuite™ software; BD Biosciences.
- BD LSRFortessa™ X-20 Flow Cytometer with BD FACSDiva™ software; BD Bioscience.
- Dionex UltiMate 3000 HPLC System; Thermo Scientific: composed of a quaternary pump, an autosampler, a column compartment and an UV-Vis detector (UltiMate™ 3000 VWD Variable Wavelength Detector).
- HPLC Column: Hypersil GOLD™ C18 Selectivity HPLC Columns - Reversed Phase; 5µm; 150mm x 4.6mm; max pressure 5800psi; Flow rate 1.25mL/min; Pore size 175A.
- Ultrasonic Processor Vibra-Cell VCX-500; Sonics.

- Mini-PROTEAN Tetra Handcast Systems; Bio-Rad.
- Mini-PROTEAN® Tetra Cell; Bio-Rad.
- Mini Trans-Blot®Electrophoretic Transfer Cell; Bio-Rad.
- PowerPac™ Basic Power Supply, Bio-Rad.
- Image Quant LAS 4000, GE Healthcare Bio-Sciences.
- Amersham Protran 0.45µm NC - Nitrocellulose Blotting Membrane; GE Healthcare Life Science.
- QuantStudio™ 5 Real-Time PCR System; A3432; Applied Biosystems™.
- T100 PCR thermal cycler; Bio-Rad.
- NanoDrop 2000 Spectrophotometer; Thermo Scientific.
- Centrifuge 5430 - Plate centrifuge; Eppendorf.
- Precisterm Water bath; JP Selecta™.
- Cell culture hood B100; Cultair.
- Heracell 150i CO_2 Incubator; Thermo Fisher Scientific.
- TC10 automated cell counter; Bio-Rad.
- Vacutainer tubes: BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube with Sodium Citrate and BD Vacutainer tube with dipotassium ethylenediaminetetraacetic acid (K2-EDTA).
- Blood extraction materials (tourniquet; gauze; alcohol swab; needle holder; double-sided needle).

- Laboratory Consumables.

3.2. Methods

3.2.1. Subjects

A total of 47 cognitively healthy subjects participated in this study, of which 32 were *APOE4*-carriers and 15 were non-carriers. *APOE4* carriers were further subdivided in heterozygous (N=19) and homozygous (N=13). All groups were comprised of both men and women, aged between 35 and 65 years, of all levels of education. All participants lived in the Province of Valencia in the Valencian Community in Spain and spoke Spanish as a native language.

The current study is a follow-up of a previous study, conducted by our research group, that lead to the doctoral thesis of doctor María Del Carmen Badía Picazo, with the title "ESTUDIO DE ESTRÉS OXIDATIVO EN HIJOS DE PACIENTES CON ENFERMEDAD DE ALZHEIMER PORTADORES DEL ALELO 4 DE LA APOLIPOPROTEÍNA E.". That study included 33 *APOE4* carriers who had a parent diagnosed with AD and 14 non-carriers without a family history of AD.

For the current study, recruitment of participants was done in two parts. First, *APOE4* carriers that participated in the previous study were contacted by telephone and informed of the present research. After the contact, 24 of the initial 33 participants acceded to participate in this follow-up. Then, as it was not possible to contact the same control group that participated in 2008, we recruited 23 age-matched subjects, who were cognitively healthy and community-dwellers. These participants were then classified according to their genotype into non-carriers and volunteer *APOE4* carriers. These *APOE4*

volunteers were included in the cross-sectional analysis and excluded from the longitudinal analysis and the non-carrier participants formed the 2019 control group for both analyses.

Ethics Statement

Participation in the study was voluntary and an informed consent was obtained from all individual participants. This study was conducted in accordance with the Helsinki declaration, UNESCO's Universal Declaration on Bioethics and Human Rights, the International Ethical Guidelines for Biomedical Research Involving Human Subjects from the Council for International Organizations of Medical Sciences', the Council of Europe's Convention on Human Rights and Biomedicine and the European Charter of Fundamental Rights. Furthermore, it was conducted in accordance with the Spanish legislation regarding personal data protection, biomedical research, and bioethics. This study was approved by the ethics committee of the University of Valencia (reference: H1542117584721).

Inclusion and Exclusion Criteria

Returnees were included if they were cognitively healthy and signed the informed consent. Newly recruited subjects were included if they were cognitively healthy, were aged between 35 and 65 years and had Spanish as a native language. Any individual, whether a returnee or a new participant, that presented any exclusion criteria were excluded from the study.

Exclusion Criteria for all groups:

I. Presented an acute inflammatory or infectious disease,
II. Used substances or medications that might affect cognitive processes,

III. Presented cognitive deficits or dementia,

IV. Suffered from severe visual or auditory handicaps,

V. Did not sign the informed consent.

The previous study excluded subjects that presented certain conditions, such as diabetes mellitus and menopause. However, this follow-up study did not exclude participants based on these factors. This decision was based on the fact that, with time and aging, many returnees could have developed chronic diseases and many women would have reached menopause.

Interview

Interviews and assessments were conducted at the office of Dr. Ana Lloret in the Department of Physiology in the Faculty of Medicine of the University of Valencia. Although it was known that the 24 returnees were *APOE4* carriers, researchers involved in the current study did not know their specific genotypes prior to the interview and neuropsychological assessment. Upon arrival, the study's protocols and aims were explained, and any questions the subject might have had were explained. After signing the informed consent, a number was assigned to the participant which was used in all subsequent testing and analysis to maintain confidentiality. Interviews and neuropsychological assessment were performed individually, with each participant. After the clinical assessment, blood samples were drawn by a registered nurse. Samples' handling and storage are described further on.

Interviews consisted of a series of questions on the subject's socio-demographic and clinical history (annex 2). Information was gathered on level of education, work history, practice of physical exercise, previous clinical diagnoses, smoking and alcohol consumption and use of chronic

medication. Furthermore, subjects were inquired about subjective memory complaints [SMC] and about current symptoms of anxiety or depression.

3.2.2. Neuropsychological Assessment

3.2.2.1. Rey Auditory Verbal Learning Test

Background

The Rey Auditory Verbal Learning Test [RAVLT] (Rey, 1964) is a verbal learning test that relies on the auditory presentation of a word list, and can be used to analyze different components of the learning process. It is a useful tool in the diagnosis of many neuropathologies and it has normative studies which take into account varying socio-demographic data (Lezak, Howieson, Bigler, & Tranel, 2012).

Procedure and Scoring

A list of 15 words (list A) is read to the subject at the rate of one per second. Immediately after, the subject is asked to recall as many words as they can, in any order (trial I – immediate recall). The same procedure is done four more times (Trials II-V). Then, a second list (list B) is presented to the subject, followed by a free-recall of the new words (Trial VI). Next, the subject is asked to freely-recall the words from the first list (Trial VII). After a 30-minutes interval, in which the subject performs other non-memory related tasks, the subject is asked again to recall the words from list A (Trial VIII - long-delay recall). Lastly, the examiner presents a list of 75 words and the subject must identify the words from list A (recognition trial). The present study used a Spanish version of the word lists A, B and recognition (Annex 3).

The raw score for each trial is the number of words correctly recalled. In this work, we evaluated immediate recall (trial I), long-delay recall (trial VIII) and total learning (expressed by the sum of trials I-V). In the cross-sectional analysis, in order to compare results between all subjects, raw scores were corrected for age, sex and education using normative data provided by the Mayo Normative Studies (Stricker et al., 2021). For longitudinal analysis, raw scores were converted into psychometric T-scores (standard score with mean of 50 and standard deviation of 10).

3.2.2.2. The Stroop Color and Word Test

Background

The Stroop test (Stroop, 1935) relies on the fact that it takes longer to name colors when they are presented with the interference of conflicting words (word and color ink do not match), than when such interference does not occur (naming the color of a square). This difference in color-naming performance between congruent and incongruent stimuli is called the Stroop Effect (Algom & Chajut, 2019).

The Stroop test has been used to measure multiple cognitive functions, but its main importance is as a measure of focused attention and of inhibitory control; thus, it is particularly valuable to identify cases of frontal lobe damage (Lezak et al., 2012)

Procedure and Scoring

In this study we used the Spanish version of the Stroop Color and Word Test (Golden, 2001) (Annex4). It consists of 3 pages with 100 elements each. The

elements on the first page are the words ROJO (red), VERDE (green) and AZUL (blue), randomly distributed and printed in black ink. The second page consists of "XXXX" elements printed in blue, green or red ink. The third page consists of the words on the first page printed in the colors of the second page, which are combined so that the names of the colors and the color ink do not match. The subject is instructed to perform three timed tasks: reading the words on the first page and naming the color of the ink on the second and third pages. Each of the tasks lasts for 45 seconds and the subject is instructed to perform them as fast as they can. The task scores are the number of correct elements named on each page.

As results can be affected by education and age, raw scores were corrected for the cross-sectional analysis using the norms devised by the Spanish Multicenter Normative Studies, NEURONORMA Project (Peña-Casanova et al., 2009) and NEURONORMA young adults project (Rognoni et al., 2013). For longitudinal analysis, raw scores were converted into psychometric T-scores (standard score with mean of 50 and standard deviation of 10).

3.2.2.3. Memory Failures in Everyday Questionnaire

Background

The Memory Failures in Everyday questionnaire [MFE] (Sunderland, Harris, & Baddeley, 1983) is a frequently used tool to investigate occurrence, frequency, and types of memory failures in everyday life, both in healthy individuals and in people with varying brain disorders (Lezak et al., 2012). The 28-item MFE presents a normative study in healthy Spanish adults that

found no significant effects due to age, years of education, or sex (Montejo Carrasco, Montenegro Peña, & Sueiro, 2012)

Procedure and Scoring

In our study, MFE was self-administered. Subjects were instructed to read each of the 28 items of the questionnaire and rate them according to the frequency with which they occurred: 0 (zero) if the situation never or rarely occurred; 1 (one) if it occurred sometimes but not often; or 2 (two) if it occurred frequently or often. If, for any reason, the subject could not read the questionnaire, the investigator would read each item to them. The professional would also clarify any doubts about the meaning of the questions. The total result of the MFE was calculated as the sum of all 28 items.

3.2.2.4. The Hamilton Depression Rating Scale

Background

The Hamilton Depression Rating Scale (Hamilton, 1960) was designed to evaluate the severity of depressive symptoms during the previous week. This scale is a useful to monitor the course of the depressive disorder and to assess the result of treatment, and has been widely used in clinical practice and research settings (Worboys, 2013).

Procedure and Scoring

Our study used the 17-item Spanish version of the Hamilton scale. During the initial interview, the subject was questioned on the presence of a previous diagnosis or of current symptoms of depressive and/or anxious disorder. In

an affirmative case, the subject was further questioned about symptoms in the previous week. The interviewer would then answer the items of the scale.

The scores for each item were then summed, generating a global severity score where higher scores denoted higher severity. Our study used the recommendations of the American Psychiatric Association (Rush, 2000) and severity was classified as follows: Not depressed ≤ 7; Mild 8-13; Moderate 14-18; Severe 19-22; Very severe ≥ 23**.** Furthermore, subjects that presented a global score ≤ 7 but were in use of antidepressants were classified as "in remission" (Grupo de trabajo de la Guía de Práctica Clínica sobre el Manejo de la Depresión en el Adulto, 2014).

3.2.3. Sample extraction and processing

Reagents

- ***N*-Ethylmaleimide (NEM310) [NEM]**– To prepare 310mM NEM, 3.88g of NEM were diluted in 100mL of Milli-Q water. Solution was stored at −20°C in 10mL aliquots.

- **Tripotassium ethylenediaminetetraacetic acid (K3-EDTA) –** K3-EDTA was prepared by adding 75mg of K3-EDTA per ml of Milli-Q water.

Blood Extraction

Blood samples were extracted by phlebotomy of the cubital vein and collected in two different vacutainer tubes: BD Vacutainer® CPT™ Mononuclear Cell Preparation Tube with Sodium Citrate and BD Vacutainer

tube with dipotassium EDTA (K2-EDTA). Samples were processed to obtain whole blood, plasma and lymphocyte pellets, as described below.

Whole blood and plasma

Immediately after extraction, two 500µL whole blood aliquots were separated from the blood sample collected in the K2-EDTA tube. One aliquot was added to an Eppendorf tube containing 11.5µL of K3-EDTA, while the other aliquot was added to a tube with 11.5µL of K3-EDTA and 50µL of NEM310. Both samples were mixed for one minute and then stored at -80°C.

The remaining blood sample was centrifuged at 2,000G for 15 minutes at 4ºC. The plasma supernatant was collected and separated into 1mL aliquots, which were stored at -80°C

Lymphocyte extraction and culture

Blood samples collected in Vacutainer® CPT™ tubes were processed to isolate lymphocytes. After centrifuging the tubes at 1,800G for 35 minutes at 22ºC, mononuclear cells were collected using a sterilized glass Pasteur pipette and transferred into a clean plastic tube. Plasma was collected in another tube, aliquoted and stored at -80°C. RPMI medium was added to the tube containing mononuclear cells, which was then centrifuged at 1,500RPM for 10 minutes; RPMI was discarded and cells resuspended in 1mL of culture medium. Cell suspension was then placed in a sterile petri dish with 5mL of culture medium (total volume of 6mL) and incubated for 3 hours at 37ºC. Although most mononuclear cells grow attached to the petri dish (adherent culture), lymphocytes grow floating in the culture medium (suspension culture); therefore, after incubation, liquid medium containing floating lymphocytes was carefully collected and lymphocytes were counted

in an automated cell counter. An aliquot containing 500,000 cells was separated and centrifuged at 1,500RPM for 10 minutes; the resulting cell pellet was resuspended in 1mL of PBS, and the sample was immediately destined for flow cytometry analysis. The remaining cells were centrifuged at 1,500RPM for 10 minutes and the supernatant medium was discarded. Afterwards, cell pellet was resuspended in 1ml of culture medium and centrifuged at the same conditions of the previous step. After discarding the supernatant, the resulting cell pellet was stored at -80°C.

Figure 17 shows the workflow of our study and the use of the samples.

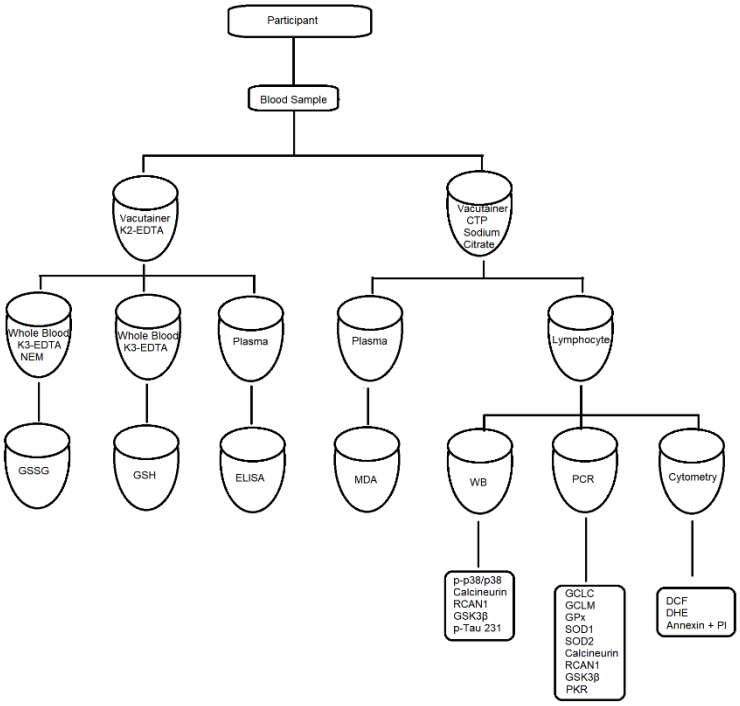


Figure 17: Workflow and use of samples in analytical procedures.

3.2.4. Analytical Methods

3.2.4.1. Glutathione

Background

The glutathione recycling assay (Tietze, 1969) is a specific method to measure the total content of reduced and oxidized glutathione, with minimal or absent interference from other thiols and disulfides. It is based on the continuous regeneration of GSH and on the reduction of 5,5'-dithio-bis-2-nitrobenzoic acid [DTNB], a water-soluble compound that reacts with free sulfhydryl groups. This reaction yields 5-thio-2-nitrobenzoic acid (TNB), a yellow-colored product that can be quantified by spectrophotometry at a 412nm wavelength. GSSG levels can be accurately estimated by pretreating samples with NEM, which prevents GSH autooxidation during sample preparation (Asensi et al., 1994) and allows only GSH that has been reduced from GSSG originally present to participate in the reaction.

Reagents

- **Disodium hydrogen phosphate solution (0.2M Na_2HPO_4)** – This solution was prepared by diluting 2.84g of Na_2HPO_4 in 100mL of Milli-Q water.
- **Potassium dihydrogen phosphate solution (0.2M KH_2PO_4)** – This solution was prepared by diluting 1.36g of KH_2PO_4 in 50mL of Milli-Q water.
- **PB200 (phosphate buffer, 0.2M, pH 7.4)** – To prepare PB200, 6.5mL of KH_2PO_4 solution was mixed with 40.5mL of Na_2HPO_4 solution. Next, pH was checked and adjusted to 7.4.

- **PB50 (phosphate buffer, 0.05M, pH 7.4) -** To prepare PB50, PB200 was diluted with Milli-Q water (1:4 ratio).
- **Trichloroacetic acid 15% (TCA15)–** First, 10mL of 60% TCA (wt/vol) was prepared by mixing 6mL of 100% TCA with 4mL of Milli-Q water. Then, 60% TCA was diluted with Milli-Q water (1:4) to obtain TCA15.
- **β-NADPH$^+$ (4.8 mM) –** First, 50mg of $NaHCO_3$ were diluted in 10mL of Milli-Q water to obtain a 0.5% $NaHCO_3$ (wt/vol) solution. Then, 8mg of $NADPH^+$ were diluted in 2mL of 0.5% $NaHCO_3$ to obtain 4.8mM β-$NADPH^+$.
- **DTNB (20mM)** – DTNB was prepared adding 15.8mg of DTNB to 2ml of PB200. Solution was prepared and stored wrapped with aluminum foil.
- **Glutathione reductase 20U/mL [GR] –** In a tube wrapped in aluminum foil, 160µL of GR were mixed with 2mL of PB50.
- **GSSG standard (10µM GSSG)** – First, 10mM GSSG was prepared by adding 24.5mg of GSSG to 4ml of PB50. Then, 10µl of 10mM GSSG were diluted in 9.99ml of Milli-Q water to obtain 10µM GSSG.
- **GSH stock solution (20mM)** – This solution was prepared by diluting 6.1mg of GSH in 1mL of Milli-Q water.
- **GSH standards (6.25-400µM)** –GSH standard solutions (400, 200, 100, 50, 25, 12.5, and 6.25µM) were freshly prepared on the day of the experiment. The 400µM GSH standard was prepared by diluting 20µL of the 20mM GSH stock solution in 980µL of Milli-Q water. The other GSH standard solutions were prepared by sequential dilution of 500µL of each standard with 500µL of Milli-Q water.

Procedure

We analyzed both GSH and GSSG using the spectrophotometric recycling assay method as described by Giustarini and colleagues (Giustarini, Dalle-Donne, Milzani, Fanti, & Rossi, 2013).

GSSG analysis

Samples containing K3-EDTA and NEM were used to measure GSSG. Samples were brought to room temperature and mixed with 500µL of TCA15 (1:1 dilution). The mixtures were shaken vigorously and centrifuged at 14,000G for 2.5 minutes at room temperature. Next, a 50µL aliquot of the supernatant was separated and diluted with 50µL of Milli-Q water (1:1 dilution). The remaining sample supernatant was stored at -80ºC. Excess NEM was extracted from the sample by adding 300µL of dichloromethane to 100µL of sample, followed by mixing on a vortex shaker at 800RPM for 5 minutes and then centrifuging for 1 minute at 14,000G at room temperature. The supernatant was carefully extracted as not to disturb the dichloromethane bellow.

After preparing the samples, the reaction was prepared by mixing the reagents in a microcuvette in the following order: 945µL of PB200, 5µL of DTNB, 20µL of sample and 20µL of β-$NADPH^{+}$. After stirring the contents, 20µL of GR were added to the cuvette and mixed. Absorbance was recorded for 1 minute at 412nm. After this 1-minute reading, 10µL of 10µM GSSG standard were added to the cuvette and mixed. Absorbance was then recorded at the same wavelength for another minute. “Blank” analyses were carried out throughout the experiment, using the same reagents but

substituting the sample with PB200. This two-step analysis was performed for each measured sample.

GSSG concentration was calculated in the following manner:

I. Calculate Slopes: The slopes of each sample (first minute), each sample after GSSG (second minute), and blank analysis (first minute) were calculated.
II. Calculate *S*: "*S*" was calculated by subtracting the blank slope from the sample slope (*S* = slope sample − slope blank)
III. Calculate *St*: "*St*" was calculated by subtracting the sample slope from the sample slope after GSSG (*St* = slope sample after GSSG − slope sample)
IV. Calculate concentration: the concentration of each sample was calculated using the equation:

$$[GSSG\ blood] = \frac{S * [standard\ GSSG]}{St * dilution\ factors}$$

Were [standard GSSG] is the concentration of the GSSG standard in the cuvette. Final concentrations were expressed in nmol/mL of blood.

GSH analysis

Samples containing only K3-EDTA were used to measure total glutathione. After bringing samples to room temperature, 500µL of TCA15 were added to each sample (1:1 dilution). The mixture was shaken vigorously and centrifuged at 14,000G for 2.5 minutes at room temperature. From the resulting supernatant, 10µL were diluted in 50µL of Milli-Q water (1:5 dilution) and the rest was stored at - 80°C. Next, reagents were placed in a microcuvette in the following order: 945µL of PB200, 5µL of DTNB, 10 µL of

sample and 20µL of β-NADPH^{+}. After stirring the contents, 20µL of GR were added to the cuvette and mixed. Absorbance at 412nm was measured at 60 seconds. The same procedure was repeated for each sample and GSH standard solutions. "Blank" analyses were carried out throughout the experiment, using the same reagents but substituting the sample with PB200.

To calculate total glutathione concentration, the absorbance of the blank analysis was subtracted from the absorbance of each GSH standard and sample. The resulting absorbances of the GSH standards were used to create a calibration curve, where the unknown samples were interpolated. The final concentration of total glutathione was obtained after multiplying the results by the dilutions used during the experiment. Then, reduced glutathione (expressed in nmol/mL of blood) was calculated using the equation:

$$GSHred = total\ glutathione - (2 * GSSG).$$

Oxidated-reduced glutathione ratio was calculated using the equation:

$$GSSG/GSH\ Ratio = \left(\frac{GSSG}{GSH\ red}\right) * 100.$$

Oxidated-reduced NADP ratio was calculated using the equation:

$$NADP/NADPH\ ratio = \frac{k * GSSG}{GSH^2}$$

where k is $1.98*10^{-2}$ M^{-1}, the equilibrium constant for the glutathione reductase reaction.

3.2.4.2. High Performance Liquid Chromatography

Background

High performance liquid chromatography [HPLC] is a form of column chromatography where a sample is dissolved in a solvent (mobile phase) and pumped at high pressure through a column with an immobilized packing material (stationary phase). As each analyte in the sample will interact differently with the stationary phase, depending on their physical and chemical properties, sample components will be separated as they move through the column. When the mobile phase exits the column, it passes through a detector that generates a signal proportional to the amount of analyte emerging from the column (Petrova & Sauer, 2017).

We measured MDA by HPLC using the method described by Wong and colleagues (Wong et al., 1987), which involves the hydrolysis of lipoperoxides present in the sample, followed by the reaction of MDA with 2 molecules of thiobarbituric acid [TBA]. This reaction leads to the formation of a pink-colored MDA-TBA adduct [MDA-TBA_2], that can be measured by HPLC at 532nm. Although other aldehydes can interact with TBA, HPLC is capable of differentiating between MDA-TBA_2 and other thiobarbituric acid reactive substances with a high specificity, accuracy and good analytical validity (Mas-Bargues, Escrivá, Dromant, Borrás, & Viña, 2021).

Reagents

- **Sodium Acetate Anhydrous Buffer (2M, pH3.5) with 0.2% TBA** - First, 119mL of 98% anhydrous acetic acid were added to 600mL of Milli-Q water, and pH was adjusted to 3.5 using a 10M sodium hydroxide (NaOH) solution. Then, 2g of TBA were added to the solution. This mixture was then stirred on a hot-plate (temperature between 50-60ºC), until TBA was fully dissolved. After checking the pH, and adjusting it back to 3.5, if

needed, Milli-Q water was added until the solution reached a total volume of 1 liter.

- **Potassium phosphate (KH_2PO_4) Buffer (50mM, pH 6.8)** – This solution was prepared by diluting 3.4g of KH_2PO_4 in 400mL of distilled water. After adjusting the pH to 6.8 using 1M potassium hydroxide (KOH), water was added to reach a final volume of 500mL.

- **Potassium phosphate (KH_2PO_4) Buffer (50mM, pH 3.5)** - 3.4g of KH_2PO_4 were diluted in 400mL of distilled water and 1M hydrochloric acid (HCl) was used to adjust the pH to 3.5. Then, water was added to reach a final volume of 500mL.

- **MDA standards (0.625 - 50nmol/mL) and Blank** – First, a 5mM MDA solution was prepared by adding 410µL of a commercial 12.2M MDA-bis solution to 1000mL of water. The first MDA standard (50nmol/mL) was obtained by performing a 1/100 dilution of the 5mM MDA solution. The other standards (25, 12.5, 6.25, 3.125, 1.562, 0.781) were obtained by serial 1:1 dilution of the previous standard with water. A blank analysis was also conducted, using milli-Q water that was processed the same way as the MDA standards.

- **Mobile phase, washing phase solution (30:70 (v/v) acetonitrile/water mix) -** 350mL of Milli-Q water were mixed with 150mL of 99% acetonitrile, and the solution was degassed with ultrasonic bath for 20 minutes.

- **Mobile phase, Elution phase solution (83:17 (v/v) KH_2PO_4 solution/acetonitrile mix)** - First, 13.6g of 50mM KH_2PO_4 were dissolved in 2 liters of Milli-Q water and pH was adjusted to 6.8 using 1M KOH.

Then, 410mL of acetonitrile were added, and the solution was fully homogenized and filtrated using a vacuum pump and a 0.22-micron filter (Millipore). Lastly, the solution was degassed with ultrasonic bath for 20 minutes.

Derivatization Procedure

With the reagents prepared, we proceed to derivatize each sample, MDA standard and "blank" using the following protocol:

I. Add 25µL of sample (or standard or blank) to 500µL of sodium acetate anhydrous buffer with TBA.
II. Heat the samples at 95ªC for 60 minutes.
III. Put the samples in ice and quickly add 500µL of the pH6.8 KH_2PO_4 buffer.
IV. Centrifuge the solution for 5 minutes, at 13,000RPM, at 4ºC.
V. Extract the supernatant and separate a 200µL aliquot.
VI. Add 200µL of the pH3.5 KH_2PO_4 buffer to the 200µL supernatant aliquot and agitate.
VII. Separate 200µL of this solution to use for HPLC analysis and store the rest.

Chromatography method

To analyze the samples, we used a reverse phase HPLC, with a C18 Selectivity HPLC Column (Hypersil GOLD™) and an Ultimate 3000 Dionek UV detector.

The HPLC system was programmed to run with a mobile phase flux of 1.25mL/minute, in an isocratic elution method (where the concentration of the mobile phase is constant), in a three-stage sequence:

I. Column preparation: 8 minutes, using the elution phase solution
II. Sample elution: 13 minutes, using the elution phase solution. In this stage, the sample passed through the column and absorbance was then recorded at a 532nm wavelength.
III. Column washing: 9 minutes, using the washing phase solution. After this stage, the system was ready to restart the cycle for a new sample.

The HPLC was programmed to repeat this cycle for all samples (and standards and blank), producing chromatographs of each sample. To quantify the MDA concentration, the area under the curve of each chromatograph was measured, and the results of the unknown samples were plotted on a calibration curve created from the results of the MDA standards.

3.2.4.3. Enzyme-linked immunosorbent assay

Background

Enzyme-linked immunosorbent assay [ELISA] is a commonly used assay to analyze quantitatively or qualitatively the presence of specific antigens. Although six variants exist, the sandwich ELISA is the most sensitive and is frequently used for the detection of large molecules. In this noncompetitive assay, two antibodies that recognize different epitopes on the same target are used to capture a specific antigen present in a sample, creating the sandwich. To reveal the presence of the antibody-antigen sandwich, a third, enzyme-conjugated antibody is used; the enzyme reacts with added substrates, producing colored products which can be measured. As these successive reactions can only occur if the sample contains the specific

antigen, the lack of color indicates the absence of the antigen (Twyman, 2019).

Reagents

- All Reagents were provided in the ELISA kits and were diluted as described in the manufactures' kit manual.

Samples

- Stored plasma samples were brought to room temperature and diluted as directed in the manuals.

Procedure and Calculations

Before the procedure, all reagents and samples were brought to room temperature. All samples and standards were analyzed in duplicate. The assays were conducted as described by the manufacturers of the Kits:

ApoE4 ELISA

First, 100µL of each standard and sample were added to the appropriate wells of the provided plate, which was then incubated for 1.5 hours at 37°C. Next, the solution was discarded and the plate was washed 3 times with 1x washing buffer A, using *Tecan Hydroflex* microplate washer. After washing, 100µl of 1x Detection Antibody solution were added to each well and the plate was incubated for 1 hour at 37°C. After incubation, the plate was washed 3 times with 1x Wash Buffer B. Then, 100µl of 1x HRP Conjugate solution were added per well and the plate was incubated for 1 hour at 37°C. Afterwards, the solution was discarded and the plate washed 4 times with 1x Wash Buffer B. Next, 100µl of TMB Substrate were added to each well, the plate was gently shaken and absorbance at 650nm was measured each 2

minutes at room temperature. Once the absorbance at 650nm was 0.5, 100µl of Stop Solution were added to each well and the plate was gently shaken. Final absorbance was read at 450nm.

We calculated apoE4 concentration and *APOE4* genotype using the method described in the manufactures' kit manual: First, the mean absorbance for each set of duplicates was calculated. Then, results of the apoE4 standards were plotted in a calibration curve, and apoE4 concentration of each sample in the wells was calculated by interpolation of the standard curve. The result was multiplied by 400 (dilution factor of the samples) to calculate the concentration of apoE4 in the sample. Results were represented in µg/mL and genotypes were defined according to ApoE4 concentration: non-carriers presented a concentration around 0µg/mL, heterozygotes around 50µg/mL and homozygotes around 100µg/mL.

IL-1β and TNF-α ELISA

First, 50µL of all samples and standards were pipetted into the appropriate wells. Then, 50µL of the Antibody Cocktail were added to each well, the plate was sealed and incubated at room temperature on a plate shaker. The incubation time was 1 hour for TNF-α and 2 hours for IL-1β. After incubation, wells were washed 3 times with 350µL of 1x Wash Buffer. Then, 100µL of TMB Development Solution were added to each well and the plate was incubated for 10 minutes, in the dark, on a plate shaker. Lastly, 100µL of Stop Solution were added to each well, the plate was shaken for 1 minute and absorbance was recorded at 450nm.

Concentrations were calculated by interpolation of the mean absorbance for each set of duplicates into a calibration curve plotted with the standards. If

the samples were diluted, results were multiplied by the dilution factor of the samples.

3.2.4.4. Flow Cytometry

Background

Flow cytometry can measure and analyze multiple physical characteristics of cells. In the flow cytometer, cells are carried in a fluid stream through a laser intercept; as they pass, cells deflect the incident laser light, causing light scattering. Forward scattering has an intensity proportional to the cell's size, and side scattering has an intensity proportional to the complexity of the cell. Their correlated measurements can be used to differentiate cell types.

Fluorescence emission can also be measured and most cytometry experiments use fluorescent dyes to identify intracellular or surface molecules. Once a marked cell passes through the light beam, the compound is excited and emits fluorescence over a range of wavelengths that are characteristic for that compound. The amount of fluorescent signal detected is proportional to the number of fluorochrome molecules (and, therefore, of particles) present in the cell. Since cells pass the laser beam one at a time, data are collected on the characteristics of each cell.

Our study used double staining with Annexin V and propidium iodide [PI] to characterize cell death. Annexin is a non-permeable protein that has a high affinity for phosphatidylserine, a phospholipid that is externalized to the surface of plasma membrane early after apoptosis onset. Conversely, PI is a nucleic acid dye that can only enter cells with disrupted membrane integrity.

Once inside the cell, it proceeds to bind to DNA, which produces a 20- to 30-fold increase in its fluorescence.

To measured intracellular reactive species production, we used H_2DCFDA and dihydroethidium [DHE]. H_2DCFDA is a cell permeant probe which is retained in the intracellular space, where it is oxidized and converted into dichlorofluorescein [DCF] by a wide array of oxidants, but especially by hydrogen peroxide (Gomes, Fernandes, & Lima, 2005). DHE is a widely used probe for detection of intracellular superoxide. Even though different compounds can oxidize DHE to form ethidium, superoxide further reacts with ethidium producing hydroxylated ethidium, which has a distinct excitation wavelength. Therefore, intracellular superoxide can be quantified by analyzing hydroxylated ethidium fluorescence using its specific excitation and emission wavelengths (Cho & Hwang, 2011). As we aimed to analyze live cells, dead cells were excluded in both experiments by double staining cells with 4', 6-diamidino-2-phenylindole [DAPI], a membrane impermeant nucleic acid stain, which can only enter cells with a disrupted membrane.

Procedure

Blood samples were collected and processed as described above (Lymphocyte culture). Sample aliquots were separated and processed according to the probe used.

Annexin V and Propidium Iodide

Samples were diluted with 95µl of 1x Annexin Binding Buffer (a mixture of HEPES/NaOH, NaCl and $CaCl_2$) and stained with 5µl of Annexin and 0.25µl of PI. After a 15-minute incubation at room temperature, 300µl of Biding Buffer were added to the sample and cell staining was analyzed. Results are shown

as relative percentage of cells according to their staining: early cell death - PI negative and Annexin positive; late cell death - PI and Annexin positive; Total Cell death - early cell death + late cell death.

Dichlorofluorescein

Samples were mixed with 0.625µl of H_2DCFDA and 0.2µl of DAPI and the solution was diluted with PBS to a total volume of 250µl. Samples were incubated for 20 minutes at 37°C. After incubation, samples were washed with PBS, by centrifuging the sample at 300G for 5 minutes, resuspending it in PBS and centrifuging again. After washing, cells were resuspended in 250µl of PBS and analyzed. Results are reported as mean fluorescence intensity arbitrary units [MFI AU] of DAPI negative cells. Longitudinal results are expressed as MFI AU relative to controls.

DHE

Samples were stained with 0.625µl of DHE and 0.2µl of DAPI. The solution was brought to a total volume of 250µl by adding PBS. Samples were incubated at 37ºC for 20 minutes, then were washed twice. After washing, samples were resuspended in PBS and fluorescence was measured. Results are reported as MFI AU of DAPI negative cells.

3.2.4.5. Lowry Protein Assay

Background

The Lowry protein assay (Lowry, Rosebrough, Farr, & Randall, 1951) is a simple and sensitive method to quantitatively measure total protein concentration. This assay relies on two reactions: the Biuret reaction and the

reduction of the Folin-Ciocalteu phenol reagent. In the first reaction, the peptide bonds of proteins react with copper ions under alkaline conditions, to form a protein-copper complex. The second step is the reduction of the Folin-Ciocalteu phenol reagent by the complex, producing an intense blue color, proportional to the sample's protein concentration, which can be measured by spectrophotometry (Noble & Bailey, 2009).

The sample's absorbance (i.e., the amount of light absorbed after it passes through the sample solution) measured by the spectrophotometer, is used calculate the protein concentration using the Beer-Lambert Law. According to this law, there is a linear relationship between the absorbance and the concentration of a sample, as expressed by the equation: $A = \varepsilon.l.c$

Where A is the absorbance, ε is the substance's molar extinction coefficient, l is the path length (i.e., the cuvette length), and c is the concentration in the cuvette.

In the case of proteins, by concomitantly analyzing standards with known concentrations of proteins, under the same conditions, a calibration curve can be plotted. As the molar extinction coefficient and the cuvette's length are the same, the concentration of unknown samples can be calculated by simple interpolation of their absorbance into the curve.

Reagents

- **Lowry reagent** - It was prepared by thoroughly dissolving the provided powder (L3540, Sigma) in 40mL of Milli-Q water, after wrapping the bottle in foil paper.
- **Folin-Ciocalteu's phenol reagent (22.5%)** - It was obtained by diluting 18mL of Folin reagent in 90mL of Milli-Q water.

- **Protein standards (0.195 – 50mg/mL)** – First, a 100mg/mL bovine serum albumin [BSA] stock solution was prepared by diluting BSA in Milli-Q water. Then, a serial dilution was performed by adding 50µL of Milli-Q water to 50µL of a previous BSA solution, to create the standards: 50; 25; 12.5; 6.25; 3.125; 1.56; 0.78; 0.39; and 0.195mg/mL.
- **Samples** – Samples were prepared as described below (Western Blot sample processing).

Procedure

To create a calibration curve, a blank analysis was performed by using only Milli-Q water which was processed the same way as the samples and standards. The assay was conducted protected from light. First, 5µL of sample (or standard or blank) were diluted in 495µL of Milli-Q water. Then, 500µL of the Lowry reagent were added to the tube, mixed gently, and the solution was incubated for 20 minutes at room temperature, in the dark. Next, 250µL of the Folin-Ciocalteu's phenol reagent were added, followed by a 30-minutes incubation at room temperature in the dark. Lastly, absorbance was measured at 660nm and each sample's protein concentration was calculated by interpolation.

3.2.4.6. Western Blotting

Background

Western blotting [WB], also called immunoblotting, is a widely used method for protein detection. It is based on the indirect detection of proteins immobilized on a nitrocellulose or polyvinylidene fluoride membrane, and it

relies on the specificity of the antigen-antibody interaction (Towbin, Staehelin, & Gordon, 1979).

It is executed in sequential steps: First, samples are treated in order to extract proteins from the cells. Then, proteins are denatured, given a negative charge, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS-PAGE]. This method uses an electric field and a porous acrylamide-based matrix to separate denatured proteins by their molecular weight. After proteins are electrophoretically resolved, they are transferred from the gel into a membrane, where they remain immobilized. This is done by applying an electric field perpendicular to the gel containing the separated proteins, which will carry molecules from the gel towards the membrane. Lastly, immobilized proteins can be detected by the sequential use of two antibodies: a primary antibody specific to the protein of interest and a secondary conjugated antibody directed against the primary antibody. Secondary antibodies are most commonly conjugated with horseradish peroxidase, an enzyme that reacts with substrates containing luminol, producing an oxidation reaction that generates a light signal. This light emission can be detected by an imager equipment and, as the signal intensity is proportional to the amount of protein on the membrane, protein can be quantitated using analysis software (Mishra, Tiwari, & Gomes, 2017).

Reagents

- **Resolving gel (12.5%)**: This gel was made by adding 2.5mL of 1.5M Tris-HCl (pH 8.8), 3.13mL of acrylamide and 0.1mL of 10% SDS to 4.27mL of distilled water. Then, 30µL of 10% APS and 15µL of TEMED were added to the solution immediately before casting the gel.

- **Stacking gel solution**: This gel was made by mixing 3.075mL of distilled water, 1.25mL of 1M Tris-HCl (pH 6.8), 0.625mL of acrylamide, 50µL of 10% SDS, 15µL of 10% APS and 7.5µL of TEMED. APS and TEMED were added to the solution immediately before casting the gel.
- **TBS 10x:** This solution was obtained by dissolving 80g of NaCl and 24.2g of Tris (Tris(hydroxymethyl)aminomethane) in 1 liter of distilled water. The solution's pH was adjusted to 7.6 using NaOH or HCL solution.
- **TBS-t:** It was made by adding 100mL of the 10x TBS solution to 900mL of distilled water, then adding 1mL of Tween 20 to the solution.
- **Running buffer [0.25M Tris, 1.9M Glycine, 1% (w/v) SDS]:** It was done by first dissolving 30g of Tris, 144g of Glycine and 10g of SDS in 1 liter of distilled water and mixing thoroughly. Then, 100mL of this solution were mixed with 900ml of distilled water.
- **Transfer Buffer [25mM Tris, 192mM Glycine, 20% (v/v) methanol]:** First, 14.4g of Glycine and 3.03g of Tris base were dissolved in 800mL of distilled water and the solution was cooled at 4ºC. Immediately before use, 200mL of methanol were added to the solution and mixed manually.
- **Tris-HCl [1M], pH 6.8:** Obtained by dissolving 60.55g of Tris in 500mL of distilled water, and adjusting pH to 6.8 with HCl solution.
- **Tris HCl [1.5M], pH 8.8:** Obtained by dissolving 90.83g of Tris in 500mL of distilled water and adjusting pH to 8.8 with HCl solution.
- **Lysis Buffer:** First, 0.927g of Tris were dissolved in 100mL of distilled water, and pH was adjusted to 6.7. Then, 10mL of glycerol and 2g of SDS were added to 87mL of the Tris solution.

- **Lysis Buffer working solution**: 10.6µL of sodium orthovanadate and 10.6µL of protease inhibitor solutions were dissolved in 1mL of Lysis Buffer.
- **Sample Loading Buffer, 2x [50mM Tris-HCl pH 6.8; 3% SDS; 10% glycerol; 0,005% bromophenol blue; 5% 2-mercaptoethanol]:** This solution was prepared by mixing 0.6mL of Tris-HCl (pH 6.8), 1.5mL of 20% SDS, 1mL of glycerol, and 50µL of 1% bromophenol blue. The resulting solution was diluted in 9.6mL of distilled water. Finally, 500µL of 2-mercaptoethanol were added to the solution.
- **5% BSA Blocking solution** - 5g of BSA were dissolved in 100mL of TBS-t, to obtain 5% BSA.
- **50% Isopropanol** – 20mL of 100% Isopropanol were diluted in 20mL of distilled water to obtain 40mL of 50% Isopropanol.
- **Primary Antibodies -** All antibodies were diluted according to manufacturer's information, as described in table 1 below.
- **Secondary antibodies** - Secondary antibodies were diluted in BSA 5% (Anti-mouse: 1:10,000 dilution; Anti-rabbit: 1:3,000 dilution). The choice between anti-rabbit or anti-mouse was done according to the source of the primary antibody.

Table 1: Primary antibodies used for WB analysis.

Antibody	Molecular Weight (kDa)	Dilution	Diluted in	Source
Calcineurin A	59	1:1000	BSA 5%	Rabbit

RCAN1	28	1:10,000	BSA 5%	Rabbit
GSK3β	46	4:1000	BSA 5%	Mouse
p38	40	1:1000	BSA 5%	Rabbit
P-p38	43	1:1000	BSA 5%	Rabbit
p-Tau (Thr 231)	48	1:1000	BSA 5%	Rabbit
β-Actin	42	1:1000	BSA 5%	Mouse

Sample Processing

In our study, we used a combination of detergent-based lysis, which uses a detergent to solubilize cell membranes, and ultrasonication, which uses high-frequency sound waves to disrupt cells' membranes.

First, lymphocyte pellets were defrosted and resuspended in 40µL of lysis buffer working solution. Immediately after resuspension, samples were sonicated using sound waves at a frequency of 20kHz via a probe inserted in the sample. Each sample was sonicated twice for 20 seconds. If viscosity persisted, the sample was cooled on ice, to avoid protein damage by overheating, and a third 10-second sonication was performed. The probe was cleaned with 70% ethanol followed by distilled water after each sample, to avoid cross-contamination. After sonication, samples were kept on ice for 5 minutes and, then were centrifuged at 10,000G for 20 minutes. Supernatant was separated, and protein concentration on samples were quantified using the Lowry method described above. After quantification, samples were stored at -20°C until use.

Western Blot Procedure

First, 12.5% SDS-PAGE gels were cast with the volumes and reagents described above. Then, processed samples were prepared for loading by diluting a sample aliquot which contained 15µL/mL of proteins with 2x Loading Buffer in a 1:1 ratio, followed by heating the samples at 95ºC for 10 minutes. Once samples were prepared, gels were placed in an electrophoresis tank filled with running buffer and samples were loaded. After assembling the remaining electrophoresis module, power was set to 100V constant and electrophoresis was run until the bromophenol blue front reached the end of the gel, which took approximately 90 minutes.

Once electrophoresis was completed, the gel was carefully removed and prepared for transfer by creating a gel "sandwich" that was set up in the following order, starting on the black side of the transfer gel holder cassette: Foam pad, filter paper, polyacrylamide gel, nitrocellulose membrane, filter paper and foam pad. After closing, the cassette was put in the transfer tank, which was then filled with transfer buffer and a cooling unit. The whole system was kept on ice throughout the procedure. Power was set up to maintain transfer at 240mA constant (120mA for each gel) for 90 minutes. The procedure was observed, so its voltage didn't surpass 100V, in which case the amperage would be lowered.

After the transfer, the system was disassembled and the membrane was stained with Ponceau red. Once the presence of the proteins was confirmed, the stain was washed off and the membrane could be probed. It started with the incubation of the membrane with 5% BSA blocking solution for 1 hour, at room temperature, with mild shaking. After incubation, the blocking solution

was discarded and a primary antibody was added, which was maintained over-night at 4ºC, with mild shaking.

On the next day, the primary antibody was removed and the membrane was washed 3 times with TBS-t. Next, the membrane was incubated with a secondary, conjugate antibody, for one hour at room temperature with mild shaking. After incubation, the membrane was washed and detection was done by uniformly adding 1mL of luminol to the membrane and detecting light signal using ImageQuant™ LAS 4000 mini biomolecular imager, GE Healthcare Bio-Sciences.

After imaging, the membrane was reprobed with an anti-β-actin antibody, which was used as a loading control. This was done by incubating the membrane with a stripping solution for 10 minutes at room temperature with gentle shaking; this eliminated the antibodies bound to the membrane, but maintained the immobilized proteins. This solution was then removed and the membrane washed, after which it is ready to be probed again using the same steps previously described.

Analysis

Once the western blot procedure was completed, images were analyzed with *ImageGauge V4.0* software, which permitted quantification of bands present in the membrane. This was done for both the protein of interest and for β-actin. In both cases, background intensity was also quantified. The result for each sample could then be calculated as: (Protein signal - protein background)/ (β-actin signal - β-actin background).

3.2.4.7. Polymerase Chain Reaction

Background

The polymerase chain reaction [PCR] (Mullis et al., 1986), is a technique used for exponential amplification of specific DNA sequences. It consists on repetitive cycles of denaturation, hybridization (annealing), and polymerase extension of the sample's DNA under thermal cycling.

Since it was first described, many variations have been described. Reverse transcription PCR [RT-PCR] is used to amplify RNA targets and generate complementary DNA [cDNA] from messenger RNA [mRNA]; the produced cDNA is more stable and can be used for PCR. Quantitative PCR [qPCR], also called real-time PCR, is a modification that allows PCR to be used as a quantitative analytical tool. It uses fluorescence to detect PCR products as they accumulate during the reaction. Our study used fluorogenic probes (TaqMan® Gene Expression Assay), which are sequence-specific oligonucleotides covalently joined to two other molecules, a fluorescent reporter and a quencher. When the probe is intact, both molecules are in close proximity and the probe cannot emit a fluorescent signal when light hits it. However, when DNA polymerase digests a probe bound to the DNA, reporter and quencher are separated, which allows the reporter to produce a fluorescent signal that the equipment can detect. Each time a new PCR product is generated fluorescence increases, which is read and recorded by the real-time instrument after each cycle. The fluorescence increase is proportional to product, and its rate of accumulation indicates how many target DNAs were present in the sample. Thus, the original gene target quantity can be deduced mathematically.

Reagents

- **Master mix solution:** This solution is prepared by mixing RT buffer, nucleotides, random primers, reverse transcriptase and RNase inhibitor as indicated in table 2 below.
- **Master mix Solution without reverse transcriptase [RT]:** This solution is prepared as described in table 2 below.
- **PCR reaction mix:** A solution containing Master Mix, nuclease-free water and Gene Assay was prepared. To a total volume of 8µL per reaction, 5µL of Master Mix, 0.5µL of the Gene assay and 2.5µL of Nuclease-Free water were mixed. The specified volumes are for each reaction and the total volume of each component is established by the total amount of reactions needed. PCR reaction mixes were prepared for each gene assayed.

Table 2: Volumes of components of the master mix solutions for RT-PCR. Volumes indicated are for each reaction and the total volume should be calculated for the total procedure.

Component	Volume/Reaction (µL)	
	Kit with RT	Kit without RT
10× RT Buffer	2.0	2.0
25× dNTP Mix (100 mM)	0.8	0.8
10× RT Random Primers	2.0	2.0
MultiScribe Reverse Transcriptase	1.0	—
RNase Inhibitor	1.0	1.0
Nuclease-free H_2O	3.2	4.2
Total per Reaction	10.0	10.0

Procedure

First, we used the NucleoSpin® TriPrep kit to extract RNA from lymphocyte pellets. The procedure was performed at room temperature, in sterile conditions, following the manufacture's protocol. All reagents were provided in the kit and were diluted as described in the manufactures' kit manual. After extraction, RNA was resuspended in RNase-free H_2O and quantified

using NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Then, the volume of sample that contained 2µg of RNA was calculated and separated; if needed, RNase-free H_2O was added to the sample, so it reached a total volume of 10µL. Next, 10µL of the master mix solution were mixed with 10µL of sample. A No-Template Control [NTC] was prepared by adding 10µL of RNase-free H_2O (substituting the sample) to 10µL of the master mix solution; a No-Reverse Transcription [NRT] control was prepared by mixing 10µL of the master mix solution without reverse transcriptase with 10µL of a random sample that contained RNA.

With the solutions prepared, tubes were quickly spined using a mini centrifuge, and samples, NTC and NRT were put in the thermocycler to undergo reverse transcription. Thermal cycler conditions were set as described in the Master Mix solution manual. After reverse transcription, cDNA was obtained.

Once cDNA was obtained, qPCR was performed. First, we prepared the PCR reaction mix for each gene assayed (GLCL, GCLM, GPx1, GSK3β, PKR, Calcineurin A, RCAN1 and SOD1) and for the housekeeper molecule, GAPDH. Then each well of an optical reaction plate were filled with 8µL of PCR Reaction Mix and 2µL of cDNA template. For NTC reactions, 2µL of Nuclease-free water were added. Each sample was run in triplicate and NTC were included for each assay. Assays with GAPDH, a housekeeper molecule, were run on the same plate to be used as normalizer. The reaction plate was sealed and centrifuged briefly, and thermal protocol was set up as described on the TaqMan® Gene Expression Assay manual.

Calculations

Once the reaction finished, the resulting amplification plot was used to calculate the original RNA quantity. Our study used the $2^{-\Delta Ct}$ method, where ΔCt refers to Ct values (threshold cycle number) normalized to GAPDH gene assays that were run in the same plate.

The fold-change in gene expression between 2019 and 2008 was calculated using the following expression as describe by Schmittgen & Livak (Schmittgen & Livak, 2008):

$$Fold\ Change = \frac{-1}{2019\ \text{mean expression}/2008\ \text{mean expression}}.$$

Longitudinal changes in gene expression were considered significant if fold-change ≥1.5 and p-value ≤ 0.05 (Dalman, Deeter, Nimishakavi, & Duan, 2012).

3.2.5. Statistical Analysis

Our study used the *IBM SPSS Statistics 22* software for statistical analysis. All variables were subjected to normality tests, specifically the Kolmogorov-Smirnov test with the Lilliefors correction, prior to statistical hypothesis testing. We used different statistical tests to analyze cross-sectional and longitudinal data.

For cross-sectional analysis, nominal and ordinal variables were analyzed with Pearson's Chi-square test. Parametrical variables were analyzed using the Student's *t*-test and Analysis Of Variance (ANOVA), for 2 variables and 3 or more variables, respectively. Conversely, 2 non-parametric variables were analyzed with Mann–Whitney *U* test, while 3 or more non-parametric scale variables were analyzed with Kruskal–Wallis *H* test.

For longitudinal analysis, paired nominal data were analyzed with McNemar's test. Parametric related variables were analyzed with paired Student's *t*-test and non-parametric related variables were analyzed with Wilcoxon signed-rank test. In all tests, a result was considered statistically significant when the p-value was lower than 0.05.

RESULTS

4 Results

4.1 CROSS-SECTIONAL

4.1.1 Sample description

Our sample consisted of a total of 47 middle-aged subjects, as described in table 3. As we can see in the table, women formed almost 60% of our sample. Although women were slightly younger than men (mean age 50.0 ± 8.0 and 53.8 ± 7.4, respectively), there was no statistical difference in age between both sexes (p=0.99). Furthermore, men and women did not differ in educational level (Table 3).

Table 3: Whole sample description. Age represented as the mean ± standard deviation. Values specify the number (N) and corresponding percentage (%) of subjects in each group.

Total	47		
Age	51.6 ± 7.9 (Range 35-65)		
		N	**%**
Genotype	*3/3*	15	31.9
	3/4	19	40.4
	4/4	13	27.7
APOE	*APOE3*	15	31.9
	APOE4	32	68.1
Education	*Primary*	10	21.3
	Secondary	14	29.8

	Tertiary	23	48.9
Sex	*men*	19	40.4
	women	28	59.6

Our study considered individuals who were 50 years or older as older adults, and individuals who were less than 50 years as younger adults. According to this classification, most of our sample was formed by older adults (61.7%; N=29). However, younger and older adults did not differ in sex nor educational attainment.

The presence of an *APOE4* allele can influence many physiological aspects and increase the risk of developing many disorders. Therefore, we divided our sample into carriers and non-carriers, according to *APOE4* status. Subjects that did not have the allele were considered as non-carriers and comprised the control group. Subjects that had at least one *ε4* allele, that is, heterozygous and homozygous carriers, were considered as *APOE4* carriers. Carriers (N=32) had a mean age of 51.59 ± 8.2, most had tertiary studies (56.3%) and were women (56.3%).

We also considered that allele dosage has an important impact on the risk of developing AD, and created a second set of groups according to genotype: the control group (N=15), the heterozygous group (N=19) and the homozygous group (N=13). Our study used the ELISA method to identify and quantify the presence of the *APOE4* allele. Therefore, it did not specify the genotype of controls and heterozygous subjects. However, as the frequency of the *APOE2* allele is very low, we chose to consider our groups as 3/3 (controls), 3/4 (heterozygous) and 4/4 (homozygous) genotypes. Table 4

shows the demographics of our subjects according to their genotype. There was no difference of sex or age between the 3 groups.

Table 4 also shows the level of education of each group. There was no difference in educational attainment between the three genotypes. However, as we can see in the table, around 30% of controls had tertiary education, while this percentage was over 50% in heterozygous and homozygous subjects. Even though this was not significant, we considered that education could affect cognitive performance and chose to correct RAVLT and Stroop results by education in our cross-sectional analysis (as described in the methods section).

Table 4: Sample description according to genotype. Age represented as the mean ± standard deviation. Values specify the number (N) and corresponding percentage (%) of subjects in each group.

Genotype		3/3 (N=15)		3/4 (N=19)		4/4 (N=13)	
Age (Mean)		51.5 ± 7.4		50.5 ± 8.3		53.2 ± 8.2	
		N	**%**	**N**	**%**	**N**	**%**
Education	*Primary*	3	20.0	3	15.8	4	30.8
	Secondary	7	46.7	6	31.6	1	7.7
	Tertiary	5	33.3	10	52.6	8	61.5
Sex	*men*	5	33.3	7	36.8	7	53.8
	women	10	66.7	12	63.2	6	46.2

4.1.2 Clinical assessment

Our current study included subjects that presented chronic disorders, such as diabetes mellitus and hypertension. However, we did not perform clinical or laboratory tests to evaluate them, as it was not our intention to diagnose these diseases. Consequently, any prevalence here indicated reflects our subjects' answers and their knowledge of having a disease.

Common chronic diseases, such as hypertension, dyslipidemia, diabetes mellitus and metabolic syndrome were present in both carriers and non-carriers. About 25% of carriers and of non-carriers referred dyslipidemia that required medication. However, 56% of carriers reported having being told before that they had high blood lipid levels, while only 33% of non-carriers reported the same information. Contrariwise, the informed prevalence of hypertension and of anti-hypertensive drug use was similar for both carriers and non-carriers (about 25% and 20%, respectively). Lastly, metabolic syndrome with diabetes mellitus was reported by 3 *APOE4* carriers and 2 non-carriers, all of which were in use of oral hypoglycemic drugs.

Certain diseases were only present in *APOE4* carriers. Inflammation-related disorders, which included autoimmune hepatitis, HLA-B27-related uveitis, vitiligo and hypothyroidism, were reported by 2 heterozygous women and 3 homozygous women. Of these, 2 women presented autoimmune diseases and were in use of immunosuppressant drugs. Unfortunately, blood results of these two women could not be used in our analysis, as the mechanism of action of these drugs directly affects lymphocytes. Other reported disorders were benign prostatic hyperplasia, chronic pain, treated breast ductal carcinoma, obsessive-compulsive disorder, arrythmia and hyperuricemia.

Regarding smoking habit, 6 heterozygous carriers referred active smoking versus two controls. Most were long-time, light to moderate smokers (less

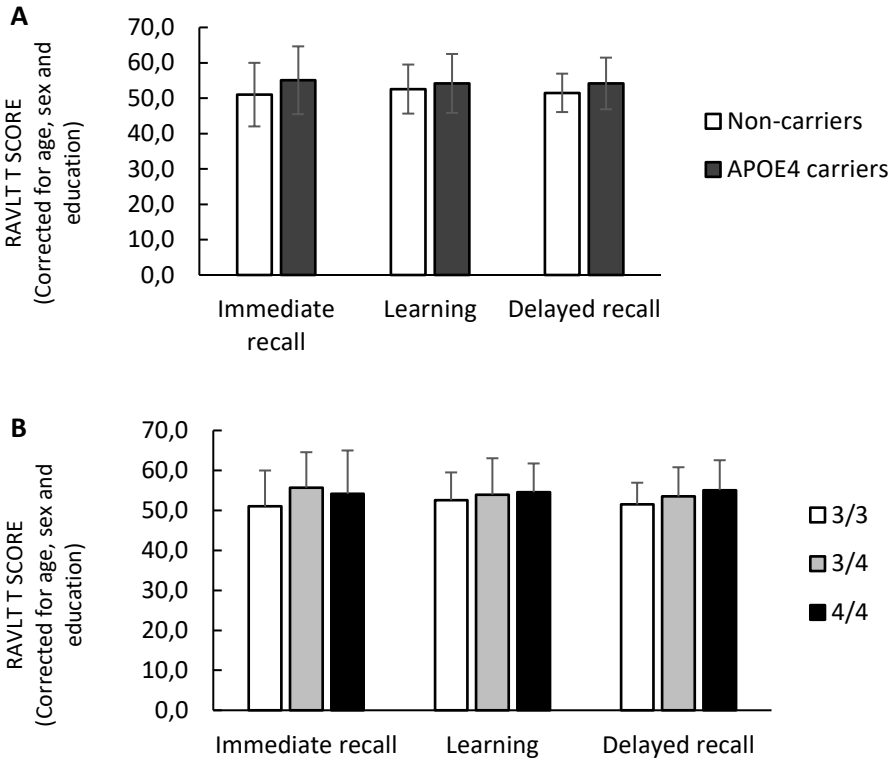
than 20 cigarettes per day, for more than 25 years), and 7 out of 8 smokers were women. Moreover, active smokers were older than non-smokers, and 7 out of the 8 active smokers were older than 50 years. Lastly, 13 *APOE4* carriers identified as former smokers, versus 8 controls.

4.1.3 Cognition and Depression

4.1.3.1 Memory

We analyzed objective memory using the immediate recall, learning and delayed recall scores of the RAVLT. Raw scores were corrected by age, education and sex, as described in the methods section. All subjects completed the test and were included in the analysis. No subject presented memory deficits and all subjects presented scores within the normal range. As expected, there was no difference between men and women, nor between subjects of all three educational levels.

With these results in mind, we compared RAVLT scores between carriers and non-carriers and between genotypes and did not find any difference (graphic 1). Lastly, we considered that both age and sex combine with the *APOE4* allele to increase the risk of developing AD. Although test scores were corrected for these factors, it was possible that this interaction could lead to a sex or age difference in RAVLT scores within *APOE4* carriers. However, we did not find any difference according to sex or age.



Graphic 1: RAVLT scores according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=32); B) 3/3 (N=15), 3/4 (N=19), 4/4 (N=13). Rey Auditory Verbal Learning Test (RAVLT) T Scores refer to scores corrected for age, sex and education; bars represent mean score ± standard deviation.

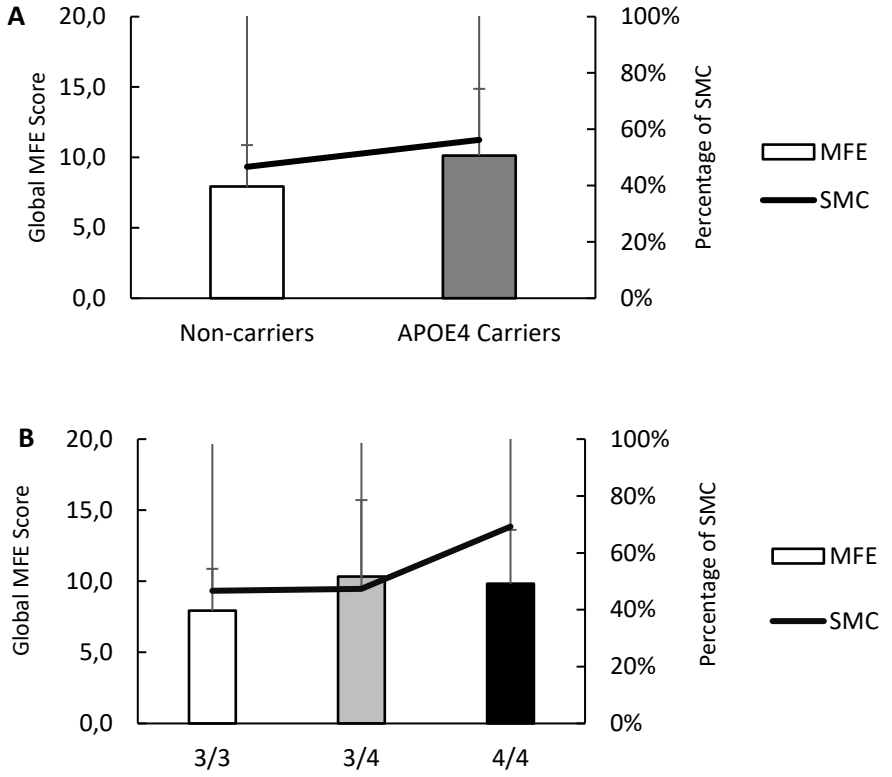
Our study analyzed SMC by asking all subjects the question "Do you think you currently have memory problems?", which was registered as a yes or no answer. Then, we used the MFE questionnaire to investigated what types of failures individuals considered memory problems. Of all the participants, 53.2% considered they had a memory problem, while the mean MFE score was 10.36 (SD 5.5; range 1-25). When considering the types of failures, we found that what most subjects considered as memory problems were, in fact,

attentional failures. This was demonstrated by the higher scores our subjects had in questions related to attention, such as “Forgetting where you have put something” and “Finding that a word is on the tip of your tongue”.

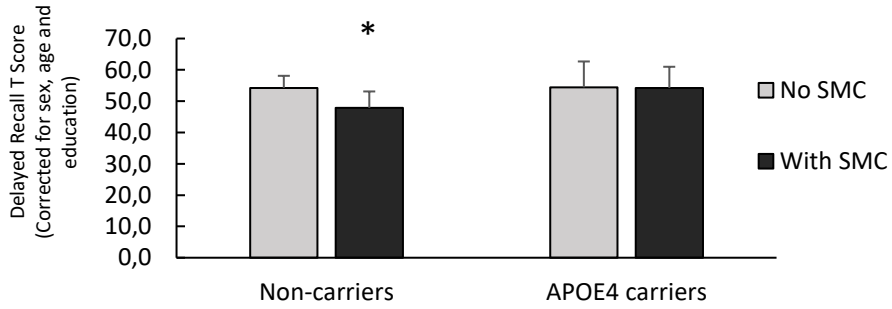
Age and sex had no significant influence on SMC and MFE. Other factors are known to influence memory and attention, such as depression. In our study, although individuals with depression had slightly higher MFE scores and SMC prevalence, it was not statistically significant.

Our study did not find any difference between carriers and non-carriers, even though carriers had a slightly higher global MFE (10.1 vs 7.9) and percentage of individuals referring SMC (56% vs 47%). We also did not find any difference between the genotypes. However, heterozygous and homozygous carriers have different views of their everyday memory. As we can see in graphic 2, homozygous carriers tend to refer more SMC than heterozygous carriers (69% vs 47%), even though they have similar MFE scores.

Lastly, we considered the relationship between subjective and objective memory. When studying all subjects, there was no relationship between RAVLT and SMC, nor between RAVLT and MFE. However, when considering individuals by *APOE4*-carriage, we found that controls that referred SMC did in fact have lower scores on RAVLT delayed recall than those that did not refer SMC. Conversely, *APOE4* carriers that referred SMC did not have lower RAVLT scores (Graphic 3). There was no interaction between *APOE4* and sex and between *APOE4* and age.



Graphic 2: Subjective memory according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=32); B) 3/3 (N=15), 3/4 (N=19), 4/4 (N=13). On the left vertical axis, bars indicate the mean Memory Failures of Everyday (MFE) score ± standard deviation. On the right vertical axis, the line represents the mean percentage of subjective memory complaints (SMC) ± standard deviation.

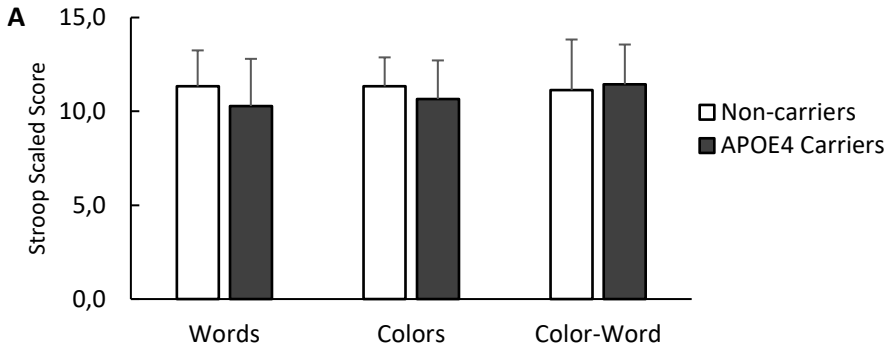


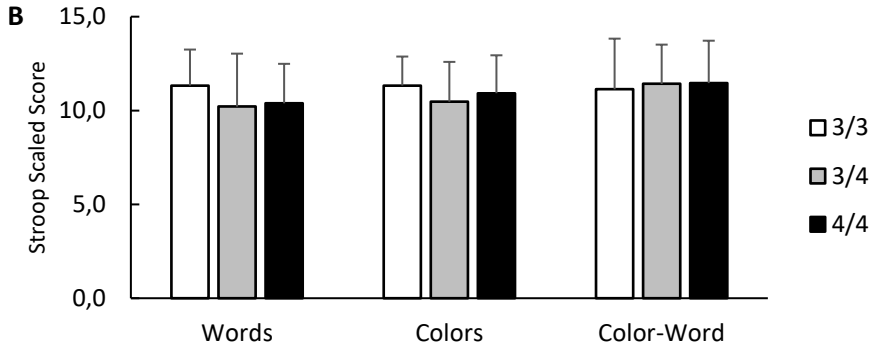
Graphic 3: Objective memory relative to subjective memory complaints (SMC) in controls and APOE4 carriers. On the left, controls (N=15) that referred SMC (dark gray bar; N=7) and those that did not refer SMC (light gray bar; N=8). On the right, APOE4 carriers (N=32) who referred SMC (dark gray bar, N=17) and those that did not refer SMC (light gray bar; N=14). Bars indicate the mean RAVLT delayed recall score T score ± standard deviation. *p < 0.05 for controls no SMC *versus* controls with SMC.

4.1.3.2 Attention

We analyzed selective attention using the Stroop test, which all subjects completed. Our study did not find any difference due to sex, age nor education. This was expected as sex does not influence test performance and we used scaled scores corrected by age and education.

Regarding *APOE4*, we did not find any significant difference between carriers and non-carriers, nor between the different genotypes (graphic 4). Furthermore, we did not find any influence of age and sex within *APOE4* carriers.





Graphic 4: Stroop scores according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=32); B) 3/3 (N=15), 3/4 (N=19), 4/4 (N=13)**.** Bars indicate the mean Stroop scaled score corrected for age and education ± standard deviation.

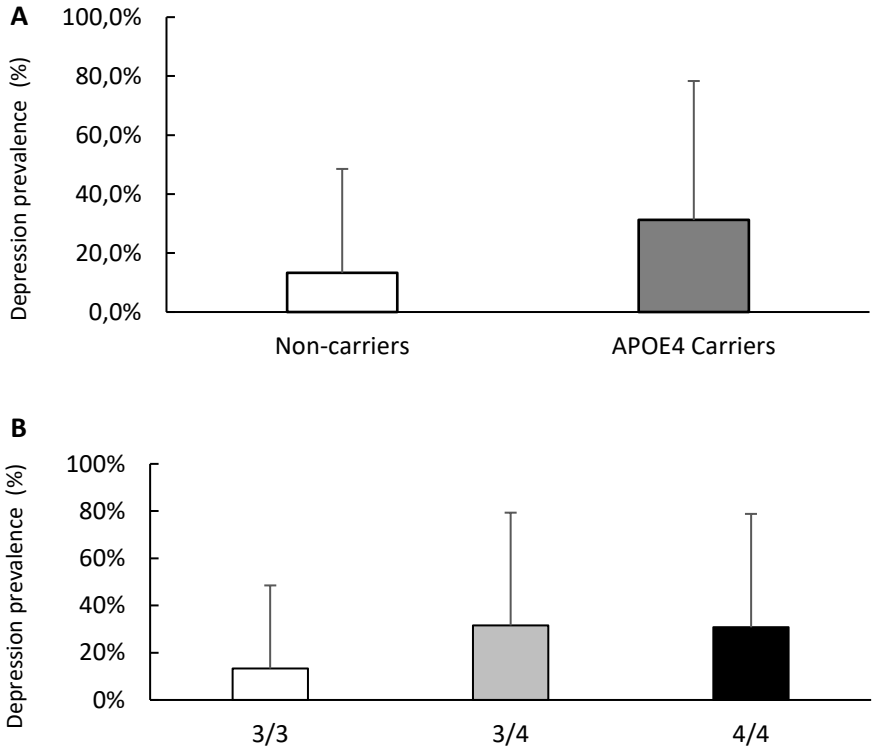
4.1.3.3 Depression

We chose to investigate the prevalence and severity of depression in our subjects using the Hamilton Depression Scale. We found a total prevalence of depression of 25.5% (N=12), which was significantly higher in women than in men. Depression occurred at all ages and had varying severity, although no subject presented severe depression.

Of notice is the fact that most individuals with clinical depression did not have any type of treatment; that is, only 4 out of 12 individuals with depression had sought psychiatric or psychological assistance and were currently under treatment. Moreover, of these 4 treated subjects, only 2 had reached remission characterized by a Hamilton score of 7.

Our study did not find a difference in prevalence (graphic 5) nor in severity between the three genotypes, although carrier groups tended to have a higher prevalence (around 30%) than controls (10.3%). However, it is

important to remark that depression in men and in younger individuals was only found amongst *APOE4* carriers.



Graphic 5: Depression prevalence according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=32); B) 3/3 (N=15), 3/4 (N=19), 4/4 (N=13)**.** Bars indicate the mean depression prevalence ± standard deviation.

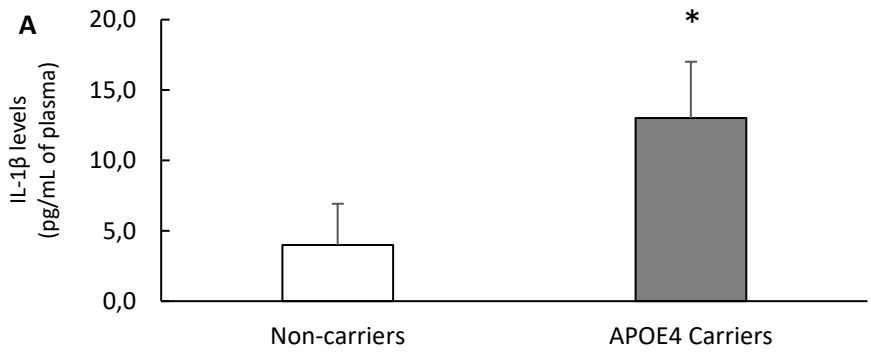
4.1.4 Analytical measures

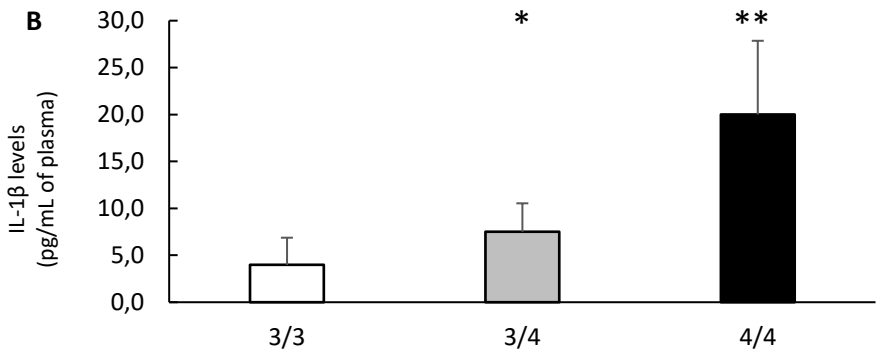
4.1.4.1 Pro-inflammatory Cytokines

IL-1β and TNF-α are important pro-inflammatory cytokines. Therefore, we investigated inflammation by measuring levels of both cytokines in plasma using sandwich ELISA kits.

Regarding TNF-α, it was not possible to analyze results as most individuals presented values that were lower than the detection limit, with only a few carriers presenting levels that were detectable.

Considering IL-1β, we did not find any difference according to sex nor age, between all subjects. This remained even when considering environmental risk factors such as smoking, diabetes and other inflammation-related diseases. However, when considering *APOE* genotype, we found an important increase in *APOE4* carriers in an allele dose-dependent manner (graphic 6); that is, heterozygous *APOE4* carriers presented higher levels compared to non-carriers, while homozygous carriers presented increased levels compared to both non-carriers and heterozygous carriers. There was no interaction between *APOE4* and sex or age.





Graphic 6: IL-1β plasma levels according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=14), *APOE4* carriers (N=25); B) 3/3 (N=14), 3/4 (N=14), 4/4 (N=11). Bars indicate the mean plasma IL-1β ± standard deviation. *p<0.05 for *APOE4* carriers *versus* non-carriers, for 3/4 *versus* 3/3, and for 4/4 *versus* 3/4 and 4/4 *versus* 3/3.

4.1.4.2 Oxidative stress parameters in blood

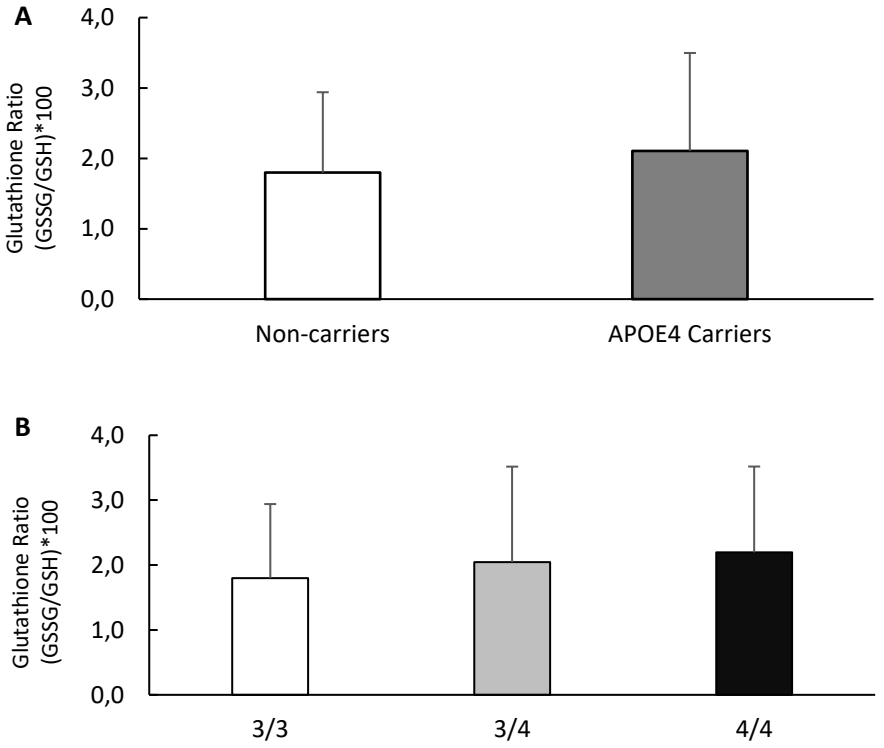
Glutathione

Glutathione is one of the most important molecules in the antioxidant defense system. Thus, we chose to initially analyze redox status by measuring GSSG and GSH levels, and glutathione and NADP oxidized-reduced ratios in whole blood.

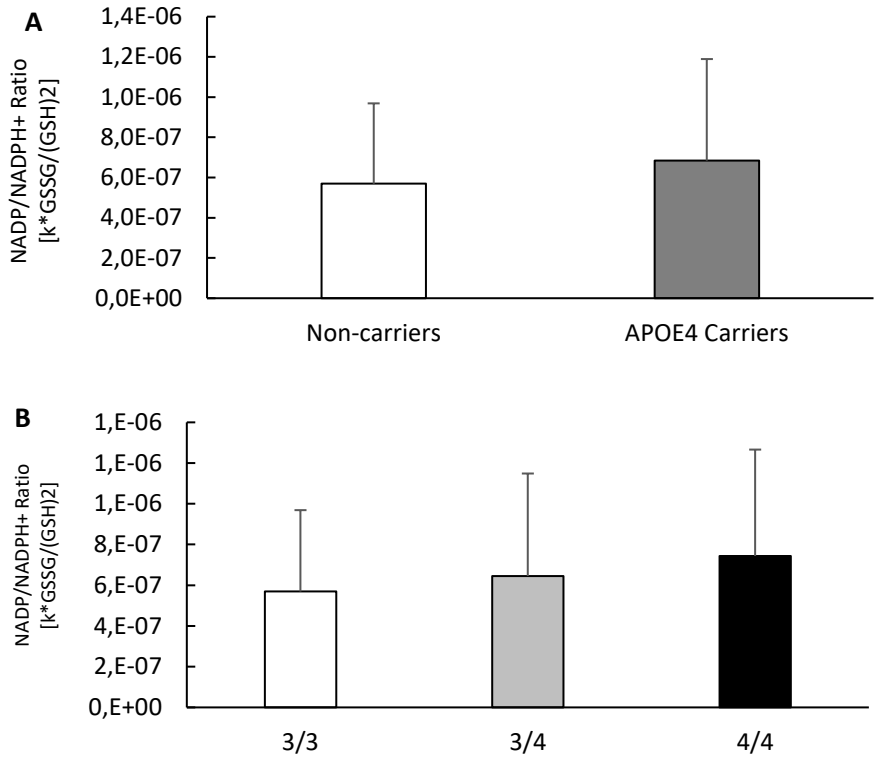
We first considered sex and age. While we did not find any difference between men and women, we found that older individuals tended to present higher oxidation levels than younger ones, although this did not reach statistical significance. This was not influenced by environmental risk factors.

Regarding *APOE4*, our study did not find any difference between carriers and non-carriers, even though *APOE4* carriers had slightly higher GSSG, GSSG/GSH ratio and NADP/$NADPH^+$ ratio (graphic 7A and 8A). There was also

no difference between the three genotypes (graphic 7B and 8B), not even when considering smoking and diabetes. Furthermore, we did not find any significant difference between both sexes nor between younger and older carriers.



Graphic 7: GSSG/GSH ratio according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=30); B) 3/3 (N=15), 3/4 (N=18), 4/4 (N=12). Bars indicate the mean GSSG/GSH ratio ± standard deviation.



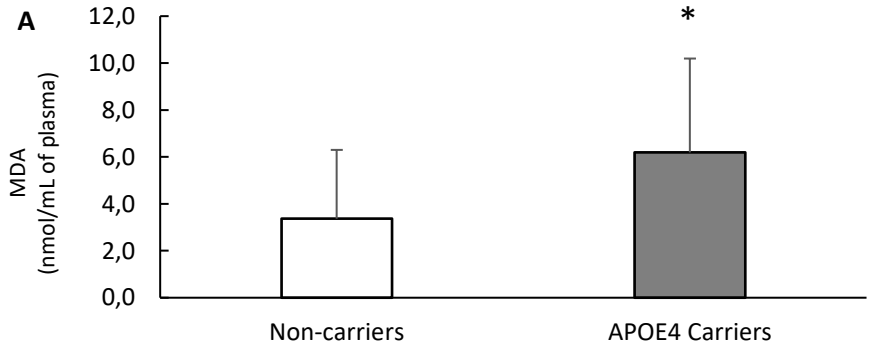
Graphic 8: NADP/NADPH^{+} ratio according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=30); B) 3/3 (N=15), 3/4 (N=18), 4/4 (N=12). Bars indicate the mean NADP/NADPH^{+} Ratio ± standard deviation.

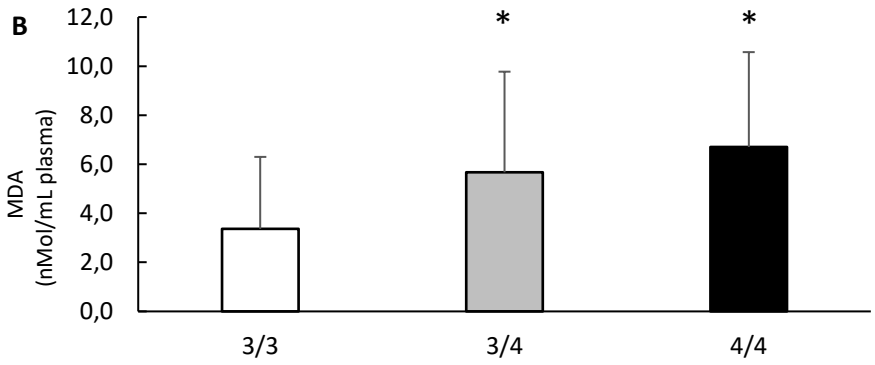
Malondialdehyde

We analyzed oxidative damage by measuring MDA plasma levels with HPLC. We did not find any difference according to sex or age, although younger subjects had slightly lower MDA levels than older subjects. As before, we also considered the possible influence of smoking and diabetes, but results remained the same.

Then, we investigated the influence of *APOE4* on MDA levels and found that carriers presented significantly higher levels of MDA when compared to non-carriers (graphic 9A). Furthermore, heterozygous and homozygous carriers had similar MDA levels, which were significantly higher than non-carriers' levels (graphic 9B). We also considered that environmental risk factors could influence MDA levels. When excluding diabetic individuals and smokers, we found that MDA levels actually increased in an allele dose-dependent manner, and only homozygous carriers presented significantly higher levels than non-carriers.

Lastly, we considered how sex and age could interact with *APOE4*. We did not find any difference between male and female carriers. However, we found that older *APOE4* carriers had higher MDA levels when compared to younger carriers, although it did not reach statistical significance (p=0.069).





Graphic 9: MDA plasma levels according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=15), *APOE4* carriers (N=29); B) 3/3 (N=15), 3/4 (N=17), 4/4 (N=12). Bars indicate the mean plasma MDA ± Standard Deviation. * p<0.05 for *APOE4* carriers *versus* non-carriers, for 3/4 *versus* 3/3, and for 4/4 *versus* 3/3.

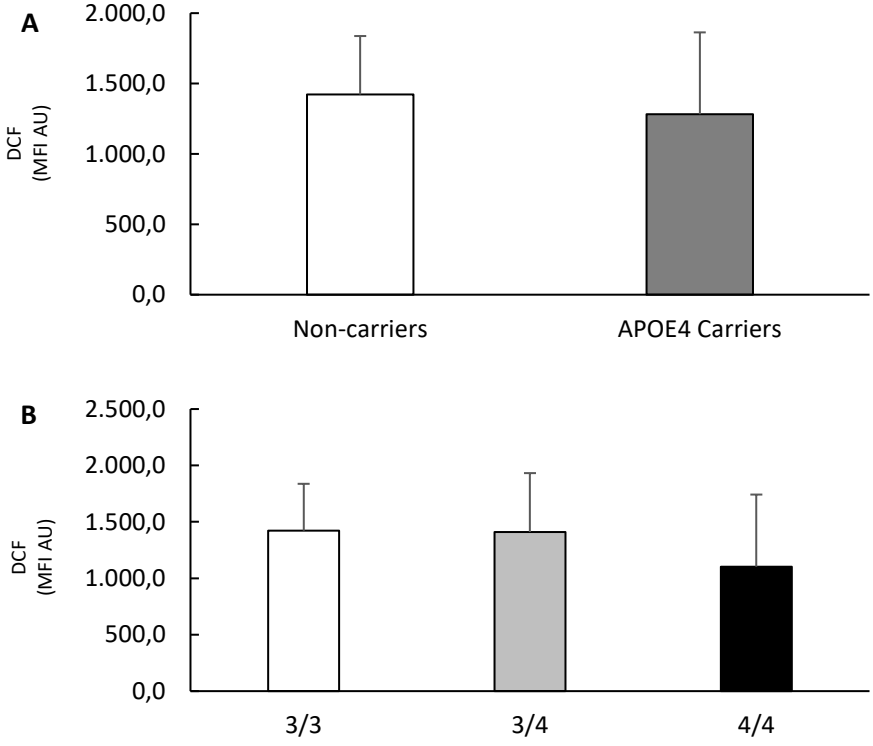
4.1.4.3 Oxygen reactive species

As we found that carriers presented increased oxidative damage, we decided to investigate if this was due to increased levels of reactive species. To that end, we analyzed intracellular reactive species using flow cytometry with DCF and DHE staining.

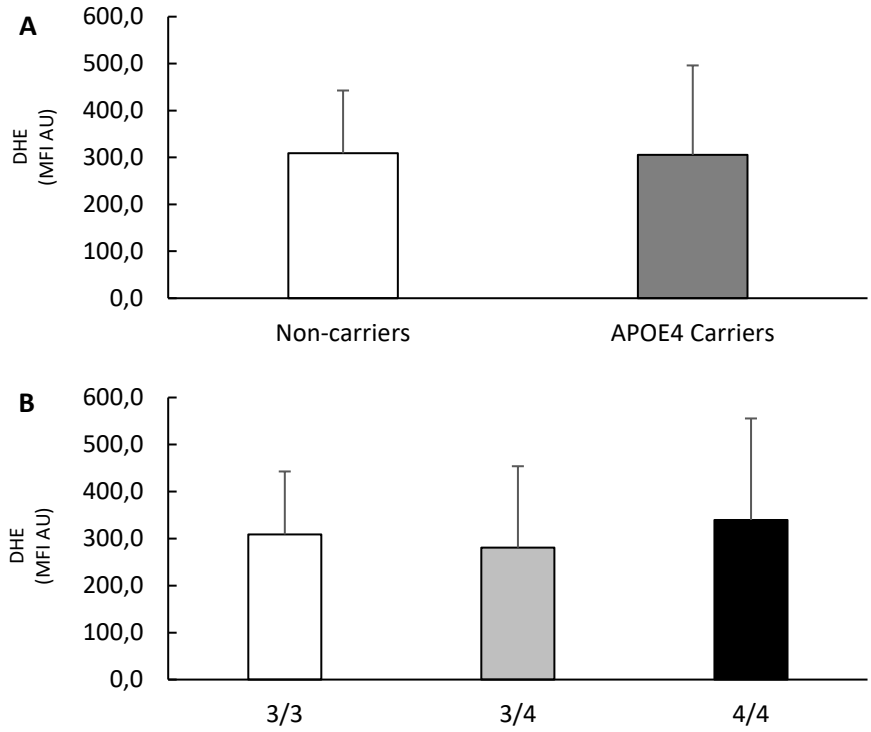
Regarding DCF, we did not find an influence of sex or age in DCF mean fluorescence. We also did not find any difference between carriers and non-carriers, nor even when considering genotypes (graphic 10). Although homozygous tended to have a slightly lower mean fluorescence when compared to controls and heterozygous carriers, this was not significant (p=0.305).

Considering DHE, as shown in graphic 11, there was no significant difference between carriers and non-carriers nor between the three *APOE* genotypes,

although homozygous carriers tended to have higher levels. Furthermore, there was no age-*APOE4* nor sex-*APOE4* interaction.



Graphic 10: DCF according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=16), *APOE4* carriers (N=24); B) 3/3 (N=6); 3/4 (N=14); 4/4 (N=10). Bars indicate the mean fluorescence intensity in arbitrary units (MFI AU) ± standard deviation.

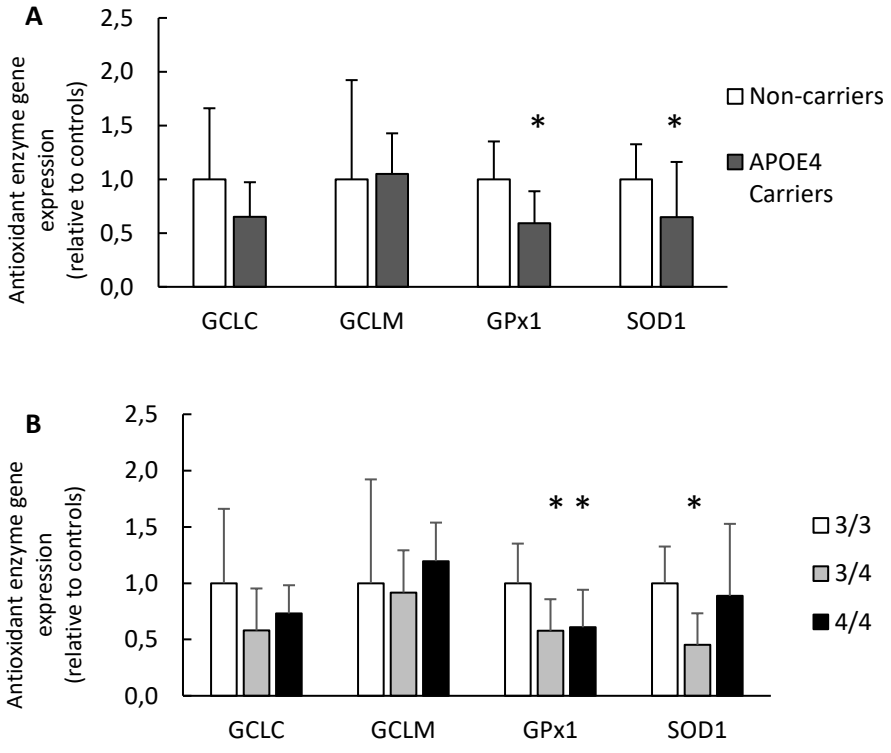


Graphic 11: DHE according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=8), *APOE4* carriers (N=26); B) 3/3 (N=8); 3/4 (N=15); 4/4 (N=11). Bars indicate the mean fluorescence intensity in arbitrary units (MFI AU) ± standard deviation.

4.1.4.4 Antioxidant enzymes

Oxidative stress involves an imbalance between oxidant species and antioxidant defense. Thus, antioxidant enzymes might be altered in our subjects. Therefore, we analyzed gene expression of enzymes involved in antioxidant defense with qPCR. In our sample, gene expression was similar between both sexes and ages. When considering *APOE* status, we found that *APOE4* carriers had significantly lower GPx1 and SOD1 gene expression than non-carriers, while there was no difference in the other enzymes'

expressions. As seen in graphic 12, we compared genotypes to evaluate if there was a gene-dose influence, which was not the case; while both heterozygous and homozygous carriers had significantly lower GPx1 expression when compared to non-carriers, only heterozygous carriers had significantly lower SOD1 expression when compared to non-carriers. There was no significant difference between both carrier groups. These results remained the same even when considering environmental risk factors, sex and age, although women carriers tend to have lower expression of both enzymes than men carriers.



Graphic 12: Gene expression of antioxidant enzymes according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=8), *APOE4* carriers (N=20); B)**.** 3/3 (N=8); 3/4 (N=11); 4/4 (N=9). Bars indicate the mean expression relative to

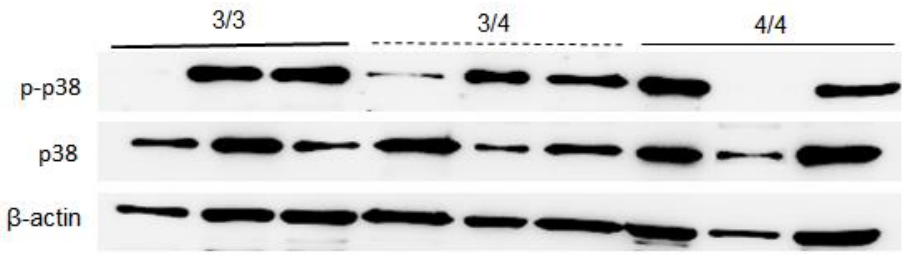
controls ± standard deviation. Expressions were normalized for GAPDH expression. GPx1 *p<0.05 for *APOE4* carriers *versus* non-carriers, 3/4 *versus* 3/3, and 4/4 *versus* 3/3; SOD1 *p<0.05 for *APOE4* carriers *versus* non-carriers, and 3/4 *versus* 3/3.

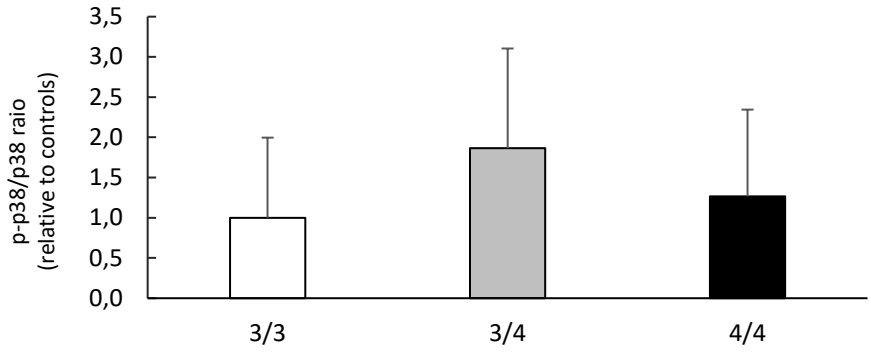
4.1.4.5 Stress-related proteins

Next, we sought to investigate if the increase in cytokine and the oxidative damage we found in *APOE4* carriers activated pathways related to cellular stress.

p-p38/p38 ratio

First, we analyzed the activation of the stress-related enzyme p38 by measuring the p-p38/p38 ratio by western blot. There was no difference between carriers and non-carriers, nor between the three genotypes, even though heterozygous carriers tended to have higher p-p38/p38 ratios than controls and homozygous carriers (graphic 13). This was influenced by sex, as women carriers of both genotypes presented higher ratios than men carriers and controls.

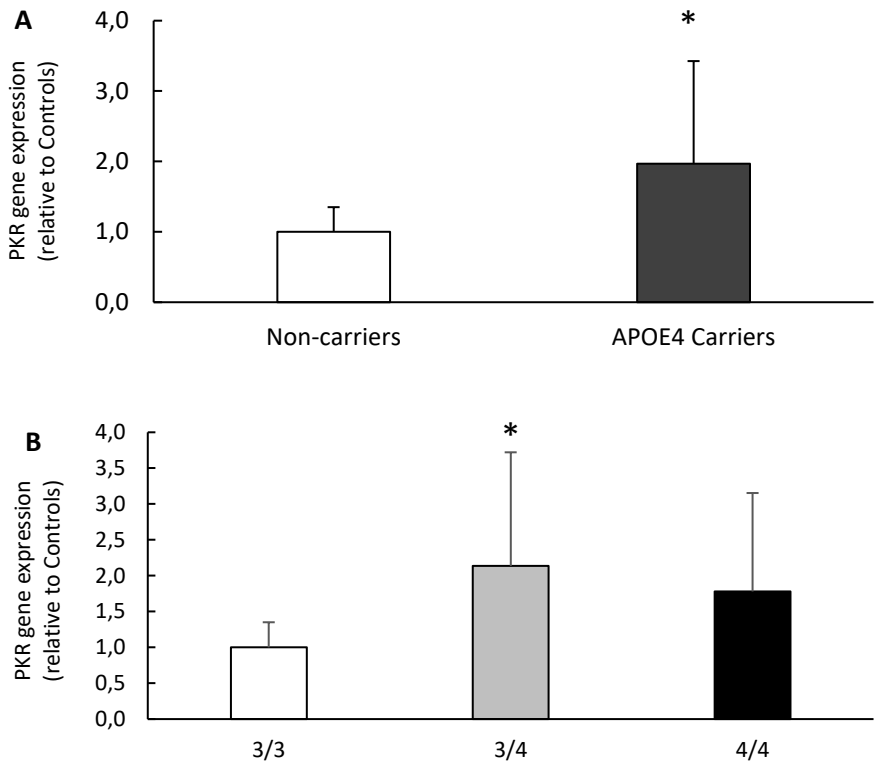




Graphic 13: p-p38/p38 ratio according to APOE genotype and representative WB. 3/3 (N=6); 3/4 (N=10); 4/4 (N=6). Bars indicate the mean ratio relative to controls ± standard deviation. P-p38 and p38 expressions were normalized for β-actin expression.

PKR

Next, we analyzed gene expression of PKR, another stress-related enzyme, with qPCR. *APOE4* carriers presented significantly higher PKR levels than non-carriers (graphic 14A). When separated by genotype, only heterozygous APOE4 carriers presented significantly higher levels than non-carriers, even though homozygous carriers presented a tendency to higher expression (p=0.135) (graphic 14B). PKR levels in *APOE4* carriers were influenced by sex, as women carriers had higher levels of PKR than men carriers and controls.

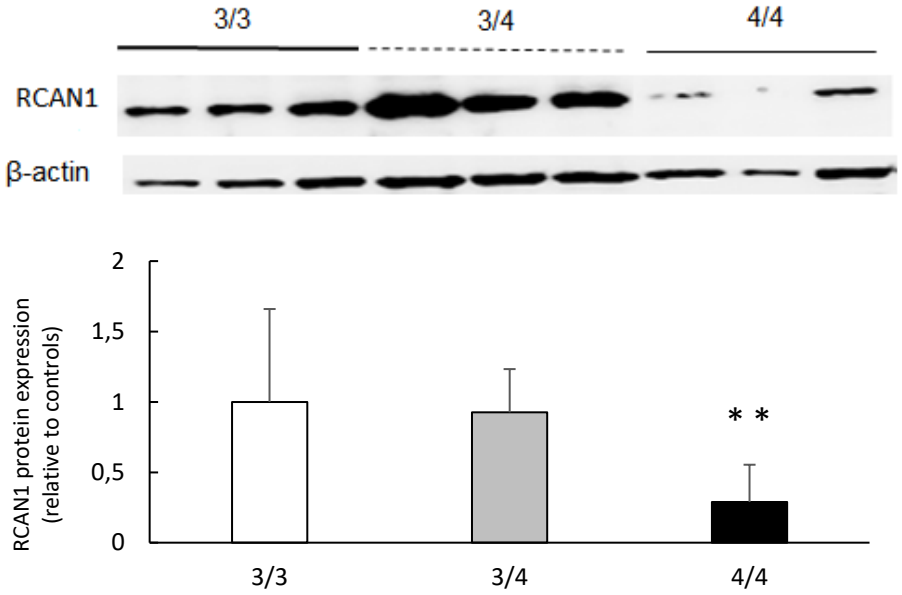


Graphic 14: PKR gene expression according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=6), *APOE4* carriers (N=19); B) 3/3 (N=6); 3/4 (N=10); 4/4 (N=9). Bars indicate the PKR gene expression relative to controls ± standard deviation. PKR expression was normalized for GAPDH expression. *p<0.05 for *APOE4* carriers *versus* non-carriers, and for 3/4 versus 3/3.

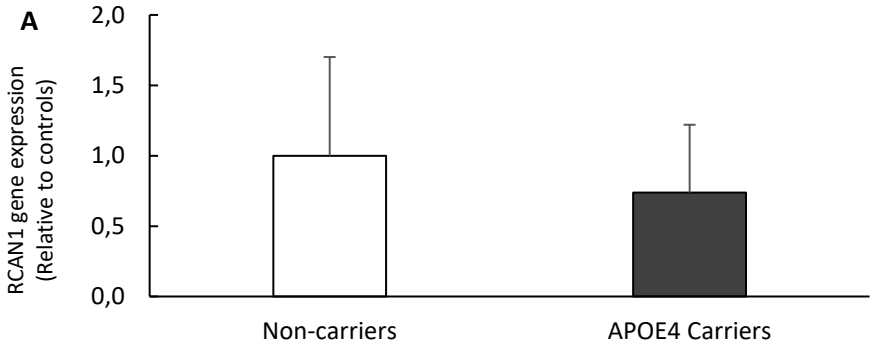
RCAN1

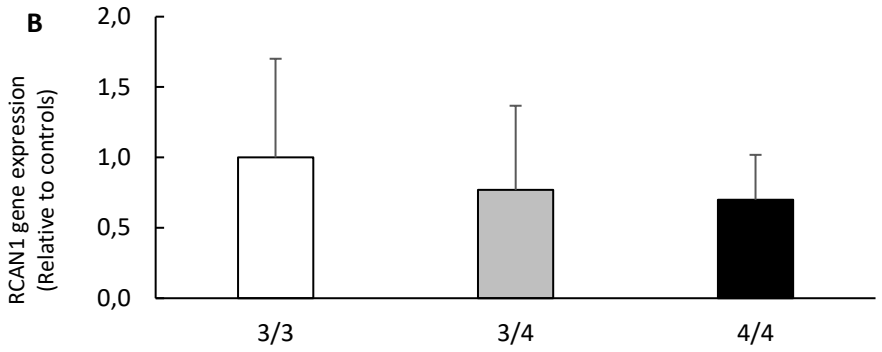
RCAN1 is a protein that can be induced by multiple stresses. Thus, we chose to analyze RCAN1 protein and gene expression with WB and qPCR, respectively. In all subjects, age and sex had no effect on RCAN1. Regarding *APOE4*, we found that homozygous carriers had significantly lower protein levels when compared to both heterozygous carriers and non-carriers

(graphic 15). This difference was not seen in gene expression, though *APOE4* carriers tended to have lower expression (graphic 16). Results were not influenced by environmental risk factors nor by age or sex.



Graphic 15: RCAN1 protein expression according to *APOE* genotype and representative WB. 3/3 (N=4); 3/4 (N=6); 4/4 (N=7). Bars indicate the RCAN1 protein expression relative to controls ± standard deviation. RCAN1 expression was normalized for β-actin expression *p<0.05 for 4/4 *versus* 3/3, and for 4/4 *versus* 3/4.

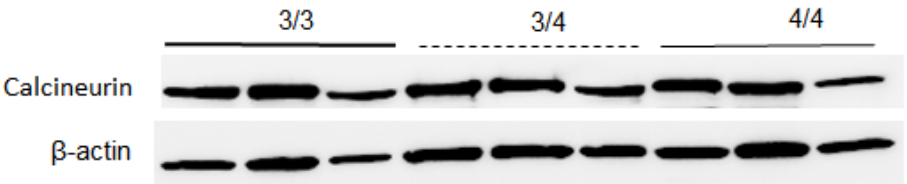


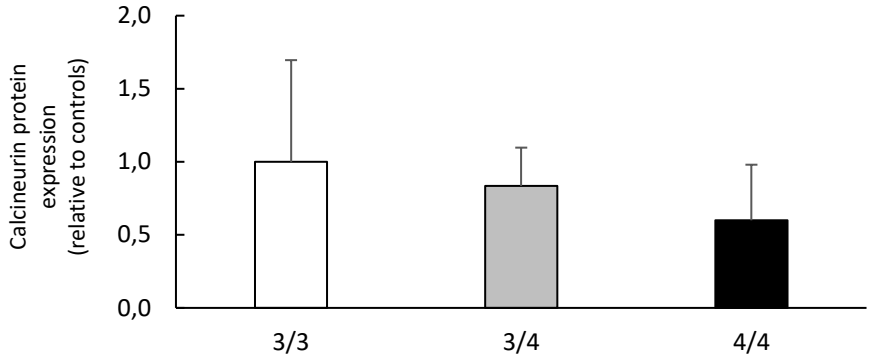


Graphic 16: RCAN1 gene expression according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=8), *APOE4* carriers (N=20); B) 3/3 (N=8); 3/4 (N=11); 4/4 (N=9). Bars indicate the RCAN1 gene expression relative to controls ± standard deviation. RCAN1 expression was normalized for GAPDH expression.

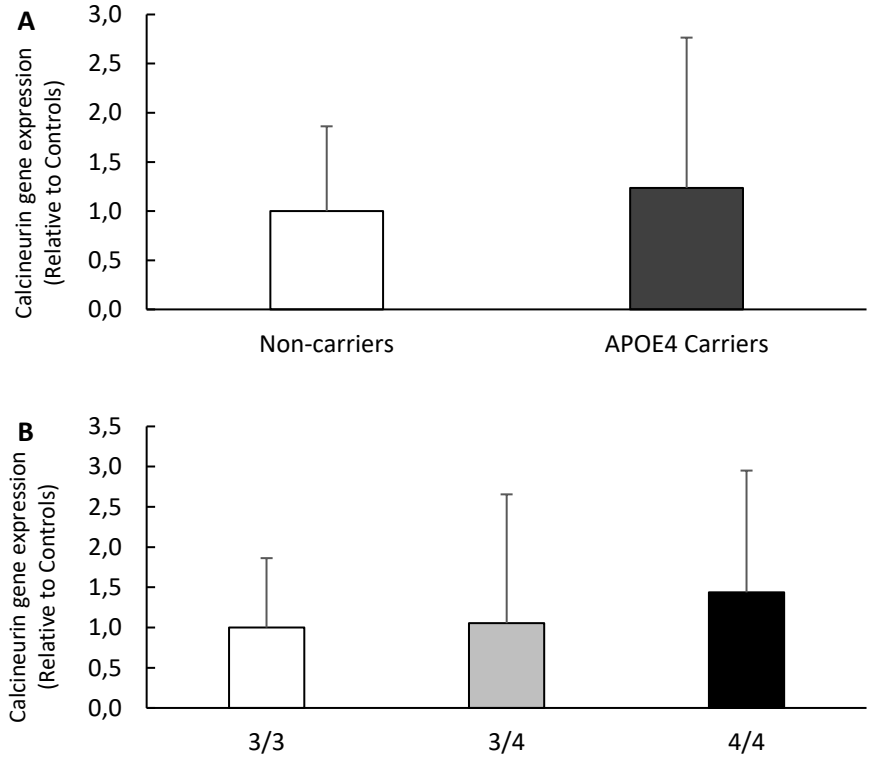
Calcineurin

Calcineurin is a protein related to inflammation which is regulated by RCAN1. Therefore, we chose to analyzed calcineurin enzyme and gene expression with WB and qPCR, respectively. As shown in graphics 17 and 18, there was no difference between *APOE4* carriers and non-carriers nor between the three genotypes, although homozygous carriers tended to have lower enzyme and higher gene expression. There was no age- or sex-*APOE4* interaction.





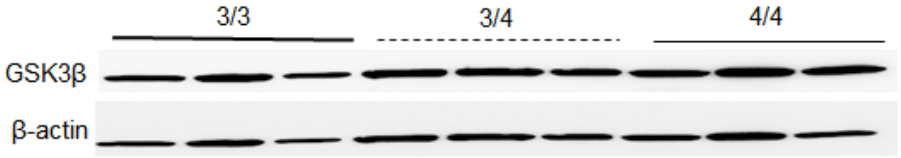
Graphic 17: Calcineurin protein expression according to APOE genotype and representative WB. 3/3 (N=5); 3/4 (N=8); 4/4 (N=4). Bars indicate the calcineurin protein expression relative to controls ± standard deviation. Calcineurin expression was normalized for β-actin expression.

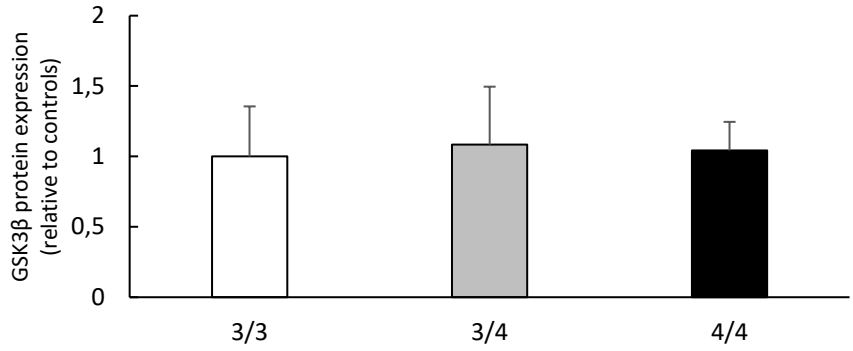


Graphic 18: Calcineurin gene expression according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=6), *APOE4* carriers (N=19); B) 3/3 (N=6); 3/4 (N=10); 4/4 (N=9). Bars indicate the calcineurin gene expression relative to controls ± standard deviation. Calcineurin expression was normalized for GAPDH expression.

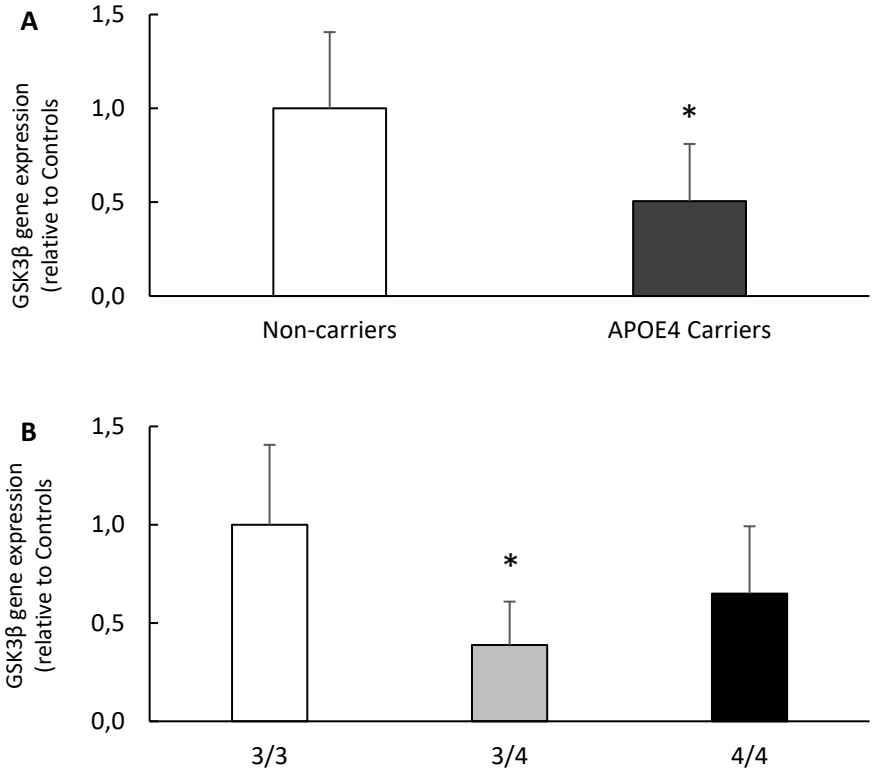
GSK3β

The last stress-related enzyme we analyzed was GSK3β. We did that by analyzing enzyme and gene expression with WB and qPCR, respectively. *APOE4* carriers had significantly lower GSK3β gene expression than non-carriers, even though they had similar enzyme expression. As we can see in graphics 19 and 20, heterozygous carriers had significantly lower gene expression than controls, even though all three genotypes have similar enzyme expression. Although heterozygous carriers also had lower gene expression than homozygous carriers, it did not reach statistical significance (p= 0.063). Similarly, although homozygous carriers had slightly lower gene expression than controls, it was not significant (p=0.124). These results remained even when we considered possible influences of sex, age and environmental risk factors.





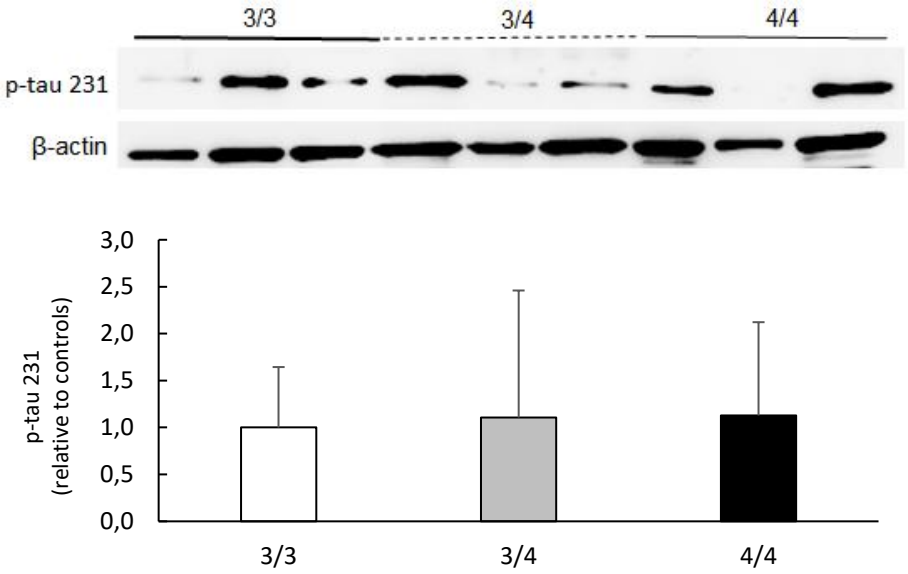
Graphic 19: GSK3β protein expression according to APOE genotype and representative WB. 3/3 (N=5); 3/4 (N=8); 4/4 (N=5). Bars indicate the GSK3β protein expression relative to controls ± standard deviation. GSK3β expression was normalized for β-actin expression.



Graphic 20: GSK3β gene expression according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=8), *APOE4* carriers (N=19); B) 3/3 (N=8); 3/4 (N=11); 4/4 (N=8). Bars indicate the GSK3β gene expression relative to controls ± standard deviation. GSK3β expression was normalized for GAPDH expression. *p<0.05 for *APOE4* carriers *versus* non-carriers, and for 3/4 *versus* 3/3.

4.1.4.6 p-tau 231

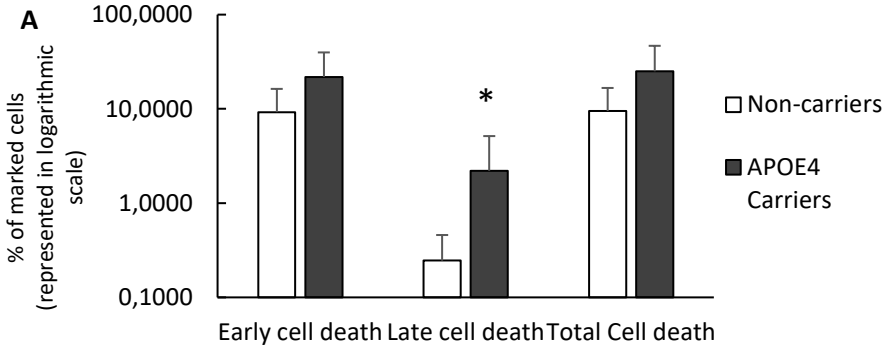
All the previous stress-related proteins are related to increased tau phosphorylation in AD. Therefore, we chose to analyze p-tau levels with WB. We did not find any difference according to *APOE4* status or genotype (graphic 21).

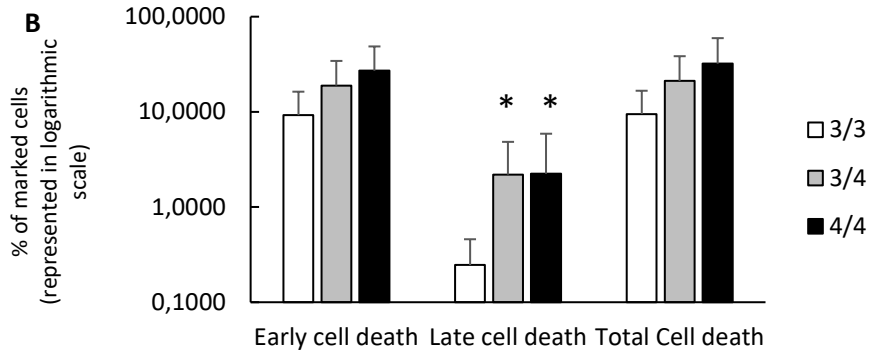


Graphic 21: p-tau 231 expression according to APOE genotype. 3/3 (N=3); 3/4 (N=3); 4/4 (N=3). Bars indicate the p-tau 231 expression relative to controls ± standard deviation. p-tau 231 expression was normalized for β-actin expression.

4.1.4.7 Cell death

Cell death is a final event caused by inflammation, oxidative stress and cell stress. Therefore, we chose to analyze cell death in our subjects by cytometry with annexin + PI staining. In our study, sex and age did not have an influence in cell death. However, the *APOE4* allele did. *APOE4* carriers had overall higher percentage of cell death, though it was only significant in late cell death. The same result was seen according to genotype, with both heterozygous and homozygous carriers presenting higher late cell death than non-carriers (graphic 22). We considered that environmental risk factors could influence these results, so we analyzed results considering factors such as smoking habits, diabetes and depression. Even so, results remained the same. Although early cell death did not show a significant difference between the three genotypes, we could see a tendency to higher levels in an allele dose-dependent manner.





Graphic 22: Early, late and total cell death according to A) *APOE4* carriage and B) *APOE* genotype. A) Non-carriers (N=7), *APOE4* carriers (N=25); B) 3/3 (N=7); 3/4 (N=16); 4/4 (N=9). Bars indicate the percentage of marked cells ± standard deviation, represented in logarithm scale. Early cell death: annexin +, PI-; Late cell death: annexin +, PI+; total cell death: early + late cell death. *p<0.05 for *APOE4* carriers *versus* non-carriers, for 3/4 *versus* 3/3, and for 4/4 *versus* 3/3.

4.2 LONGITUDINAL ANALYSIS

4.2.1 Sample description

Our study conducted a follow-up after 11 years of the first investigation. Of the original 33 subjects, 9 (27.3%) did not participate in this follow-up analysis. Table 5 describes the returnees (which are our current longitudinal *APOE4*-carrier group) and the drop-outs. As we can see, those that did not return were slightly older and had lower levels of education than the ones that did return (8 out of the 9 drop-outs had secondary and primary studies), although these differences were not statistically significant. Finally, 3 participants that chose not to return had unknown genotype; however, they were known *APOE4* carriers as they had at least one parent who was *APOE4* homozygous according to the first study's recruitment criteria.

Table 5: Longitudinal sample description of APOE4 carriers according to return status. Age represented as the mean ± standard deviation. Values specify the number (N) and corresponding percentage (%) of subjects in each group.

		Returnees (N=24)		Drop-out (N=9)	
Age (Mean)		39.96 ± 8.30		44.89 ± 5.00	
		#	%	#	%
Education	*Primary*	5	20.8	4	44.4
	Secondary	9	37.5	4	44.4
	Tertiary	10	41.7	1	11.1
Sex	*Men*	9	37.5	5	55.6
	Women	15	62.5	4	44.4
Genotype	*3/4*	14	58.3	3	33.3
	4/4	10	41.7	3	33.3
	Unknown	.	.	3	33.3

Regarding the control groups, unfortunately our current study could not recruit the same individuals that participated in the previous study. Thus, we recruited new non-carrier, control subjects that were matched in age, sex and education to our carriers' group. Table 6 shows the sample description

of the control groups of the previous and the current studies. The age difference between both groups is of approximately 11 years, which is the time difference between both studies. These control groups present slightly different educational level and percentage of women; however, this difference was not significant.

Table 6: Sample description of control groups. Age represented as the mean ± standard deviation. Values specify the number (N) and corresponding percentage (%) of subjects in each group.

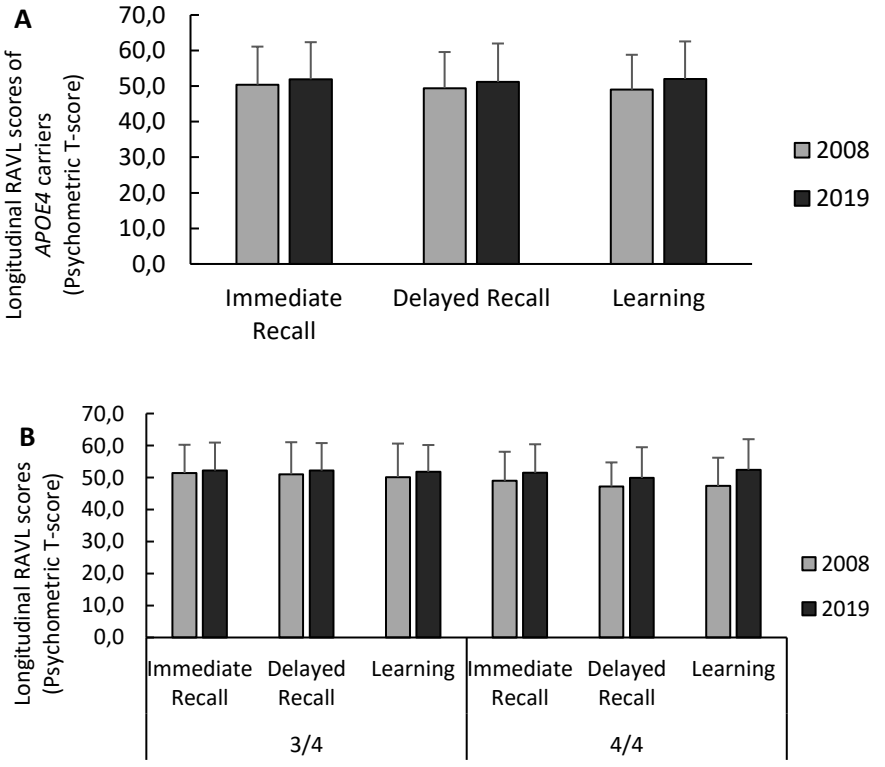
		Control 2008 (N=14)		**Control 2019 (N=15)**	
Age (Mean)		39.85 ± 9.0		51.29 ± 7.7	
		#	%	#	%
Education	*Primary*	2	14.3	3	20.0
	Secondary	4	28.6	7	46.7
	Tertiary	8	57.1	5	33.3
Sex	*men*	7	50.0	5	37.5
	women	7	50.0	10	62.5

4.2.2 Cognition and Depression

4.2.2.1 Memory

When analyzing longitudinal results in objective memory (RAVLT), we first evaluated a possible return bias. In order to do that, we compared 2008 results between returnees and drop-outs. Scores were corrected for age, sex and education. We did not find any significant difference between both groups, even though drop-outs had slightly lower results. Next, we sought to see if there was any difference between our control groups but did not find any.

Then, we proceeded to analyze the evolution of RAVLT scores in *APOE4* carriers, but did not find any significant difference through time, not even when considering subjects by genotype (graphic 23).

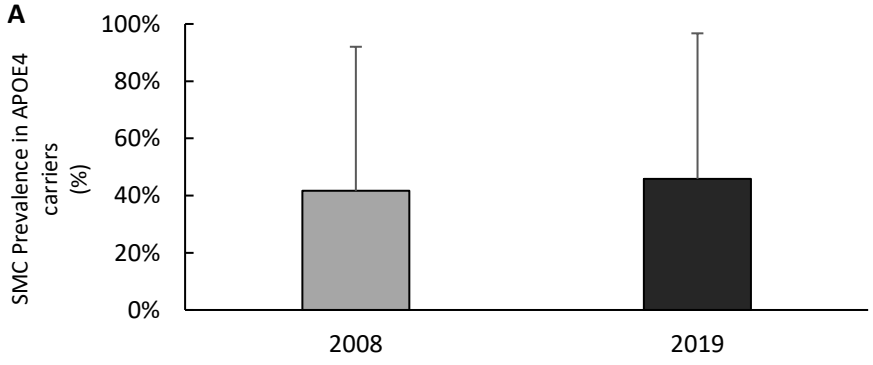


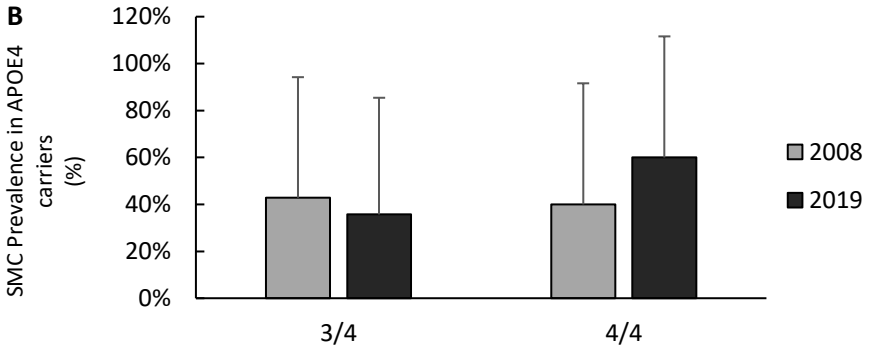
Graphic 23: Longitudinal RAVLT scores according to A) *APOE4* carriage and B) *APOE* genotype. A) *APOE4* carriers (N=24); B) 3/4 (N=14), 4/4 (N=10). Psychometric T Scores refer to scores with a mean of 50 and standard deviation of 10. Values represent the mean score ± standard deviation.

Regarding subjective memory, our longitudinal analysis was done using the "yes" or "no" answers of SMC. Unfortunately, MFE could not be analyzed longitudinally because it was not a part of the first study.

As we did with objective memory, we first excluded return bias by analyzing SMC between returnees and drop-outs and, as expected, did not find a difference between both groups.

Next, we considered controls and *APOE4* carriers. Current controls presented more SMC than previous controls. On the other hand, *APOE4* carriers did not show a difference in the prevalence of SMC between both studies, not even when considering genotype (graphic 24), sex and current age. However, although there was no general difference, there were individual changes in answers, where some individuals that previously had referred SMC, now reported not having it, and vice versa.



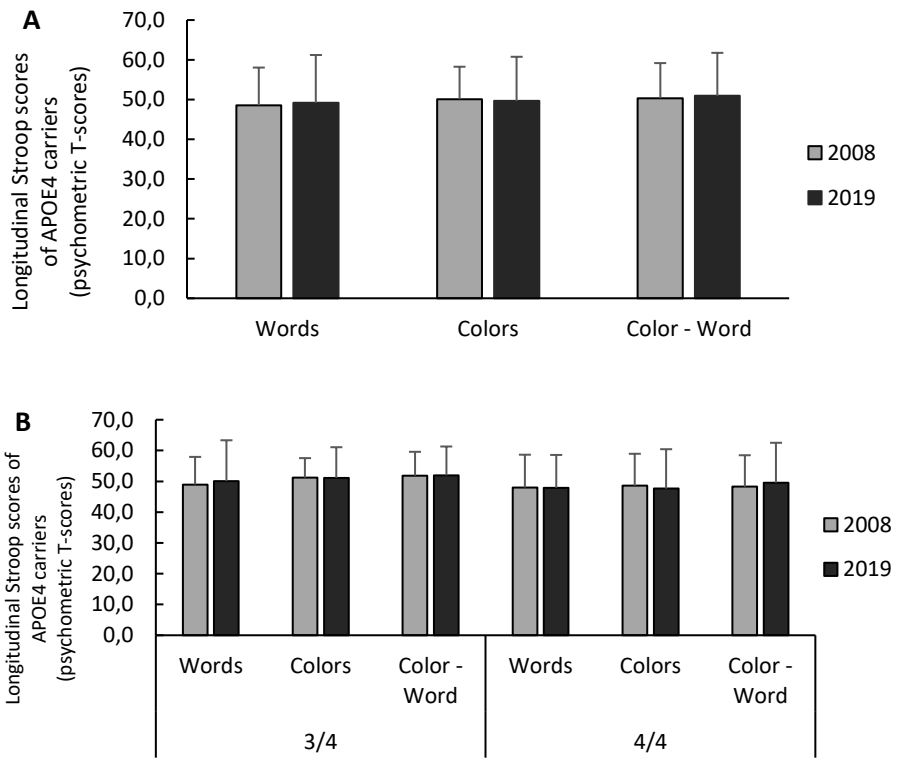


Graphic 24: Longitudinal prevalence of SMC according to A) *APOE4* carriage and B) *APOE* genotype. A) *APOE4* carriers (N=24); B) 3/4 (N=14); 4/4 (N=10). Values represent the mean prevalence ± standard deviation.

4.2.2.2 Attention

As we did with memory, we first analyzed Stroop scores between returnees and drop-outs and between control groups. Although drop-outs tended to have lower scores, there was no difference between both groups. There was also no difference between both control groups.

Longitudinal results of homozygous and heterozygous *APOE4* carriers are displayed in graphic 25. As we can see, the scores of homozygous and heterozygous *APOE4* carriers remained similar through time.



Graphic 25: Longitudinal Stroop scores according to A) *APOE4* carriage and B) *APOE* genotype. A) *APOE4* carriers (N=24); B) 3/4 (N=14); 4/4 (N=10). Psychometric T-scores refer to scores with a mean of 50 and standard deviation of 10. Values represent the mean score ± standard deviation.

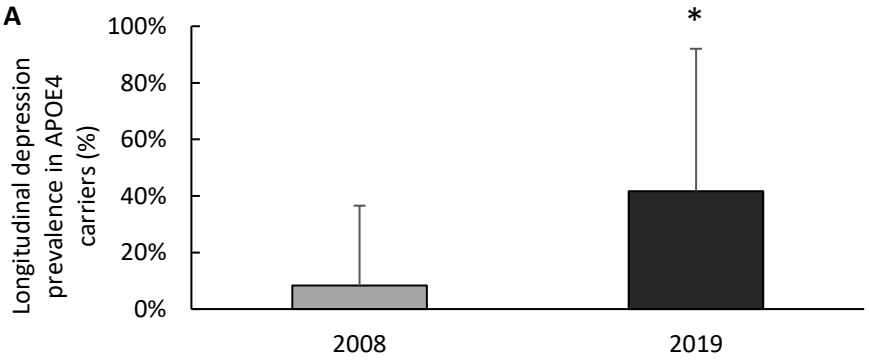
4.2.2.3 Depression

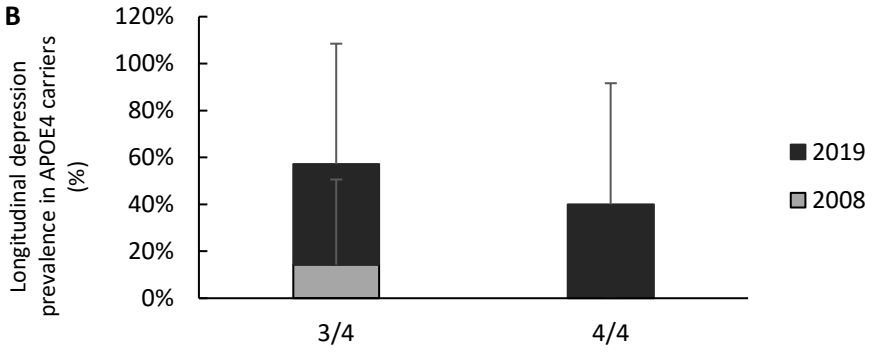
In 2008, four (4) *APOE4* carriers presented depression. Of these, two subjects returned for this follow-up. Overall, there was no difference in depression prevalence between returnees and drop-outs. Longitudinal analysis was conducted considering only those subjects that did return.

Control groups did not differ regarding depression prevalence, even though no individuals presented depression in the 2008 control group, while in our current study two women presented mild depression.

On the other hand, there was a significant increase in depression prevalence within *APOE4* carriers (graphic 26A). In our current study, 10 out of 24 carriers presented depression, while in 2008 it was only present in 2 of these *APOE4* carriers. Although this increase could be seen in both 3/4 and 4/4 carriers, when analyzing subjects by genotype, this rise did not reach statistical significance (graphic 26B).

In our current study, women carriers presented higher prevalence of depression than men, while younger and older carriers presented similar prevalence. Thus, we expected to see an influence of sex but not of age in the longitudinal analysis and, indeed, that is what we found. Both younger and older carriers had a similar increase in depression prevalence, while women presented a significant increase that men did not, even though men had slight rise in depression prevalence.





Graphic 26: Longitudinal prevalence of depression in A) all *APOE4* carriers and B) *APOE4* carriers according to *APOE* genotype. A) *APOE4* carriers (N=24); B) 3/4 (N=14); 4/4 (N=10). Values represent the mean prevalence ± standard deviation. *p<0.05 for 2019 *versus* 2008.

4.2.3 Analytical Measures

4.2.3.1 Pro-inflammatory cytokines

We could not evaluate changes in IL-1β nor in TNF-α levels through time, because the previous study analyzed TNF-α levels and did not measure IL-1β; while, our current study measured both cytokines, but could only analyze IL-1β, as TNF-α plasma levels were below the detection limit.

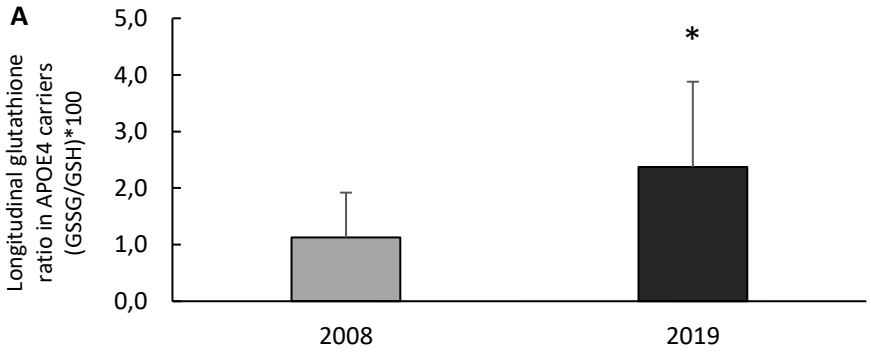
4.2.3.2 Oxidative stress parameters in blood

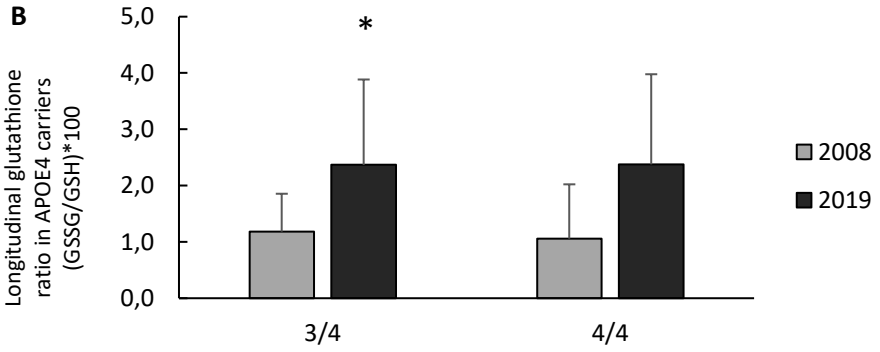
Glutathione

The previous study found that *APOE4* subjects presented a reductive stress, with lower GSSG and oxidized/reduced ratio than non-carriers. Thus, we sought to see if this reductive stress had changed over time to the oxidative stress characteristic of AD. To that end, we compared both control groups to

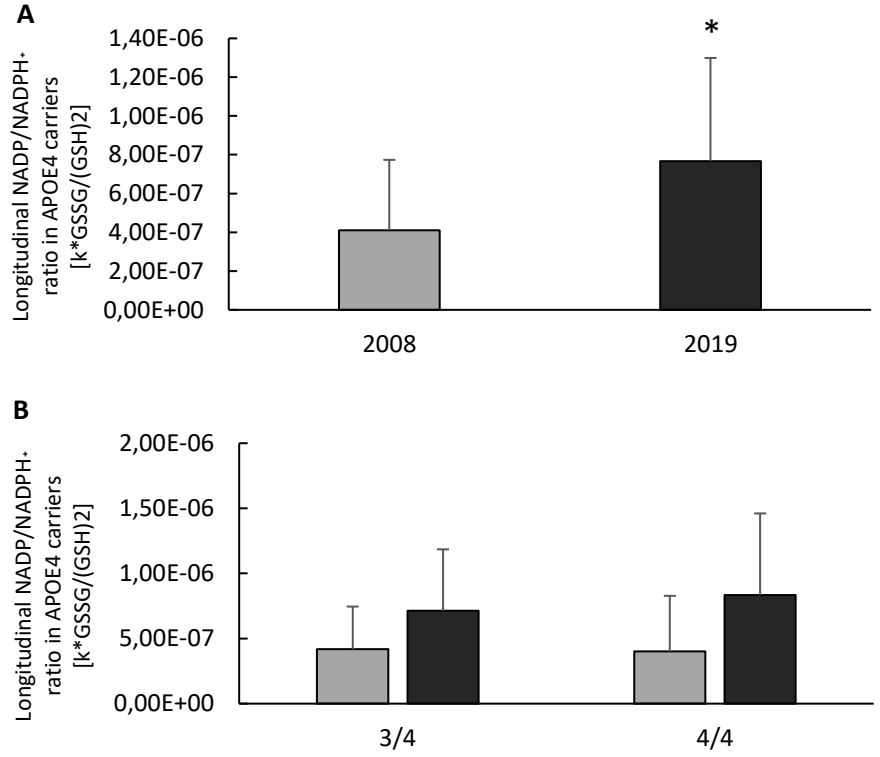
see if a possible increase in oxidation could be due to age and/or environmental risk factors. However, we did not find any difference in GSSG and GSH levels, nor in GSSG/GSH or NADP/NADPH^{+} ratios between both control groups. Conversely, *APOE4* carriers presented a significant increase in GSSG, GSSG/GSH ratio (graphic 27A) and NADP/NADPH^{+} ratio (graphic 28). Regarding GSSG/GSH ratio this increase occurred in both homozygous and heterozygous carriers, though it only reached significance in the later (graphic 27B). Conversely, there was a tendency to increased NADP/NADPH^{+} ratio in both homozygous and heterozygous carriers, that did not reach significance due to the low number of subjects in each group. On the other hand, GSH levels were maintained between both studies.

Lastly, we analyzed the interactions age-*APOE4* and sex-*APOE4*. We found that, although both sexes presented a rise in GSSG levels, these were only significantly increased in men. Similarly, while younger carriers had slightly higher oxidation levels, which was not significant, older carriers presented an important increase in GSSG and oxidized/reduced ratios.





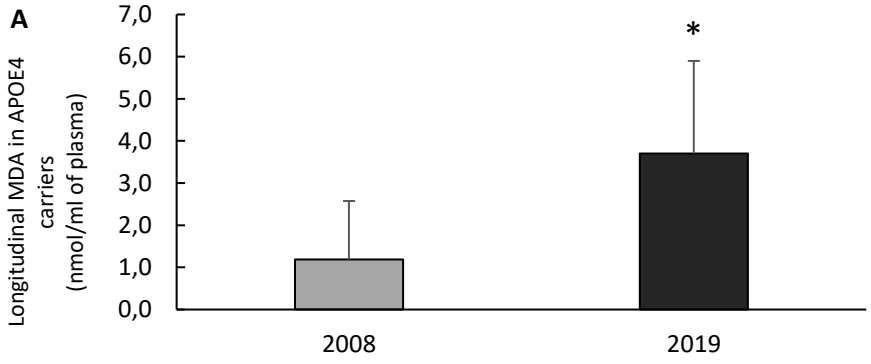
Graphic 27: Longitudinal oxidized/reduced glutathione ratio of A) all APOE4 carriers and B) APOE4 carriers according to APOE genotype. A) APOE4 carriers (N=18); B) 3/4 (N=10); 4/4 (N=8). Values represent the mean ratio ± standard deviation. *p<0.05 for 2019 versus 2008.

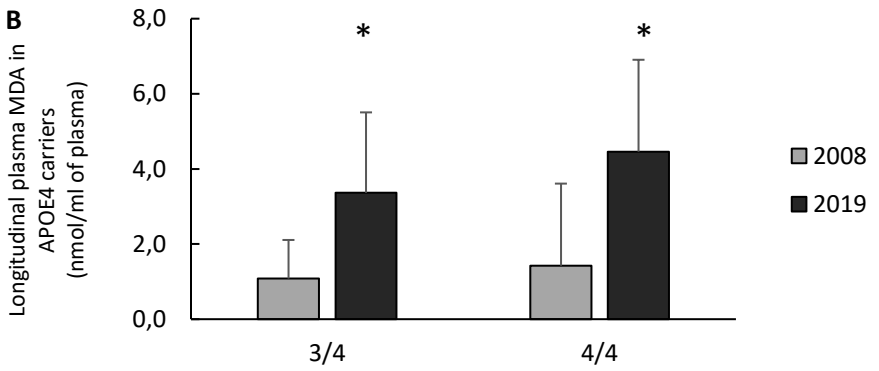


Graphic 28: Longitudinal NADP/NADPH$^+$ ratio of A) all *APOE4* carriers and B) *APOE4* carriers according to *APOE* genotype. A) APOE4 carriers (N=18), B) 3/4 (N=10); 4/4 (N=8). Values represent the mean ratio ± standard deviation. *p<0.05 for 2019 versus 2008.

Malondialdehyde

To investigate oxidative damage, we analyzed longitudinal changes in plasma MDA. First, we compared controls groups and found that they had similar plasma MDA. Then, we analyzed *APOE4* carriers and found that they had an important increase in MDA levels in the last 11years (graphic 29A), which was also significant when considering homozygous and heterozygous carriers (graphic 29B). This significant increase in oxidative damaged occurred in all *APOE4* carriers, regardless of sex or age. However, age did interact with *APOE4*, as older carriers presented a higher mean increase in MDA concentration than younger carriers. Lastly, environmental risk factors also interacted with *APOE4*, increasing oxidative damage, although there was no significant difference between carriers that had environmental risk factors and those that did not.

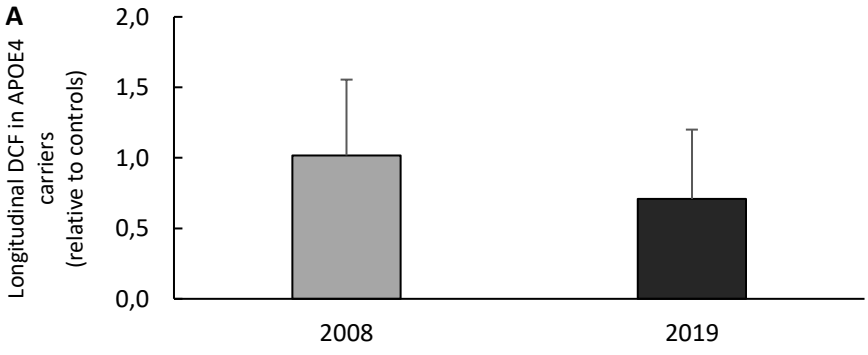


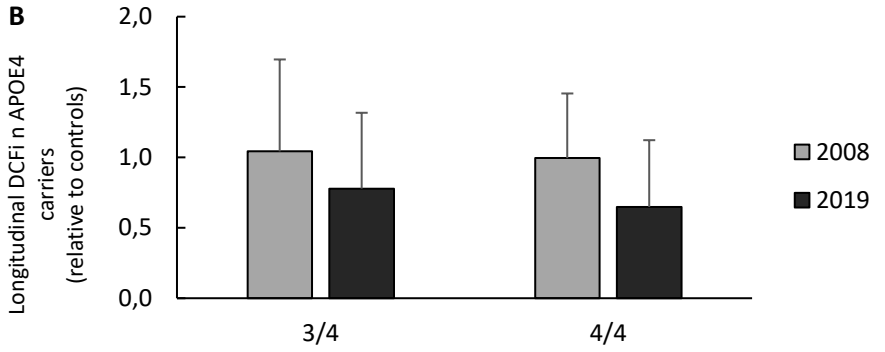


Graphic 29: Longitudinal MDA plasma concentrations of A) all APOE4 carriers and B) APOE4 carriers according to APOE genotype. A) APOE4 carriers (N=13). B) 3/4 (N=9); 4/4 (N=4). Values represent the mean MDA ± standard deviation. *p<0.05 for 2019 versus 2008.

4.2.3.3 Intracellular reactive species

We analyzed longitudinal differences in intracellular reactive species in *APOE4* carriers using DCF levels relative to controls. There was no overall difference in DCF levels in *APOE4* carriers (graphic 30A), nor when considering genotype (graphic 30B), although there was a tendency to current lower DCF (p=0.198).





Graphic 30: Longitudinal DCF levels in A) all APOE4 carriers and B) APOE4 carriers according to APOE genotype. A) APOE4 Carriers (N=15), B) 3/4 (N=7), 4/4 (N=8). Values represent the mean DCF relative to controls ± standard deviation.

4.2.3.4 Antioxidant Enzymes

In the previous study, homozygous and heterozygous carriers had higher levels of GCLC, GCLM and GPx1 than non-carriers. However, they did not present any difference in SOD1 levels. Results from the previous study are displayed in annex 5. Table 7 shows the fold-change in gene expression and their correspondent p values. As we can see, there was a reduction in the expression of all antioxidant genes in APOE4 carriers, of both genotypes. However, significant changes (defined as fold-change ≥1.5 and p-value< 0.05) was only reached in GCLC, GCLM and GPx1 expression in all APOE4 carriers; GCLC, GPx1 and SOD1 in 3/4 carriers; and in GCLM and GPx1 in 4/4 carriers. This shows that, when compared to current results, carriers do not overexpress antioxidant enzymes any longer. In fact, GPx1 expression pattern has inverted from a previous overexpression to a current underexpression. Furthermore, heterozygous carriers changed from a

normal SOD1 expression to an underexpression and levels of GCLM and GCLC have returned to a normal expression.

Table 7: fold-change in gene expression of antioxidant enzymes and their correspondent p values.

	GCLC	GCLM	GPx1	SOD1
Fold-change APOE4 carriers	-4,66	-3,96	-3,59	-2,66
p-value	0,02	0,00	0,01	0,33
Fold-change 3/4 carriers	-4,08	-2,07	-2,52	-2,81
p-value	0,03	0,14	0,06	0,00
Fold-change 4/4 carriers	-2,39	-1,77	-2,23	-1,32
p-value	0,22	0,00	0,07	0,01

4.2.3.5 Stress Response

p-p38/p38 ratio

The previous study found that both heterozygous and homozygous carriers had significantly lower p-p38/p38 ratio when compared to non-carriers. In our current study we found that there was no difference between the 3

genotypes, and APOE4 carriers tended to have a slightly higher ratio. Thus, we saw that, as they reached middle-age, carriers had a change of p38 activation.

RCAN1

In the previous study, homozygous carriers presented a significant increase in RCAN1 protein when compared to non-carriers, while heterozygous carriers did not. Currently, homozygous carriers have lower protein levels. This represents an inversion of the protein's expression pattern in homozygous carriers, as they reached middle-age. Conversely, the previous study found that RCAN1 gene expression was increased in both homozygous and heterozygous carriers, a pattern that was no longer expressed as they reached middle-age. However, as we can see in table 8, although there was a decrease in expression in all APOE4 carriers and in heterozygous carriers, this only reached significance in homozygous carriers.

Calcineurin

Similarly, previous results showed that both 3/4 and 4/4 subjects had higher calcineurin enzyme and gene expression than 3/3 subjects, an overexpression that normalized as subjects reached middle-age. The decrease in gene expression occurred in all carriers, however, it did not reach significance in homozygous carriers (table 8).

GSK3β

Eleven years ago, *APOE4* carriers of both genotypes presented higher GSK3β enzyme levels when compared to non-carriers, while there was no difference in gene expression. As it happened with other proteins, enzyme levels normalized as subjects reached middle-age. Conversely, gene expression

changed from a normal expression to an underexpression in heterozygous carriers, as we can see in table 8.

PKR

Previous results showed that 4/4 subjects presented a higher PKR gene expression when compared to non-carriers. Currently, *APOE4* carriers present higher PKR than controls, especially heterozygous carriers. As we can see in table 8, there was no significant reduction in PKR gene expression through time in APOE4 carriers, not even when considering genotypes.

Table 8: fold-change in gene expression of stress-related enzymes and their correspondent p values.

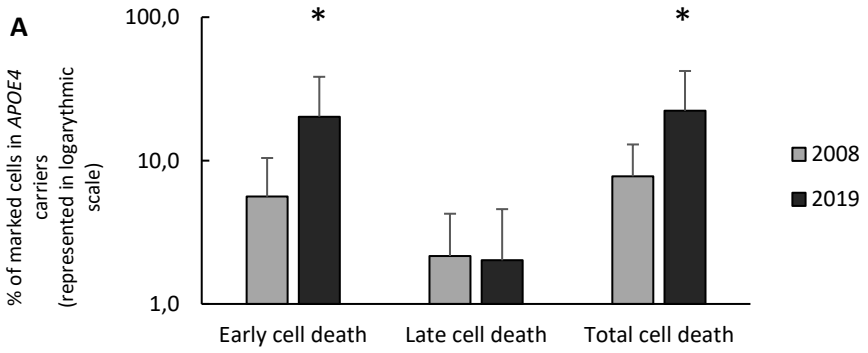
	GSK3β	**RCAN1**	**Calcineurin**	**PKR**
Fold-change APOE4 carriers	-2,62	-1,90	-18,33	-1,24
p-value	0,00	0,39	0,01	0,93
Fold-change 3/4 carriers	-3,57	-1,90	-3,18	-0,53
p-value	0,00	0,92	0,03	0,27
Fold-change 4/4 carriers	-2,81	-3,27	-4,40	-1,23
p-value	0,12	0,02	0,11	0,56

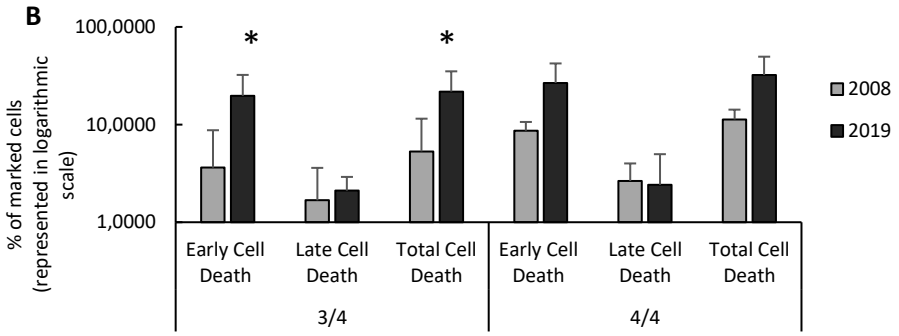
4.2.3.6 p-tau 231

Previous results showed that homozygous carriers presented higher levels of p-tau 231 when compared to the other groups. This overexpression has normalized with time, and now all groups present similar p-tau levels.

4.2.3.7 Cell death

Lastly, we analyzed cell death. Control groups presented similar early and total cell death. On the other hand, *APOE4* carriers presented a significant increase in early and total cell death in the last 11 years (graphic 31). Although this increase occurred in both heterozygous and homozygous, it only reached significance in the former.





Graphic 31: Longitudinal cell death in A) all APOE4 carriers and B) APOE4 carriers according to APOE genotype. A) APOE4 Carriers (N=15), B) 3/4 (N=7), 4/4 (N=8). Bars represent the mean % of marked cells ± standard deviation, represented in logarithmic scale. *p<0.05 for 2019 *versus* 2008.

DISCUSSION

5 Discussion

Apolipoprotein E is a multifunctional protein involved in many cellular processes. It acts in lipid homeostasis, the response to intracellular calcium, energetic metabolism, the modulation of intracellular pathways, endothelial activation and repair, maintenance of the BBB integrity, elimination of cellular debris, and the control of inflammatory response (Martínez-Martínez et al., 2020). It is also involved in redox homeostasis due to its antioxidant function, eliminating oxidated molecules, neutralizing oxidant metals and regulating the cell's response to stress (Miyata & Smith, 1996). However, its apoE4 isoform presents altered conformation and function, which increases the risk of many diseases.

In our study, we found that the number of individuals that had dyslipidemia requiring pharmacological treatment was around 25% in both carriers and non-carriers. This prevalence is similar to that found in Spain and in the Valencian community (Vegazo et al., 2006). However, the percentage of individuals referring ever having blood lipid alterations was higher in carriers than non-carriers. This agrees with studies that found that *APOE4* carriers have higher total cholesterol levels and higher LDL-cholesterol, which can be seen even at young ages (Hanh et al., 2016; Zende, Bankar, Kamble, & Momin, 2013).

Although diabetes mellitus and hypertension are risk factors for cognitive decline, the relationship between *APOE4* and the risk of diabetes or hypertension is equivocal in the literature (Chaudhary et al., 2012; Lumsden, Mulugeta, Zhou, & Hyppönen, 2020; Rao, Wu, Yu, & Huang, 2022; Shi et al., 2018). This matches our study, that found that carriers and non-carriers

presented similar prevalence of diabetes and hypertension and that these were close to that found in Spain and in the Valencian community (Menéndez Torre, Ares Blanco, Conde Barreiro, Rojo Martínez, & Delgado Alvarez, 2021; Menéndez et al., 2016). However, although *APOE4* might not increase the risk of these diseases, it does interact with them to increase pathology (Bangen et al., 2016; Oberlin et al., 2015).

Certain diseases were only seen in *APOE4* carriers in our study, such as those related to immune system dysregulation and increased inflammation. *APOE4* has not been conclusively linked to an increased risk of these disorders, however, it may influence them as it alters the inflammatory profile and increases cytokine production (Zhang, Wu, & Zhu, 2010a; Zhang, Wu, & Wu, 2011)Furthermore, *APOE4* is known to impact processes such as autophagy and efferocytosis (Cash et al., 2012; Eran & Ronit, 2022), functions that are critical for the prevention of inflammation and autoimmunity (Deretic, 2021).

5.1 *APOE4* and Cognition

Subjective memory complaints are frequently analyzed in studies because they can be a prodromal signal of future memory decline and dementia, even though many people with SMC will not develop cognitive impairment (Lee et al., 2020). In clinical settings, SMC is associated with *APOE4* (van der Flier et al., 2008) and it could serve as an indicator of preclinical AD, especially among carriers (Jessen et al., 2014). Thus, we chose to inquire about SMC in our study as it was possible that *APOE4* carriers would refer more complaints.

We did not find a difference in SMC between carriers and controls cross-sectionally. Longitudinally, we found a significant difference in SMC only

between the control groups from the first and the current studies. This could be due to several factors, including age, as older individuals present more general memory complaints than younger ones (Ginó et al., 2010). Furthermore, memory complaints are common across the life span; it is referred by 53% of adults, although the prevalence of complaints that cause concern is lower (Luck et al., 2018). Lastly, a person's perception on memory problems reflects individual characteristics and can change due to many factors, including daily stressors, anxiety and depression (Neupert, Almeida, Mroczek, & Spiro, 2006; Sharifian & Zahodne, 2021). This could be seen in our study when we analyzed MFE results, which showed that our subjects had lower MFE than the mean found in Spanish normative studies (Montejo Carrasco et al., 2012). Moreover, most memory failures seen in our study were, in fact, attentional failures which are the most frequent memory failures in healthy individuals (Montejo, Montenegro, Sueiro-Abad, & Huertas, 2014).

Even though subjective cognitive decline might be a risk factor for clinical progression to AD (Ebenau et al., 2020), its ability to predict progression to objective decline remains low (Dubois et al., 2021). In our study we found that SMC was only related to lower scores on RAVLT delayed recall in controls and not in *APOE4* carriers. This could be related to many factors, including lower effort by those controls who already though they had a poor memory, or an increased effort by carriers due to their family history of AD.

Although *APOE4* is a risk factor for many diseases, it is specially related to AD (Michaelson, 2014). The most common AD phenotype is the amnestic, which initially affects episodic memory and focused attention, and is strongly associated with *APOE4* and a positive family history of AD (Snowden et al.,

2007), factors present in our subjects. In our study, individuals are cognitively normal and, thus, have no objective cognitive deficit. However, it was possible that they could present slight differences when compared to non-carriers or they could have had increased longitudinal decline. Therefore, we evaluated objective memory and attention using the RAVLT and the Stroop test.

We did not find any difference between the three genotypes, nor a difference through time, in RAVLT and Stroop scores. Studies analyzing the connection between *APOE4* and cognition in healthy individuals have found divergent results, and many studies, like ours, did not find any difference between carriers and non-carriers (Henson et al., 2020; Jack et al., 2015). It is possible that modifying variables may play a role on the effects of *APOE4* on cognition. In our study, most individuals had a high educational attainment and were bilingual, speaking both Spanish and Valencian as their native language; and both factors are known to increase cognitive reserve and resilience to AD pathology (Gonneaud et al., 2020; Perani & Abutalebi, 2015). Also, the influence of *APOE4* in healthy individuals may be relatively minor and specific to determined cognitive domains (O'Donoghue et al., 2018) and, as such, might not have been seen in our relatively small sample, even though ours is a longitudinal study.

Lastly, our study evaluated depression, which is a frequent neuropsychiatric symptom related to AD that can be found in individuals even before they develop MCI (Berger, Fratiglioni, Forsell, Winblad, & Bäckman, 1999; Lyketsos et al., 2011). Furthermore, *APOE4* had been associated with increased risk of late-life depression in healthy elders (Skoog et al., 2015). However, the relationship between *APOE4* and overall life-time depression

is still equivocal; while some studies found increased overall prevalence in *APOE4* carriers, other studies did not find this relationship (Burns, Andrews, Cherbuin, & Anstey, 2020; Evans & Rajan, 2015).

Cross-sectionally, our study did not find a significant difference in depression prevalence between subjects of any genotype, although *APOE4* carriers tended to have higher prevalence. However, longitudinally, we found a significant increase in depression prevalence in *APOE4* carriers, especially women. Furthermore, we found that women carriers had a higher prevalence than their male counterparts, and that depression was only present in men and younger individuals who were *APOE4* carriers. It is known that women have a higher susceptibility to depressive disorders than men (Labaka, Goñi-Balentziaga, Lebeña, & Pérez-Tejada, 2018). Our results suggest that *APOE4* carriers could also have increased susceptibility to depression. This was also suggested by Chhibber and Zhao (Chhibber & Zhao, 2017), who found that, in female mice, apoE4 interacts with estrogen receptor β to decrease the expression of brain-derived neurotrophic factor and of serotonin receptor, increasing the risk for depression.

Lastly, our study also found that most individuals with depressive symptoms did not have any form of treatment, which could have had some effect on the longitudinal increase in prevalence. Because depressive symptoms can interact with *APOE4* to increase tau accumulation in regions related to affective regulation (Gonzales et al., 2021), the lack of treatment would further increase depressive symptoms in our subjects.

5.2 *APOE4* and Inflammation

Inflammation and immune dysregulation are characteristics of AD and also of *APOE4*. In this sense, our results showed that homozygous *APOE4* carriers presented increased IL-1β when compared to non-carriers and to heterozygous carriers. Furthermore, heterozygous carriers presented higher IL-1β levels than non-carriers. As IL-1β is a pro-inflammatory cytokine, this suggests that *APOE4* is related to increased inflammation even in healthy middle-aged individuals, in an allele dose-dependent manner. This concurs with the finding that only *APOE4* carriers presented certain inflammatory diseases.

It is known that APOE4 is related to increased inflammation and higher levels of pro-inflammatory cytokines due to shift to inflammatory immune phenotype (de Leeuw et al., 2022; Zhang et al., 2011). Our results agree with studies in AD patients, where *APOE4* is related to increased levels of pro-inflammatory cytokines in a dose-dependent manner (Fan et al., 2017). Conversely, in healthy *APOE4* carriers, a recent study by Wang and colleagues (Wang, Y. et al., 2022) did not find a correlation between inflammatory cytokines and *APOE4*. This could be because, unlike us, they analyzed both homozygous and heterozygous *APOE4* carriers in the same group; this could affect results as the increase is much higher for the homozygous genotype.

This current increased inflammation contrasts with our previous study, as our subjects did not present increased inflammation 11 years ago. This would indicate that pathology might be different at different ages. However, the previous study analyzed TNF-α levels and did not measure IL-1β; conversely, our current study measured both cytokines, but could only analyze IL-1β, as TNF-α plasma levels were below the detection limit. It is possible, however, that TNF-α levels were similarly low in all three genotypes, which would

indicate a preferential increase of IL-1β over TNF-α in *APOE4* carriers. Both cytokines are pro-inflammatory and increased by the transcription factor NFκβ, which, in turn, is activated by apoE4 (Arnaud et al., 2022). However, studies have also shown that, unlike TNF-α, IL1-β is also increased by CCAAT/enhancer-binding protein [C/EBP] (Kalb et al., 2021). C/EBP is a transcription factor which increases apoE4 levels, but that is also activated by apoE4, in a vicious cycle (Wang, Z., Xia, Liu, Liu, Edgington-Mitchell, Lei, Yu, Wang, & Ye, 2021; Wang, Z., Xia, Wu, Kang, Zhang, Liu, Liu, Song, Huin, Dhaenens, Yu, Wang, & Ye, 2022; Xia et al., 2021). This differential activation of immune pathways by apoE4 might explain why homozygous carriers have higher IL-1β levels, but TNF-α levels were too low to be measured. Furthermore, it might be related to the finding that, at middle-age, heterozygous carriers had lower levels of IL-1β than homozygous, as they also express apoE3. However, it is possible that this might change with time, as individuals age and pathology increases.

5.3 *APOE4* and Oxidative Damage

ApoE4 is known to have altered antioxidant properties and to influence the oxidative status (Jofre-Monseny et al., 2007). Oxidative stress is also an important pathological mechanism in AD, with elevated oxidative stress and damage being seen in both blood and brain of MCI and AD patients (Cervellati et al., 2016; Peña-Bautista, Baquero, Vento, & Cháfer-Pericás, 2019), which is more important in *APOE4* carriers (Liou et al., 2021). However, oxidative stress is most important as an early event in the pathology and it occurs even in cognitively healthy individuals (Butterfield &

Mattson, 2020; Nunomura et al., 2001). Therefore, we chose to analyze oxidative markers in our study.

We found that *APOE4* carriers of both genotypes presented increased MDA levels and decreased GPx1 gene expression when compared to non-carriers. Furthermore, heterozygous carriers present lower SOD1 gene expression, with a significant reduction through time. This altered oxidative status was further supported by the longitudinal increase in MDA that was present only in carriers and not in non-carriers. Thus, in our subjects, middle-aged *APOE4* present oxidative damage, which was not present when they were younger. This agrees with other studies in healthy *APOE4* carriers that found elevated oxidative damage (Smith et al., 1998; Tsuda et al., 2004), reduced plasma antioxidant capacity and altered signaling of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), which controls the expression of antioxidant genes (Piccarducci et al., 2021). It also agrees with *in vitro* and mouse studies which found that *APOE4* was related to a decrease in expression and activity of antioxidant enzymes, including GPx1 (Ben Khedher et al., 2021; Khan et al., 2022; Persson et al., 2017).

As our results showed increased oxidative damage in *APOE4* carriers, we also expected to find increased levels of ROS. However, that was not the case; although there was a tendency to higher DHE levels and lower DCF levels in homozygous carriers, this was not significant. Nevertheless, these results suggest that homozygous carriers might present an altered handling of reactive species. This was also seen by Marottoli et al (Marottoli et al., 2021) who found decreased DCF in *APOE4* cells compared to *APOE3* cells, which was associated with decreased heme content and increased levels of other free radicals. We also expected to find increased GSSG/GSH and

NADP/NADPH^{+} ratios, as these are characteristic of oxidative stress. However, levels were only slightly increased in *APOE4* carriers compared to non-carriers. The lower levels of GPx1 might account for the absence of an important increase in GSSG in carriers, as without this enzyme reactive species cannot be coupled with GSH (Lubos, Loscalzo, & Handy, 2011). Thus, our results suggest that *APOE4* after middle-age might induce the beginning of oxidative stress by decreasing antioxidant defenses, which allows normal or slightly elevated levels of ROS to cause oxidative damage. This was further influenced by the reductive stress previously seen in our subjects, which is known to cause increased ROS production and oxidative damage (Korge, Calmettes, & Weiss, 2015), ER stress, proteotoxicity, activation of the GSK3β/Tau cascade (S Narasimhan et al., 2020) and accelerated senescence (Qiao et al., 2022) even in the presence of increased reducing equivalents. Therefore, the previous reductive stress could have caused oxidative damage without a significant increase in oxidized/reduce ratios in our subjects; and it could also explain the slight activation of p38 MAPK seen in our *APOE4* carriers, as p38 is activated by ROS during stress response (Ashraf et al., 2014).

In our sample, diabetic subjects and active smokers were only present in the non-carrier and in the heterozygous groups. However, neither factor had an important influence on MDA levels of non-carriers, while they increased MDA levels of heterozygous carriers. Environmental risk factors, such as smoking and diabetes, are related to increased oxidative stress and can, themselves, increase plasma MDA levels (Lykkesfeldt, Viscovich, & Poulsen, 2004; Slatter, Bolton, & Bailey, 2000). However, our results show that environmental and genetic risk factors combine to influence oxidative status

and increase pathology and disease risk (Dose et al., 2016; Zhang, N., Ranson, Zheng, Hannon, Zhou, Kong, Llewellyn, King, & Huang, 2021).

We also found that, when considering the environmental risk factors, the increase in MDA was allele dose-dependent. This was similar to the increase in IL-1β we found, suggesting a relationship between inflammation and oxidative damage in our subjects. This is conceivable as IL-1β causes an imbalance between oxidants and antioxidant (Chekaoui et al., 2018) and influences the production of reactive species (Boota et al., 1996); conversely, ROS and altered oxidative status can activate IL-1β (Abais, Xia, Zhang, Boini, & Li, 2015).

Lastly, our study found increased lipid peroxidation and decreased antioxidant defenses, which are biochemical features of ferroptosis (Chen, Comish, Tang, & Kang, 2021). Although we did not evaluate iron or GPx4 levels, it is conceivable that they might also be altered. Ferroptosis is a type of programmed cell death related to oxidative damage, which results from an imbalance between iron-induced production of lipid peroxides and antioxidant defenses (Dixon et al., 2012). It has been described in AD and it could be related to *APOE4* as proposed in very recent study by Belaidi and colleagues (Belaidi et al., 2022). They suggested that *APOE4* carriers present an increased susceptibility to ferroptosis due to the association of higher levels of oxidation-sensitive PUFAs and lower levels of apoE protein present in ε4 carriers; which would lead to increased ferritinophagy-dependent iron release. Therefore, in our subjects, apoE4 might be increasing oxidative damage through multiple pathways. However, more mechanistic studies are needed to define the relationship between *APOE4* and ferroptosis (Jakaria et al., 2021).

5.4 *APOE4* and Cellular Stress

Cells are constantly exposed to perturbations of their homeostasis which can induce different types of cellular stress. These, in turn, activate specific response pathways that are meant to manage stress and repair damage; or to cause cellular senescence or death, when the repair of normal functions is no longer possible (Galluzzi, Yamazaki, & Kroemer, 2018). Both *APOE4* and AD are known to cause cellular stress and both activate multiple stress response pathways (Dose et al., 2016; Weidling & Swerdlow, 2019). The activation of these pathways creates a vicious pathological cycle, as it increases the levels and /or activation of several kinases which induce tau phosphorylation, including GSK3β, RCAN1 and PKR (Badia et al., 2013). Therefore, we chose to analyze cell stress and death in our subjects.

We found increased PKR gene expression in *APOE4* carriers, especially heterozygous carriers and women; and levels remained increased in the last 11 years. This concurs with studies which found *APOE4*-related upregulation of PKR activity and activation of stress responses (Oliveira & Lourenco, 2016; Ramakrishna et al., 2021; Segev et al., 2015). PKR is a kinase that phosphorylates the eukaryotic translation initiation factor 2α (eIF2α) (Donnelly, Gorman, Gupta, & Samali, 2013). This occurs when the integrated stress response is activated by cellular stress signals, causing transcriptional changes and protein synthesis attenuation (Pakos-Zebrucka et al., 2016). ApoE4 can lead to cellular stress, especially ER stress, through different mechanisms: the altered structure of apoE4 can directly cause ER stress (Zhong et al., 2009); its altered structure affects its trafficking through the secretory pathway (Brodbeck et al., 2011); and it is also more prone to

proteolytic cleavage in neurons, which leads to increased neurotoxic apoE4 fragments (Rohn, 2013). ApoE4 can also cause increased ER stress through alteration of other intracellular pathways, including oxidative damage and inflammation (Dose et al., 2016), which we found to be altered in our subjects and might be responsible for the increased PKR.

Different types of cellular stress might be related to *APOE4* in our subjects. Initially, as young carriers, our subjects presented reductive stress. Reductive stress is known to decrease the physiological oxidative state of the ER lumen necessary for correct protein folding (Handy & Loscalzo, 2017). Furthermore, it activates the GSK3β/Tau cascade, promoting tau phosphorylation and proteotoxicity; thus, it leads to the protein aggregation and accumulation of misfolded proteins (S Narasimhan et al., 2020). Protein synthesis and turn-over are also higher, which further increases the accumulation of misfolded proteins (Handy & Loscalzo, 2017). This increased cellular stress could be seen in the previous study by the higher levels of stress-related proteins, such as PKR, GSK3β and RCAN1 found in *APOE4* carriers. However, this *APOE4*-related ER stress causes cellular dysfunction in an age-dependent manner (Zhong et al., 2009). Chronic reductive stress in our subjects could have led to the exhaustion of the antioxidant system and oxidative damage (Lloret et al., 2016; Xiao & Loscalzo, 2020) that we now see in these same individuals. Furthermore, chronic ER stress leads to an exhaustion of the stress response that tries to maintain cell function, and turns metabolic pathways towards other stress response mechanisms and programmed cell death (Lourenco, Ferreira, & De Felice, 2015). This was seen in our current study, which found higher apoptosis in carriers when compared to non-carriers and an increase in apoptosis through time. It could also be seen by the increase in the

phosphorylated form of the stress kinase p38 MAPK in women who were *APOE4* carriers, which was reduced in the previous study. Moreover, the increased MDA levels we found in *APOE4* carriers can induce lipid bilayer stress; this new type of stress, in turn, activates other stress-induced responses with different transcriptional and non-transcriptional programs (Fun & Thibault, 2020).

Our study also found alterations in other stress-related enzymes. We found significantly decreased RCAN1 protein expression and slightly lower RCAN1 gene expression in homozygous carriers when compared to heterozygous carriers and non-carriers. Conversely, there were no significant differences in calcineurin expression, although homozygous carriers tended to have lower protein expression. Longitudinally, homozygous *APOE4* carriers presented a shift from increased expression of RCAN1 and calcineurin, to decreased expression. These results are opposite to what we expected, as chronic overexpression of RCAN1 in AD is associated with cell damage and pathology progression (Ermak, Morgan, & Davies, 2001). However, most studies in AD patients have not accounted for the presence of *APOE4*. In fact, a recent study found reduced RCAN1 gene and protein expression in postmortem frontal lobes of *APOE4* AD individuals, in a dose-dependent manner (Delikkaya, Moriel, Tong, Gallucci, & de la Monte, 2019), which concurs with our results. RCAN1 is a stress-inducible protein, which provides short-term protection against acute cellular stress (Lin et al., 2003). However, RCAN1 is downregulated during chronic stress, which changes cell survival responses and increases stress-related cell death (Bartoszewski et al., 2020); this was seen in our study as increased cell death.

Homozygous *APOE4* carriers, in our study, present lower RCAN1 expression and increased IL-1β levels compared to heterozygous carriers and to non-carriers, which could indicate a relationship between inflammation and stress response. This is conceivable, since sustained lymphocyte activation, which increases IL-1β, is achieved by increasing RCAN1 degradation through lysosome-associated membrane protein type 2a (LAMP2A)-dependent autophagy (Valdor et al., 2014). Interestingly, this same pathway is used to degrade apoE protein (Fote et al., 2022). As apoE4 is retained in the intracellular space and causes autophagy dysfunction, this might further influence RCAN1 levels in *APOE4* carriers (Parcon et al., 2018; Persson et al., 2017). However, the same stimulus can have divergent effects in RCAN1 expression in different organs (Li et al., 2018; Peiris et al., 2012); thus, it is possible that, while we saw decreased RCAN1 levels in lymphocytes, its levels might be differently affected in the brain.

Our study also found decreased gene expression of GSK3β in heterozygous carriers, while protein expression was similar between the three groups. Furthermore, protein levels were increased in the previous study, which has now reversed back to normal. GSK3β is a multifunctional enzyme that engages in almost all aspects of development and functioning (D'Mello, 2021). It plays a central role in AD, where its increased activity and expression are involved in tau hyperphosphorylation and increased Aβ production (D'Mello, 2021; Hooper, Killick, & Lovestone, 2008). Although levels are reported to be elevated in brain samples of MCI and AD patients, studies in peripheral samples found divergent results and did not evaluate the effect of *APOE* genotype (Hye et al., 2005; Marksteiner & Humpel, 2009). However, mouse models found that *APOE4* is related to increased GSK3β activation

without an increase in total levels of GSK3β protein (Salomon-Zimri et al., 2019; Zhao et al., 2017; Zhou et al., 2016). In our study we did not evaluate GSK3β activation, but it is possible that it might be elevated irrespective of the normal protein levels. Furthermore, increased activation might also trigger a compensatory mechanism, which could explain the lower GSK3β gene expression in heterozygous carriers. As GSK3β induces increased levels of IL-1β (Green & Nolan, 2012), the reduced GSK3β gene expression in heterozygous carriers might also be reason for the lower elevation of IL-1β compared to homozygous carriers. As apoE3 and apoE4 are related to the differential activation and inhibition of many pathways, it is conceivable that heterozygous and homozygous *APOE4* carriers might present distinct regulation of certain pathways when compared to non-carriers (Ojo et al., 2021).

The studied enzymes are related to the phosphorylation of tau (Badia et al., 2013; D'Mello, 2021). Phosphorylated-tau231 is the earliest identified p-tau species to become elevated in AD, including pre-clinical AD (Holper, Watson, & Yassi, 2022); thus, it was possible that its levels would be increased in our middle-aged carriers. However, p-tau levels, which were previously increased in homozygous carriers, are now similar between the three genotypes. This agrees with the current enzyme levels seen in our study; that is, decreased RCAN1 protein in homozygous carriers and lower GSK3β gene expression in heterozygous carriers, even though both had slightly elevated PKR and p-p38. Plasma levels p-tau231 are higher in cognitively unimpaired older adults and elders than in young adults (Ashton et al., 2021); thus, it is possible that p-tau might not be elevated yet in our subjects. As carriers become older, however, further alterations in enzyme and p-tau levels may

be seen, as *APOE4* interacts with age to increase pathology (Saddiki et al., 2020). In fact, animal studies have demonstrated that apoE4 leads to a differential protein expression and phosphorylation pattern related to age both in the CNS and in the periphery (Yong, Lim, Low, & Wong, 2014; Zhao et al., 2017).

Lastly, our study found that PKR and p-p38 are higher in *APOE4* carriers that are women, who also tended to have lower antioxidant enzyme expression. Conversely, *APOE4* carriers that are men had higher longitudinal increase in GSSG and glutathione ratio. This sex-difference might indicate a possible defect in redox homeostasis and increased cellular stress in women carriers compared to men carriers. This would agree with the increased AD risk in women carriers and with the increased pathology and biomarkers seen in them (Altmann, Tian, Henderson, & Greicius, 2014; Farrer et al., 1997; Hohman et al., 2018). *APOE4* has also shown to act synergically with sex to alter proteome, with female *APOE4* mice showing reduced brain expression of several proteins, including antioxidant enzymes, and increased oxidative stress compared to male *APOE4* mice (Shi et al., 2014). As these differences did not occur in the previous study, 11 years ago, our results show that age, *APOE* genotype, and sex act together to impact many pathways that lead to increased AD risk (Jack et al., 2015; Zhao et al., 2020).

To summarize, our study found a reversal of the previous reductive stress present in young *APOE4* carriers, which now present increased oxidative damage and cell death, compared with non-carriers. This was seen as increased levels of lipid peroxides associated with decreased levels of antioxidant defenses. Moreover, the previous increase in stress-related proteins, including calcineurin and GSK3β, has now reversed and is decreased

in the case of RCAN1 (figure 18). However, PKR remains increased in *APOE4* carriers.

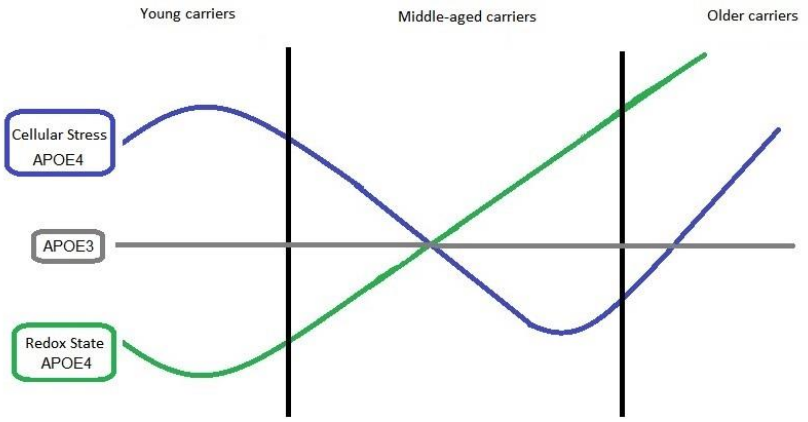


Figure 18: Redox state and cellular stress at younger adulthood and at middle-age in APOE4 carriers, according to our results. Young APOE4 carriers presented reductive stress and increased cellular stress compared to non-carriers. After 11 years, at middle-age, there is an exhaustion of the antioxidant system, a beginning of oxidative damage, and an alteration of stress pathways. These alterations could lead in the future to increased oxidative and cellular stress characteristics of AD.

Furthermore, we showed that middle-aged *APOE4* carriers present increased inflammation and sustained lymphocyte activation, when compared to non-carriers, in an allele dose-dependent manner. This was not associated with alterations in subjective or objective cognition. Figure 19 shows results according to our study. Thus, our results show that inflammation, redox homeostasis and cellular stress pathways change with age in *APOE4* carriers, and that this is influenced by sex, environmental risk factors for AD and *APOE4* allele-dose.

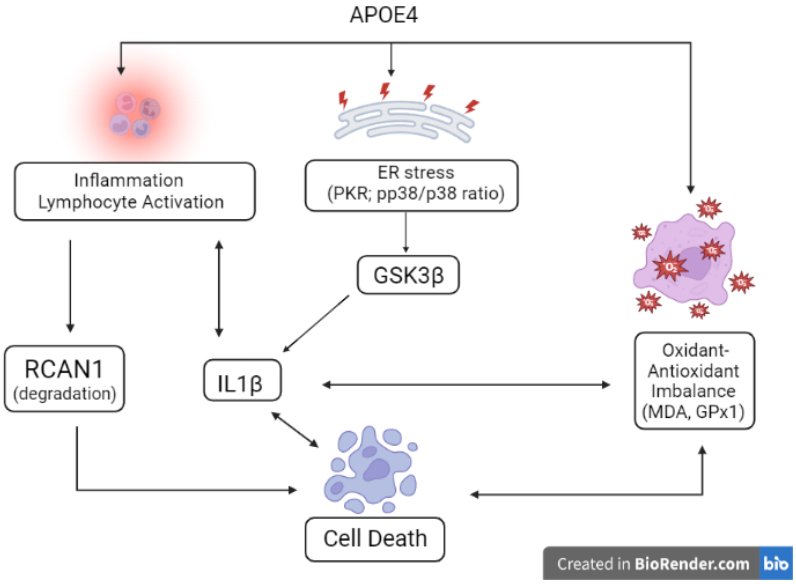


Figure 19: APOE4 effects in the periphery according to our results. *APOE4* activates inflammation and sustained lymphocyte activation (increased IL-1β, decreased RCAN1). It also causes a slight ER stress (increased PKR and tendency to increased p-p38/p38 ratio), which might affect GSK3β activity, without altering protein levels. Both inflammation and ER stress activate IL-1β, which, in turn, alters oxidant-antioxidant balance (increased MDA, decreased GPx1). *APOE4* itself may also alter redox regulation. These alterations lead to increased cell death. Furthermore, these pathways can also influence each other and most are two-way influences. In heterozygous carriers, it is possible that lower GSK3β and SOD1 gene expression might compensate these alterations. Created with BioRender.com

However, our study has some limitations. First, it is important to note that our study presents a relatively reduced sample, which did not allow for a better evaluation of other influencing factors. Second, our longitudinal study included *APOE4* carriers with a family history of AD, which is also a risk factor for AD in itself. This was necessary in our study because the frequency of the

ε4 allele is not high and randomly finding homozygous carriers would imply evaluating a large number of individuals. Third, it was not possible to evaluate quantitatively longitudinal results of WB. Lastly, we could not evaluate the same control individuals and, thus, used a new, comparable control group. Even so, we found important results that further elucidate changes related to *APOE4* that occur years before dementia.

CONCLUSION

6 Conclusions

1) There was a reversal of the previous reductive stress present in *APOE4* carriers in 2008.

2) There was also a reversal of the increase in calcineurin, GSK3β and RCAN1, which was present in *APOE4* carriers in 2008.

3) Middle-aged *APOE4* carriers in our study present higher inflammation levels, decreased antioxidant defenses and greater oxidative damage when compared to non-carriers.

4) The effects of *APOE4* on inflammation are allele-dose dependent.

5) There is a greater activation of cellular stress pathways related to PKR in *APOE4* carriers, especially heterozygous women.

6) Middle-aged *APOE4* carriers do not present an elevation in the expression of calcineurin, GSK3β and RCAN1, proteins related to tau pathology, when compared to non-carriers.

7) *APOE4* carriers do not present longitudinal cognitive alterations.

RESUMEN DE LA TESIS

7 Resumen de La Tesis

7.1 Introducción

Apolipoproteína E [apoE] es una proteína multifuncional, cuya función principal es el mantenimiento de la homeostasis lipídica (Marais, 2019; Phillips, 2014), pero que también está involucrada en diversas funciones fisiológicas, como control de la inflamación, de la inmunidad, repuesta antioxidante entre otras (Kockx et al., 2018).

El gene de la apolipoproteína E [*APOE*], localizado en el cromosoma 19q13.3, presenta 3 alelos principales, ε2, ε3 and ε4, (Abondio et al., 2019), los cuales codifican sus respectivas proteínas, apoE2, apoE3 y apoE4.

Estas isoformas se diferencian entre sí por sustituciones de aminoácidos en las posiciones 112 y 158 (Mahley, 2016a). ApoE3, la isoforma más común, contiene cisteína en la posición 112 y arginina en la posición 158; apoE2, la isoforma menos común, presenta cisteína en ambos sitios; y apoE4, con una frecuencia mundial del alelo ε4 de aproximadamente 14%, presenta arginina en ambas posiciones (Abondio et al., 2019; Marais, 2019; Semenkovich et al., 2016).

La sustitución de un único aminoácido cambia la estructura y la función de la proteína (Mahley et al., 2009). En el caso de la apoE4, el cambio implica la pérdida de funciones normales y la ganancia de funciones tóxicas, que causan diversas alteraciones patológicas (Liu et al., 2013). Esto hace que portadores de *APOE4* tengan una mayor predisposición a padecer algunas enfermedades, siendo especialmente relevante la Enfermedad de Alzheimer [EA] (Michaelson, 2014; Smith et al., 2019). La EA es la enfermedad neurodegenerativa más común relacionada con la edad. Su principal clínica

es un deterioro progresivo de las funciones cognitivas, que eventualmente llevan a la completa dependencia. Por eso, la EA tiene un gran impacto socio-sanitario, económico y personal (Alzheimer's Association, 2022; World Health Organization, 2017).

La forma esporádica de la enfermedad es la más común y presenta diversos factores de riesgo. No obstante, el principal factor de riesgo genético es la presencia de *APOE4*, la cual induce o aumenta diversas alteraciones patológicas relacionadas con la EA, incluyendo procesamiento alterado de péptido β amiloide [Aβ] y tau, estrés oxidativo y celular y muerte celular (Steele et al., 2022). Sin embargo, estas alteraciones patológicas también ocurren en personas cognitivamente sanas portadoras de *APOE4*, de modo que, conocer las alteraciones causadas por *APOE4* en estos sujetos puede ayudar a comprender mejor la EA y a crear mejores métodos preventivos.

7.2 Objetivos

Objetivo General

Se pretende conducir un estudio longitudinal prospectivo de una cohorte de 11 años de sujetos portadores de al menos un alelo 4 de la *APOE* y compararlos con sujetos no-portadores

Objetivos Específicos

1) Evaluar el estado cognitivo, oxidativo e inflamatorio actual y los niveles de proteínas relacionadas al estrés en sujetos portadores de al menos un alelo 4 de la *APOE* cuando comparados a no-portadores.

2) Comparar el estado cognitivo, oxidativo e inflamatorio y los niveles de proteínas relacionadas al estrés a lo largo de los últimos 11 años en sujetos portadores de al menos un alelo 4 de la *APOE*.

7.3 Metodología

Sujetos y Evaluación Neurocognitiva

El estudio actual es un seguimiento de una investigación anterior que culminó con la tesis doctoral de la Dra. María Del Carmen Badía Picazo, con el título "ESTUDIO DE ESTRÉS OXIDATIVO EN HIJOS DE PACIENTES CON ENFERMEDAD DE ALZHEIMER PORTADORES DEL ALELO 4 DE LA APOLIPOPROTEÍNA E."

Para el estudio longitudinal, reclutamos los portadores de *APOE4* que participaron en el estudio anterior. De estos, 24 accedieron a participar en este seguimiento, 14 heterocigotos y 10 homocigotos. No fue posible contactar los sujetos que formaron el grupo control en 2008, de modo que reclutamos 23 nuevos sujetos con las mismas características de nuestros participantes. De estos, 15 no eran portadores de *APOE4* y formaron el grupo control de 2019. Los otros 8 individuos fueron considerados portadores voluntarios e incluidos en el análisis transversal, lo cual incluyó un total de 47 sujetos cognitivamente sanos, 15 de los cuales eran no portadores (grupo control) y 32 eran portadores de *APOE4*, 19 heterocigotos y 13 homocigotos. Todos los grupos incluían sujetos de ambos sexos, con edad entre 35 y 65 años, de todos los niveles educativos. Todos los participantes viven en la Provincia de Valencia en la Comunidad Valenciana y hablan español como

lengua nativa. Los criterios de exclusión fueron: presentar una enfermedad aguda inflamatoria o infecciosa, usar sustancias que pueden afectar el proceso cognitivo, presentar déficits cognitivos o demencia, presentar importante discapacidad visual o auditiva o no firmar el consentimiento informado.

La participación en el estudio fue voluntaria y un consentimiento informado fue firmado por todos los participantes. En todo momento se respetaron los principios fundamentales establecidos en la Declaración de Helsinki, en el consejo de Europa relativo a los Derechos Humanos y la Biomedicina, en la Declaración de la UNESCO sobre el genoma humano y los Derechos Humanos, así como los requisitos establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética. El estudio fue aprobado por el comité de ética de la Universidad de Valencia (referencia: H1542117584721).

La entrevista y las pruebas cognitivas fueron realizadas individualmente en el despacho de la Dra. Ana Lloret, en el departamento de Fisiología de la Facultad de Medicina de la universidad de Valencia. La entrevista consistió en una serie de preguntas sobre la historia clínica y socio-demográfica de los sujetos. Además, sujetos fueron preguntados sobre la presencia de quejas subjetivas de memoria [QSM] y sobre la presencia de síntomas de ansiedad o depresión.

La versión española del test de aprendizaje auditivo verbal de Rey [RAVLT] fue usada para analizar memoria objetiva. Consta de un listado de 15 sustantivos que son leídos al paciente 5 veces y justo después de cada lectura, el paciente debe decir todas las palabras que sea capaz de recordar. De estas repeticiones, el número de palabras recordadas tras el primer

intento compone el recuerdo inmediato y la suma de todos los 5 intentos será el ítem aprendizaje. Pasados 30 minutos, se evalúa el recuerdo libre de la primera lista y el número de palabras recordadas compone el recuerdo diferido. Para el análisis transversal, la puntuación fue corregida por la edad, sexo y educación usando datos normativos de la clínica Mayo (Stricker et al., 2021). Para el análisis longitudinal, la puntuación fue convertida en T-score psicométrico (media de 50 y desviación estándar de 10).

Utilizamos el test de colores y palabras de Stroop (Stroop, 1935) para evaluar atención y control inhibitorio. La versión española normalizada (Golden, 2001) consta de tres páginas cada una con 100 elementos. En la primera página cada elemento es el nombre de un color (rojo, verde o azul) impreso en tinta negra. La segunda página consiste en 100 elementos iguales ("XXXX") impresos en tinta azul, verde o roja. La tercera página consiste en las palabras de la primera página impresas en los colores de la segunda, mezclados de modo que el color de la tinta y el significado de la palabra no coinciden en ningún caso. Los sujetos deben leer lo más rápido que puedan las palabras en la primera página, y el color de la tinta en la segunda y tercera páginas. La puntuación obtenida se compone de la cantidad de lecturas correctas en 45 segundos en cada página. Para el análisis transversal, la puntuación fue corregida por la edad y educación de acuerdo con las normas del Proyecto NEURONORMA y NEURONORMA adultos jóvenes (Peña-Casanova et al., 2009; Rognoni et al., 2013). Para el análisis longitudinal, la puntuación fue convertida en T-score psicométrico (media de 50 y desviación estándar de 10).

Utilizamos el cuestionario de fallos de memoria de la vida cuotidiana [MFE] (Sunderland et al., 1983) para investigar el acontecimiento, la frecuencia y

los tipos de fallos de memoria. En nuestro estudio, los sujetos leyeron y contestaron los 28 ítems según su experiencia personal y el posible acontecimiento de la imposibilidad de recordar algún suceso. El resultado total es la suma de la puntuación de los 28 ítems.

La escala de Depresión de Hamilton (Hamilton, 1960), es una escala desarrollada para evaluar la gravedad de los síntomas de depresión en la última semana. La escala contiene 17 ítems, cada uno con puntuación de 0-2 ó 0-4, dependiendo de la variable analizada. En nuestro estudio utilizamos las definiciones de gravedad propuestas por la Asociación Americana de Psiquiatría (Rush, 2000).

Obtención y procesamiento de las muestras

Tras la entrevista clínica, se recolectaron muestras de sangre de cada paciente en dos tubos, uno con EDTA y otro para separación de células. Todas ellas fueron procesadas para obtener sangre total, plasma y pellets de linfocitos.

En el caso de las muestras de sangre total, inmediatamente después de la extracción, una alícuota de 500µL de sangre total fue mezclada con K3-EDTA solamente y otra alícuota fue mezclada con K3-EDTA y N-etilmaleimida. Ambas fueron congeladas a -80°C para uso posterior. El resto de la muestra fue centrifugada y se recolectó el plasma por separado, que fue guardado en alícuotas a -80°C.

Respecto a la muestra en el tubo de para separación de células (Vacutainer® CPT™ tubes), ésta fue procesada para aislar linfocitos. Tras centrifugar las muestras, las células mononucleares fueron aisladas y lavadas con RPMI. Después, se añadió medio de cultivo a las células (hecho a partir de RPMI,

suero bovino fetal inactivado y antibiótico ((Penicillin/Streptomycin (P/S) 10,000 U/mL Antibiotic - GIBCO))). Las células se dejan en suspensión en una placa de Petri con medio de cultivo durante 3 horas a 37ºC. Tras la incubación, los linfocitos se aíslan y se recogen en dos alícuotas, una alícuota fue destinada a la citometría de flujo y lo demás fue lavado y centrifugado, resultando en un pellet que fue congelado a -80°C para posterior análisis.

Métodos Analíticos

Glutatión

Analizamos glutatión reducido [GSH] y glutatión oxidado [GSSG] con el protocolo descrito por Giustarini y colaboradores (Giustarini et al., 2013).

Para el análisis de GSSG, utilizamos las muestras mezcladas con K3-EDTA y N-etilmaleimida. Después de descongeladas, las muestras fueron mezcladas con ácido tricloroacético y centrifugadas. Una alícuota del sobrenadante fue separada, diluida con agua Milli-Q y mezclada con diclorometano. El sobrenadante resultante fue utilizado en la reacción: 945µL de PB200 (tampón fosfato, 0,2M, pH 7.4), 5µL de 20mM DTNB, 20µL de la muestra, 20µL de 4,8mM β-$NADPH^{+}$ y 20µL de 20U/mL glutatión reductasa [GR]. La reacción fue realizada en una microcubeta y la absorbancia medida por 1 minuto a 412nm. Después del minuto inicial, 10µL de 10µM GSSG estándar fueron añadidos a la cubeta y absorbancia fue medida por más 1 minuto. La concentración de GSSG fue calculada como se describe en el trabajo de Giustarini y colaboradores (Giustarini et al., 2013).

Respecto al GSH, utilizamos las muestras mezcladas con K3-EDTA. Tras descongelar las muestras, fueron mezcladas con ácido tricloroacético y centrifugadas. Una alícuota del sobrenadante fue separada, diluida con agua

Milli-Q y utilizada en la reacción: 945µL de PB200, 5µL de DTNB, 10 µL de la muestra, 20µL de β-$NADPH^+$ y 20µL de GR. La reacción fue realizada en una microcubeta y la absorbancia fue medida a 412nm pasados 60 segundos. El mismo procedimiento fue realizado para cada muestra y para los estándares de GSH. Una recta patrón fue utilizada para calcular la concentración de glutatión total. La concentración de glutatión reducido (expresada en nmol/mL de sangre) fue calculada utilizando la ecuación:

$$GSHred = glutatión\ total - (2 * GSSG).$$

La ratio glutatión oxidado-reducido fue calculada usando la ecuación:

$$GSSG/GSH\ Ratio = (GSSG/GSHred) * 100.$$

La ratio NADP oxidado-reducido fue calculada con la ecuación:

$$NADP/NADPH + ratio = k * GSSG/(GSH)2,$$

donde k es $1{,}98*10^{-2}\ M^{-1}$, la constante de equilibrio para la reacción de la GR.

Cromatografía líquida de alta eficacia [HPLC]

La cromatografía líquida de alta eficacia [HPLC] fue utilizada para medir la concentración de malondialdehído [MDA] en plasma. Utilizamos el método descrito por Wong y colaboradores (Wong et al., 1987), que implica la hidrolisis de lipoperóxidos presentes en la muestra, seguido de la reacción de MDA con 2 moléculas de ácido tiobarbitúrico [TBA]. Esta reacción forma una molécula colorida que puede ser medida por HPLC a 532 nm.

Utilizamos HPLC de fase reversa (Dionex UltiMate 3000 HPLC System, Thermo Scientific), con un flujo de la fase móvil de 1.25mL/minuto y un método isocrático. Los reactivos y la muestra fueron preparados de acuerdo con el protocolo de nuestro laboratorio. Para calcular la concentración de

MDA, el área bajo la curva de cada muestra fue medida y los resultados fueron interpolados en una recta patrón.

Ensayo por inmunoadsorción ligada a enzima [ELISA]

El ensayo por inmunoadsorción ligada a enzima [ELISA] es una de las técnicas más utilizadas para detectar antígenos específicos. Utilizamos este método para analizar la presencia de apoE4 (Apolipoprotein E4 (human) ELISA Kit - K4699-100 – BioVision) y los niveles plasmáticos de interleuquina-1β [IL-1β] (Human IL-1 beta ELISA Kit - 2ab214025 – Abcam) y del factor de necrosis tumoral alfa [TNF-α] (Human TNF alpha Simple Step ELISA® Kit - ab181421-Abcam). Todas las muestras y estándares fueron analizados en duplicado. Los reactivos y el procedimiento fueron realizados de acuerdo con los manuales de los fabricantes.

Citometría de Flujo

Utilizamos el citómetro de flujo para determinar niveles de muerte celular utilizando anexina (ANXVF-200T- immunostep) e yoduro de propidio (81845 - sigma-aldrich). Además, utilizamos H_2DCFDA (D399 - Molecular Probes) y dihidroetidio (37291 - Sigma-Aldrich) para medir especies reactivas y superóxido, respectivamente.

Para ello, separamos diferentes muestras de linfocitos y las procesamos de acuerdo con las indicaciones de los fabricantes en el servicio de citometría de flujo de la Unidad Central de Investigación de Medicina (UCIM) en la Facultad de Medicina de la Universidad de Valencia.

Western Blotting

Se llevó a cabo la técnica del Western Blotting [WB] para determinar la expresión en linfocitos de distintas proteínas: calcineurina (2614 - Cell Signaling), RCAN1 (ab140131- abcam), GSK3β (ep 7194 - MBL), p38 (9212S - Cell Signaling), p-p38 (9211S - Cell Signaling) y p-Tau231 (A00406 - GenScript).

El procedimiento empezó con el procesamiento de las muestras de linfocitos que fueron sometidas a lisis utilizando un tampón de lisis (una mezcla de Tris, glicerol, SDS, ortovanadato de sodio e inhibidor de proteasa) y ultrasonicación (Ultrasonic Processor Vibra-Cell VCX-500; Sonics). Después de esto, muestras fueron centrifugadas y el sobrenadante separado.

La concentración de proteínas en el sobrenadante fue medida con el método de Lowry (Lowry et al., 1951), en el cual la muestra diluida en agua Milli-Q es mezclada e incubada con el reactivo de Lowry (L3540-1VL - Sigma-Aldrich). A esto se sigue una segunda reacción e incubación con el reactivo de Folin-Ciocalteu (F9252-500mL- Sigma-Aldrich), tras la cual se forman complejos proteicos capaces de absorber luz a una longitud de onda de 660 nm. Al analizar estándares con concentraciones conocidas de proteína en las mismas condiciones de las muestras, se puede crear una recta patrón en la cual se interpolan los valores de absorbancia de las muestras y se obtiene su concentración proteica.

Una vez conocida la concentración de proteínas existente en las muestras, seguimos el procedimiento con la técnica del western blot. Para este, utilizamos Mini-PROTEAN Tetra Handcast Systems, Mini-PROTEAN® Tetra Cell; Mini Trans-Blot®Electrophoretic Transfer Cell y PowerPac™ Basic Power Supply, de *Bio-Rad*. Primero preparamos geles de poliacrilamida SDS-PAGE de 12,5%. Después, añadimos tampón de carga 2X (una mezcla de 50mM

Tris-HCl pH 6.8; 3% SDS; 10% glicerol; 0,005% azul de bromofenol; 5% 2-mercaptoetanol) a un volumen de muestra que contenía 15µL/mL de proteínas, seguido de una incubación a 95ºC durante 10 minutos. Seguidamente, se cargó las muestras en los pocillos del gel de poliacrilamida, el cual se colocó en la cubeta de electroforesis con el Tampón de electroforesis (0.25M Tris, 1.9M Glicina, 1% (w/v) SDS). Se sometió los geles a un voltaje continuo de 100 voltios hasta que el frente de las muestras llegara al final del gel.

Una vez terminada la electroforesis, se recogió el gel de poliacrilamida y se preparó la electrotransferencia creando un "sándwich", el cual contiene el gel de poliacrilamida y una membrana de nitrocelulosa. Una vez preparado el "sándwich" se coloca en la cubeta de transferencia junto con el Tampón de transferencia (25mM Tris, 192mM Glicina, 20% (v/v) metanol) y un acumulador de frio. Todo el sistema fue mantenido en hielo durante el procedimiento. Se programó la fuente de alimentación con un amperaje constante de 240 mA (120 mA por cada gel), durante 90 minutos y se vigiló el voltaje para que no superara los 100 voltios.

Terminada la transferencia, la membrana fue secuencialmente incubada con una solución de bloqueo, un anticuerpo primario específico para la proteína de interés y un anticuerpo secundario. Seguidamente, se reveló la membrana añadiendo 1mL de Luminol (Immobilon Classico Western HRP Substrate - WBLUC0500 - Millipore) en la superficie de la membrana y detectando la señal luminosa con ImageQuant™ LAS 4000 mini biomolecular imager, GE Healthcare Bio-Sciences.

También se determinaron los niveles de β-actina (A1978 - Sigma-Aldrich) como control de carga. Para ello, la membrana fue sometida a una

incubación con la solución de Stripping (Restore Western Blot STRIPPING BUFFER – 21059 - Thermo Scientific) que elimina los anticuerpos unidos a la membrana, pero mantiene las proteínas. Después de quitar la solución y lavar la membrana, esta era analizada con otro anticuerpo.

Finalmente, las imágenes obtenidas de las membranas fueron analizadas con el software *ImageGauge V4.0,* que permite analizar las bandas presentes en cada membrana.

Reacción en cadena de la polimerasa [PCR]

Utilizamos la reacción en cadena de la polimerasa [PCR] para analizar la expresión de diversos genes de interés en linfocitos. Para esto, primero realizamos la extracción de ARN de los pellets de linfocitos utilizando el kit NucleoSpin TriPrep (74096650 - Macherey-Nagel), siguiendo el protocolo del fabricante. Seguidamente, realizamos la reacción en cadena de la polimerasa con transcriptasa inversa (RT-PCR) para obtener ADN complementario a partir del ARN aislado. Para esto, utilizamos el High capacity cDNa Reverse Transcription Kit (4368814 - Thermo Fisher Scientific) y las muestras y controles fueron sometidos al termociclador (T100 PCR thermal cycler - Bio-Rad) en las condiciones indicadas por el fabricante.

Una vez obtenido ADN complementario, realizamos la PCR cuantitativa. Nuestro estudio utilizó las sondas de TaqMan® Gene Expression Assay (Thermo Fisher Scientific) para analizar la expresión de los siguientes genes: GCLC (Hs00155249_m1), GCLM (Hs00157694_m1), GPx1 (Hs00829989_gH), GSK3β (Hs00275656_m1), PKR (Hs00169345_m1), calcineurina (Hs00330865_s1), RCAN1 (Hs01120954_m1), SOD1 (Hs00533490_m1) y GAPDH (Hs02786624_g1). Todos los ensayos fueron realizados de acuerdo

con las instrucciones de los fabricantes. Cada muestra fue analizada en triplicado y GAPDH fue analizado en cada placa para ser usado como normalizador. Se utilizó el termociclador (QuantStudio™ 5 Real-Time PCR System - A3432 - Applied Biosystems™) en las condiciones descritas en el manual del fabricante. El resultado fue calculado utilizando el método $2^{-\Delta Ct}$, en el que ΔCt se refiere a los valores de Ct (threshold cycle number) normalizados con la expresión de GAPDH.

Los cambios en la expresión génica entre 2019 y 2008 fueron calculados como "fold-change", utilizando la siguiente expresión descrita por Schmittgen & Livak (Schmittgen & Livak, 2008):

$$Fold\ Change = \frac{-1}{2019\ \text{mean expression}/2008\ \text{mean expression}}.$$

Cambios longitudinales fueron considerados significativos si fold-change ≥1.5 y p-value ≤ 0.05 (Dalman, Deeter, Nimishakavi, & Duan, 2012).

Análisis Estadístico

Para el análisis estadístico se usó el programa *IBM SPSS Statistics 22*. Fueron realizadas pruebas de normalidad en todas las variables, específicamente el test de Kolmogorov-Smirnov con la corrección de Lilliefors. Para el análisis transversal, las comparaciones de 2 medias independientes en muestras paramétricas se realizaron mediante el test T de Student y en muestras no paramétricas se compararon con el test Mann-Whitney y el test Chi cuadrado para variables categóricas. Cuando la comparación de medias implica más de 2 se usaron el test ANOVA para muestras paramétricas y el test Kruskal-Wallis para las no paramétricas. Para el análisis longitudinal, muestras nominales

pareadas fueron analizadas con el test de McNemar. Muestras paramétricas pareadas fueron analizadas con el test T de Student pareado y las no paramétricas con el test de Wilcoxon. En todos los test se consideró un resultado estadísticamente significativo cuando el p-valor es menor a 0,05.

7.4 Resultados y Discusión

Los sujetos que participaron en nuestro estudio son de mediana edad (media de edad 51,55 ± 7,9), aproximadamente 60% son mujeres y casi el 50% tiene estudios terciarios. No hay diferencias de sexo, edad o nivel de estudios entre portadores de *APOE4* y no-portadores, ni entre los 3 genotipos.

APOE4 y Cognición

Las quejas subjetivas de memoria [QSM] pueden ser una señal prodrómica de deterioro cognitivo, aunque no todas las personas que las presenten tendrán alteraciones futuras (Lee et al., 2020). En nuestro estudio, evaluamos las QSM con una pregunta de respuesta dicotómica (sí o no) y no encontramos diferencias entre portadores y no portadores. No obstante, en el análisis longitudinal, vimos que los sujetos controles actuales presentan más QSM que los controles de hace 10 años. Esto puede deberse a diversos factores, incluyendo edad, ya que personas mayores presentan más quejas de memoria generales que personas más jóvenes (Ginó et al., 2010). Además, las QSM son comunes en todas las edades, y la percepción de problemas de memoria por un individuo puede cambiar diariamente debido a factores estresantes, ansiedad y depresión (Neupert et al., 2006; Sharifian & Zahodne, 2021). Nosotros vemos esto en nuestro estudio cuando analizamos los resultados de MFE, que mostraron que nuestros sujetos presentan un MFE

más bajo que la media española (Montejo Carrasco et al., 2012). Además, la mayoría de fallos de memoria en nuestro estudio eran, en realidad, fallos de atención, que son los más frecuentes en individuos sanos (Montejo et al., 2014).

Nuestro estudio incluyó solamente individuos cognitivamente sanos. No obstante, era posible que hubiese pequeñas diferencias a lo largo del tiempo entre controles y portadores de *APOE4* en evaluaciones cognitivas objetivas. Por esto, evaluamos memoria objetiva con RAVLT y atención con el test de Stroop, pero no encontramos diferencias entre los 3 genotipos ni a lo largo del tiempo.

La depresión es un síntoma neuropsiquiátrico frecuente en la EA (Lyketsos et al., 2011). No obstante, la relación entre *APOE4* y depresión sigue siendo una incógnita, ya que algunos estudios encontraron un aumento de prevalencia entre portadores mientras que otros estudios no muestran los mismos resultados (Burns et al., 2020; Evans & Rajan, 2015). Transversalmente, nuestro estudio no muestra diferencias significativas en la prevalencia de depresión entre los sujetos de los 3 genotipos, aunque los portadores de *APOE4* muestran tendencia a una mayor prevalencia. Sin embargo, encontramos un aumento significativo a lo largo del tiempo en portadores de *APOE4*, especialmente entre las mujeres portadoras que presentan una mayor prevalencia que hombres portadores. Además, la prevalencia de depresión en jóvenes y en hombres solo aparece entre portadores de *APOE4*. Es conocido que las mujeres tienen una mayor susceptibilidad a padecer depresión comparadas con hombres de la misma edad (Labaka et al., 2018). Nuestros resultados sugieren que los portadores de *APOE4* pueden igualmente tener una mayor susceptibilidad. Esto también fue propuesto por

Chhibber y Zhao (Chhibber & Zhao, 2017), quienes encontraron que apoE4 interactúa con receptores β de estrógeno en ratones hembras para disminuir la expresión del factor neurotrófico derivado del cerebro y de receptores de serotonina, aumentando el riesgo de depresión.

APOE4 e Inflamación

La inflamación y las alteraciones del sistema inmune son características comunes de la EA y de la *APOE4*. En este sentido, nuestros resultados muestran que sujetos homocigotos *APOE4* presentan niveles de IL-1β mayores comparado con sujetos heterocigotos y sujetos no portadores. De forma similar, los sujetos heterocigotos presentan niveles más elevados de IL-1β que los sujetos no portadores. IL-1β es una citoquina proinflamatoria, lo que sugiere que *APOE4* está relacionada a una mayor inflamación, que es dosis-dependiente incluso en sujetos sanos de mediana edad.

Este actual aumento de inflamación contrasta con el estudio anterior, que mostraba que los portadores no presentaban alteraciones inflamatorias. Esto indicaría que la patología relacionada a *APOE4* puede variar con la edad. No obstante, el estudio anterior analizó solamente niveles de TNF-α; mientras que nuestro estudio actual midió tanto IL-1β como TNF-α, pero solo pudo analizar IL-1β, puesto que los niveles de TNF-α estaban por debajo del límite de detección. Sin embargo, es posible que los niveles de TNF-α fueran igualmente bajos en los 3 genotipos, lo que indicaría un aumento preferencial de IL-1β en sujetos portadores de *APOE4*. Ambas citoquinas son proinflamatorias y elevadas por el factor de transcripción NFκβ, que es, por su vez, activado por apoE4 (Arnaud et al., 2022). Sin embargo, existen estudios que muestran que únicamente IL-1β se aumenta mediante la ruta C/EBP, un factor de transcripción que eleva los niveles de apoE4 y que es a

su vez activado por apoE4 (Wang, Z. et al., 2021; Wang, Z. et al., 2022; Xia et al., 2021). Esta activación diferencial del sistema inmune por apoE4 puede ser la causa del aumento exclusivo de IL-1β en portadores de *APOE4* que mostramos en este trabajo. Además, considero importante destacar que los portadores heterocigotos tienen niveles más bajos de IL-1β que portadores homocigotos, puesto que los primeros también expresan otra isoforma de apoE que no estaría directamente implicada en la elevación de los niveles de IL-1β.

APOE4 y Daño Oxidativo

El estrés oxidativo es un mecanismo patológico importante en la EA, y es especialmente relevante en portadores de *APOE4*, puesto que este alelo presenta propriedades antioxidantes alteradas (Jofre-Monseny et al., 2007; Liou et al., 2021). Además, se han descrito alteraciones oxidativas en sujetos portadores sanos (Butterfield & Mattson, 2020; Nunomura et al., 2001). Por lo tanto, decidimos analizar marcadores oxidativos en nuestro estudio.

Encontramos que los portadores de *APOE4* presentan un aumento de los niveles plasmáticos de MDA y una disminución de la expresión génica de glutatión peroxidasa 1 [GPx1], comparados con los sujetos no portadores. Además, portadores heterocigotos presentan menor expresión génica de superóxido dismutasa 1 [SOD1] comparados con no portadores, la cual ha disminuido a lo largo del tiempo. Esta alteración del estado oxidativo fue apoyada por el aumento longitudinal de MDA que ocurrió solamente en sujetos portadores de *APOE4*. De modo que de nuestros resultados se puede deducir que, a mediana edad, los portadores de *APOE4* presentan daño oxidativo que no estaba presente cuando eran más jóvenes. Esto coincide con otros estudios publicados que muestran un aumento del daño oxidativo

en sujetos portadores sanos (Smith et al., 1998; Tsuda et al., 2004). Nuestros resultados también coinciden con estudios *in vitro* y en ratones que encontraron una relación entre *APOE4* y una reducción de la expresión o de la actividad de enzimas antioxidantes, incluyendo GPx1 (Ben Khedher et al., 2021; Khan et al., 2022; Persson et al., 2017).

Como nuestros resultados mostraron un aumento de daño oxidativo en portadores de *APOE4*, también esperábamos un aumento de los niveles de especies reactivas. No obstante, esto no ocurrió. También esperábamos encontrar un aumento de las ratios GSSG/GSH y $NADP/NADPH^{+}$, que son características de estrés oxidativo. Sin embargo, los sujetos portadores solo presentaron una tendencia a la elevación de las ratios, cuando comparados a los no portadores. Esto puede deberse a los niveles reducidos de GPx1, ya que, sin esta enzima las especies reactivas no pueden reaccionar con la GSH (Lubos et al., 2011). Por lo tanto, nuestros resultados sugieren que, en sujetos de mediana edad, *APOE4* puede inducir un inicio de estrés oxidativo por la reducción de defensas antioxidantes, lo que permite que niveles normales o levemente aumentados de especies reactivas puedan causar daño oxidativo. Además, el estrés reductivo presente anteriormente en nuestros sujetos puede haber incrementado el daño, visto que este tipo de estrés puede causar un aumento de la producción de especies reactivas y puede causar daño oxidativo incluso en la presencia de equivalentes de reducción (Korge et al., 2015; Qiao et al., 2022; S Narasimhan et al., 2020).

Nuestros resultados también mostraron que factores de riesgo ambientales para la EA, como tabaquismo y diabetes, interactúan con apoE4, aumentando los niveles de MDA solamente en sujetos portadores. Esto muestra que factores ambientales y genéticos se combinan para influenciar

el estado oxidativo e incrementar la patología (Dose et al., 2016; Zhang, N. et al., 2021).

APOE4 y Estrés Celular

APOE4 y la EA causan estrés celular y activan diversas vías de respuesta al estrés (Dose et al., 2016; Weidling & Swerdlow, 2019). La activación de estas vías crea un bucle patológico, ya que aumenta los niveles y/o la activación de varias enzimas que inducen la fosforilación de tau, incluyendo GSK3β, RCAN1 y PKR (Badia et al., 2013). Por lo tanto, decidimos analizar estrés y muerte celular en nuestros sujetos.

Nuestros resultados mostraron un aumento de la expresión génica de PKR en portadores de *APOE4*, en especial los sujetos heterocigotos y las mujeres, cuando comparados con los sujetos no portadores. Además, la expresión génica de PKR permaneció aumentada a lo largo del tiempo. Esto coincide con estudios que encontraron un aumento de la actividad de PKR y de la activación de respuestas al estrés relacionadas a *APOE4* (Oliveira & Lourenco, 2016; Ramakrishna et al., 2021; Segev et al., 2015).

Distintos tipos de estrés celular pueden estar relacionados a *APOE4* en nuestros sujetos. En su juventud estos sujetos presentaban estrés reductivo, que altera el estado redox necesario para el correcto plegamiento de proteínas (Handy & Loscalzo, 2017). Este tipo de estrés también activa la cascada de GSK3β/tau, promoviendo la fosforilación y acumulo de la proteína tau y su toxicidad (S Narasimhan et al., 2020). Además, hay un aumento de la síntesis y de la renovación de proteínas, lo que aumenta aún más el acumulo de proteínas mal plegadas (Handy & Loscalzo, 2017). Este elevado estrés celular pudo ser visto en el estudio anterior en el aumento de

PKR, GSK3β y RCAN1, proteínas relacionadas al estrés, encontrado en portadores de *APOE4*. No obstante, el estrés celular relacionado a la *APOE4* causa disfunción celular de un modo dependiente de la edad (Zhong et al., 2009). El estrés reductivo crónico en nuestros sujetos pudo haber llevado, con el tiempo, al agotamiento del sistema antioxidante y al daño oxidativo (Lloret et al., 2016; Xiao & Loscalzo, 2020) que vemos ahora en nuestros sujetos. Además, el estrés celular crónico causa un agotamiento de las respuestas al estrés que intentan mantener la función celular e inician vías metabólicas de muerte celular programada (Lourenco et al., 2015). Esto vemos en nuestros sujetos portadores, que presentan mayor apoptosis que los no portadores, y un aumento de apoptosis a lo largo del tiempo.

Nuestro estudio también encontró una disminución significativa de la expresión proteica y una tendencia a menor expresión génica de RCAN1 en portadores homocigotos cuando comparados a portadores heterocigotos y a no-portadores. Por otro lado, no encontramos diferencias significativas en la expresión de calcineurina, aunque portadores homocigotos tienden a presentar menores niveles de proteína. Longitudinalmente, portadores homocigotos presentaron un cambio en la expresión de estas proteínas; de un aumento de la expresión de ambas proteínas, pasaron a una expresión reducida de RCAN1.

Estos resultados son opuestos a lo esperado, ya que la sobreexpresión crónica de RCAN1 en la EA está asociada al daño celular y a la progresión patológica (Ermak et al., 2001). No obstante, la mayor parte de los estudios en pacientes con EA no consideró la influencia de *APOE4*. De hecho, un estudio reciente encontró una reducción en la expresión génica y proteica de RCAN1 en el lóbulo frontal de sujetos con EA portadores de *APOE4*, de un

modo dependiente de la cantidad de alelos (Delikkaya et al., 2019), lo que está de acuerdo con nuestros resultados. Además, RCAN1 es una proteína inducida por el estrés que protege la célula del estrés agudo. Pero durante el estrés crónico, sus niveles son reducidos, lo que cambia las respuestas celulares y llevan a la muerte celular (Bartoszewski et al., 2020), como vimos en nuestro estudio.

Además, encontramos que los portadores homocigotos de *APOE4* presentan expresión reducida de RCAN1 y niveles aumentados de IL-1β cuando se comparan con portadores heterocigotos y a no-portadores; lo que indicaría una relación entre inflamación y respuesta celular al estrés. Esto tiene sentido, visto que la activación mantenida de linfocitos necesaria para aumentar los niveles de IL-1β, se consigue al aumentar la degradación de RCAN1 (Valdor et al., 2014) por la misma vía utilizada en la degradación de la proteína apoE (Fote et al., 2022). La mayor retención intracelular de apoE4 y la disfunción de la autofagia que esta causa, pueden ser la causa los niveles de RCAN1 que encontramos en los portadores de *APOE4* (Parcon et al., 2018; Persson et al., 2017). No obstante, un mismo estímulo puede tener efectos distintos en diferentes órganos (Li et al., 2018; Peiris et al., 2012); por lo que es posible que los niveles de RCAN1 en el cerebro sean distintos a los encontrados en linfocitos.

Encontramos en nuestro estudio que los portadores heterocigotos presentan una expresión génica reducida de GSK3β, mientras que la expresión proteica de esta proteína es similar entre los 3 genotipos. Además, los niveles de esta proteína se encontraban aumentados hace 10 años, lo que ha revertido al normal actualmente. GSK3β tiene un papel central en la EA, donde el aumento de su actividad y expresión están involucradas en la

hiperfosforilación de tau y en la producción aumentada de Aβ (D'Mello, 2021; Hooper et al., 2008). No obstante, pocos estudios evaluaron el efecto del genotipo *APOE* con respecto a esta enzima. En modelos animales se ha visto una relación entre *APOE4* y un aumento de la actividad de GSK3β, sin un aumento de los niveles de la proteína (Salomon-Zimri et al., 2019; Zhao et al., 2017; Zhou et al., 2016). Aunque no hemos evaluado la activación de GSK3β en nuestro estudio, consideramos que es posible que estuviera elevada, independiente de los niveles normales de proteína. Un aumento de activación podría desencadenar un mecanismo compensatorio, el cual podría explicar la menor expresión génica de GSK3β en heterocigotos. Además, como GSK3β induce un aumento de IL-1β (Green & Nolan, 2012), la disminución de su expresión génica en heterocigotos podría ser el motivo de la menor elevación de IL-1β, respecto a los portadores homocigotos. ApoE3 y apoE4 causan una activación e inhibición distinta en diversas vías intracelulares, de modo que es posible que portadores homocigotos y heterocigotos presenten una regulación distinta de ciertas vías, cuando comparados a no-portadores (Ojo et al., 2021).

Las enzimas que analizamos en este estudio están relacionadas con la fosforilación de la proteína tau (Badia et al., 2013; D'Mello, 2021). Los niveles de p-tau, que estaban elevados en el estudio anterior, ahora se encuentran similares entre sujetos de los 3 genotipos. Esto está de acuerdo con los niveles de enzimas vistos en nuestro estudio; es decir, niveles reducidos de la proteína RCAN1 en homocigotos y reducción de la expresión génica de GSK3β en heterocigotos, aunque ambos tuvieran una tendencia a mayores niveles de pp38 y heterocigotos tuvieran mayores niveles de PKR.

Finalmente, nuestro estudio encontró que PKR y p-p38 están más elevadas en mujeres portadoras de *APOE4* que en hombres portadores. Además, éstas tienden a tener niveles más bajos de antioxidantes. Por otro lado, hombres portadores de *APOE4* tienden a tener un mayor aumento de GSSG y de la ratio GSSG/GSH a lo largo del tiempo que mujeres portadoras. Esta diferencia puede indicar un posible defecto de la homeostasis del sistema redox y mayor estrés celular en mujeres portadoras que en hombres portadores de *APOE4*. En modelos animales, *APOE4* actúa sinérgicamente con sexo para alterar el proteoma, reduciendo la expresión de diversas proteínas y aumentando el estrés oxidativo en aminales *APOE4* hembras, respecto a los *APOE4* machos (Shi et al., 2014). Esto es congruente con el riesgo más aumentado de padecer EA y con mayor patología en mujeres portadoras que en hombres portadores (Altmann et al., 2014; Farrer et al., 1997; Hohman et al., 2018).

En resumen, nuestro estudio encontró una reversión del estrés reductivo que presentaban nuestros sujetos portadores de *APOE4* en el estudio anterior. Además, estos sujetos ahora presentan un aumento de daño oxidativo, caracterizado por aumento de MDA y reducción de GPx1, y muerte celular, cuando se comparan con los sujetos no portadores. Asimismo, el aumento que existía en el estudio previo en los niveles de proteínas relacionadas al estrés se ha revertido y ahora estas proteínas se encuentran en niveles normales o incluso reducidos, como es el caso de RCAN1. Igualmente, mostramos que los portadores de *APOE4* cognitivamente sanos de mediana edad presentan un aumento de inflamación, cuando los comparamos con los no portadores. Además, este aumento es dosis dependiente según a la cantidad de alelos *APOE4*. Esto no estuvo asociado a alteraciones cognitivas

objetivas ni subjetivas. Por lo tanto, nuestros resultados muestran que la inflamación, la homeostasis redox y las vías de estrés celular cambian con la edad en portadores de *APOE4*, y que el genotipo *APOE*, el sexo, la edad y factores de riesgo ambientales actúan en conjunto para afectar diversas vías que llevan a la EA (Jack et al., 2015; Zhao et al., 2020).

No obstante, nuestro estudio tiene algunas limitaciones. La primera, es importante notar que nuestra muestra es relativamente reducida. Segundo, el estudio longitudinal incluyó portadores de *APOE4* que tenían una historia familiar de EA en un padre o una madre, lo que es un factor de riesgo para la EA. Esto fue necesario porque la frecuencia del alelo ε4 no es muy alta y encontrar homocigotos de forma aleatoria implicaría evaluar una gran cantidad de individuos. Tercero, no fue posible evaluar cuantitativamente los resultados longitudinales de WB y PCR. Por último, no pudimos evaluar el mismo grupo control que se utilizó en el primer estudio y, por lo tanto, utilizamos un nuevo grupo control, comparable al primero. A pesar de eso, encontramos resultados importantes que nos ayudan a aclarar los cambios relacionados a *APOE4* que ocurren años antes del inicio de los síntomas.

7.5 Conclusiones

1) Ocurrió una reversión del estrés reductivo presentado por nuestros sujetos portadores de *APOE4* en el año 2008.
2) Ocurrió una reversión del aumento de calcineurina, GSK3β y RCAN1, presentado por nuestros sujetos portadores de *APOE4* en el año 2008.

3) Los sujetos portadores de *APOE4*, en nuestro estudio, tienen mayores niveles de inflamación, una reducción de las defensas antiinflamatorias y un mayor daño oxidativo, cuando comparados a sujetos no-portadores.

4) Los efectos de la *APOE4* en la inflamación son dependientes de la cantidad de alelos.

5) Hay una mayor activación de vías de estrés celular relacionadas con PKR en sujetos portadores de *APOE4*, especialmente en mujeres heterocigotos.

6) Portadores de *APOE4* de mediana edad no presentan una elevación de la expresión de calcineurina, GSK3β y RCAN1, proteínas relacionadas con la fosforilación de la proteína tau, cuando comparados a no-portadores.

7) Portadores de *APOE4* no presentan alteraciones cognitivas a lo largo del tiempo.

ANNEX

8 Annex

8.1 ANNEX 1 – Alzheimer's Disease Diagnostic Criteria.

Criteria defined by the International Working group for the clinical diagnosis of Alzheimer's disease (Dubois et al., 2021)**:** The diagnosis of AD is clinical–biological and requires the presence of a specific clinical phenotype of AD and both Aβ and tau positive biomarkers.

Alzheimer's disease phenotypes:

1. the amnestic syndrome of the hippocampal type (typical)
2. the posterior cortical atrophy variant
3. the logopenic variant primary progressive aphasia
4. Uncommon phenotypes, which include the behavioral variant or dysexecutive variant, the corticobasal variant and other variants of primary progressive aphasia.

Isolated subjective memory complaints and subjective cognitive decline without objective cognitive impairment, are not considered part of the AD phenotype.

Recommended biomarker measures:

1. Aβ
    a) Low CSF Aβ42
    b) Increased CSF Aβ40–Aβ42 ratio (preferred to low CSF Aβ42)
    c) High tracer retention in amyloid PET.
2. Tau pathology

a) High CSF phosphorylated tau
b) Increased ligand retention in tau PET.

Plasma biomarkers and the investigation of biomarkers in cognitively unimpaired individuals are not currently recommended in clinical practice.

If pathophysiological biomarkers are not available, patients should have a clinical syndromic diagnosis.

8.2 ANNEX 2 – Clinical Questionnaire

CUESTIONARIO

NUMERO: _____

SEXO:

EDAD:

DATOS FAMILIARES:

MADRE O PADRE:

EDAD DE INICIO:

OTROS FAMILIARES AFECTADOS:

ANTECEDENTES PERSONALES:

☐ DIABETES MELLITUS

☐ DISLIPIDEMIA

☐ HTA: ☐ CONTROLADA ☐ GRAVE O MAL CONTROLADA

☐ INSUFICIENCIA RENAL: ☐ LEVE ☐ MODERADA ☐ GRAVE

☐ TABAQUISMO: ☐ ACTIVO ☐ EXFUMADOR. CIG/ DIA____

☐ ALCOHOLISMO: ☐ ACTIVO ☐ EXBEBEDOR COPA/DIA

☐ ENFERMEDAD NEOPLÁSICA

☐ MENOPAUSIA

☐ ENFERMEDAD DEGENERATIVA: ELA, ETC.

☐ ENFERMEDADES AUTOINMUNES

☐ SIDA

☐ HEPATITIS CRÓNICA ACTIVA

☐ DEPRESIÓN O SÍNDROME ANSIOSO

☐ OTROS:

MEDICACIÓN ACTUAL: _____

NIVEL DE ESCOLARIZACIÓN: _____

HISTORIA LABORAL : tipo de trabajo, contacto con tóxicos.....

_____

¿EJERCICIO FÍSICO REGULAR?

☐ Sí

☐ No

¿PRESENTA PROBLEMAS DE MEMORIA EN LA ACTUALIDAD?

☐ Sí

☐ No

¿PRESENTA SÍNTOMAS DE ANSIEDAD Y/O DEPRESIÓN EN LA ACTUALIDAD?

8.3 ANNEX 3 – RAVLT, Spanish Version

LISTA A:

TRIAL I	**TRIAL II**	**TRIAL III**	**TRIAL IV**	**TRIAL V**
Tambor	Tambor	Tambor	Tambor	Tambor
Café	Café	Café	Café	Café
Sombrero	Sombrero	Sombrero	Sombrero	Sombrero
Color	Color	Color	Color	Color
Cortina	Cortina	Cortina	Cortina	Cortina
Pariente	Pariente	Pariente	Pariente	Pariente
Granjero	Granjero	Granjero	Granjero	Granjero
Casa	Casa	Casa	Casa	Casa
Timbre	Timbre	Timbre	Timbre	Timbre
Luna	Luna	Luna	Luna	Luna
Nariz	Nariz	Nariz	Nariz	Nariz
Río	Río	Río	Río	Río
Escuela	Escuela	Escuela	Escuela	Escuela
Jardín	Jardín	Jardín	Jardín	Jardín
Pavo	Pavo	Pavo	Pavo	Pavo

Lista B

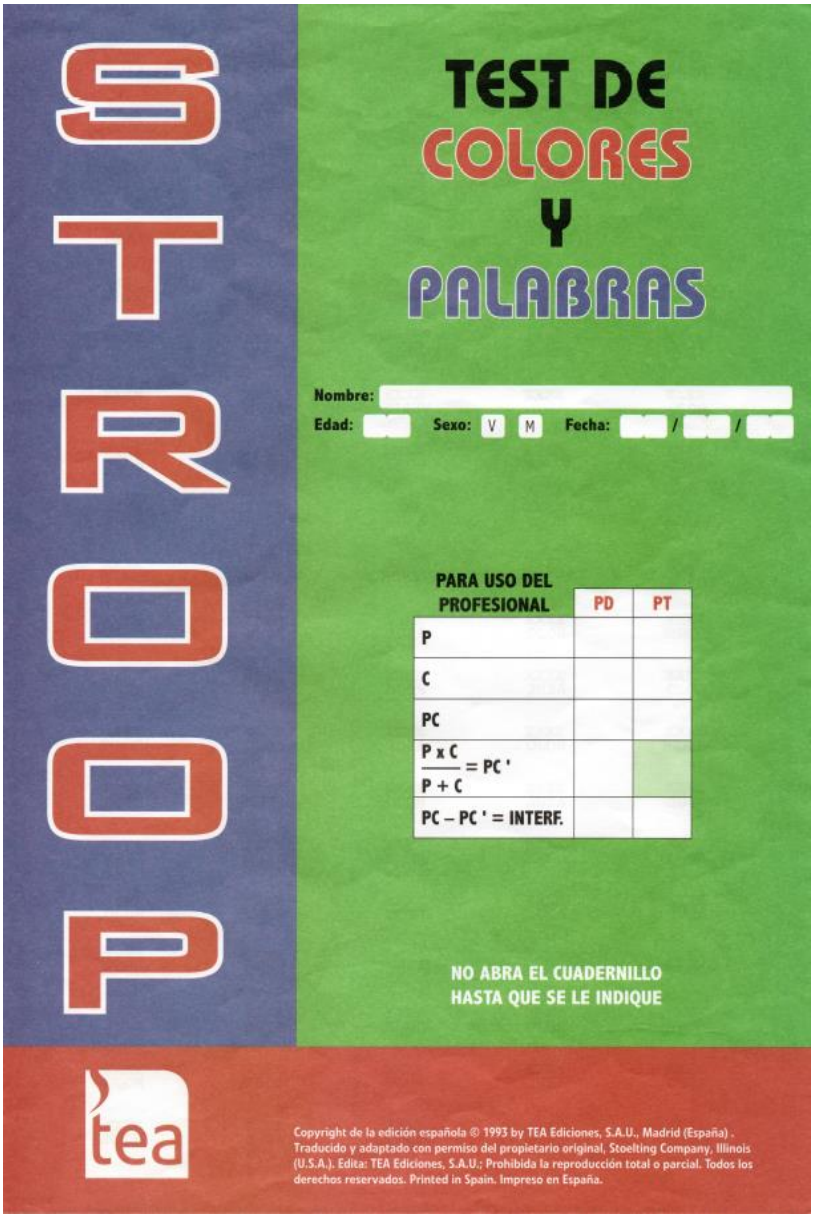
Escritorio	Vaso	Lápiz	Estufa
Montaña	Revólver	Zapato	Bote
Cordero	Pájaro	Nube	Pez
Colador	Toalla	Iglesia	

RECONOCIMIENTO (RAVLT

Picazón	Drenaje	Tambor	Duende	Piano
Tapiz	Reto	Paseo	Cortina	Viaje
Grito	Azul	Timbre	Cero	Red
Raíz	Café	Leche	Colegio	Horno
Conejo	Edificio	Ruta	Escuela	Zorrillo
Agua	Tiro	Pariente	Padre	Pareja
Sol	Mono	Luna	Esquina	Mano
Perdón	Jardín	Lluvia	Arveja	Encaje
Rata	Llave	Sombrero	Bufanda	Tortuga
Media	Canal	Extra	Granjero	Grano
Saber	Boca	Teléfono	Nariz	Sofá
Turno	Cerveza	Brazo	Pavo	Gallina
Cuerda	Columna	Color	Arco	Felpudo
Casa	Espacio	Poema	Hogar	Altar
Hueso	Rico	Remolque	Río	Riachuelo

8.4 ANNEX 4 – Stroop Color Word Test

Normalized Spanish Version - TEA Ediciones.



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VERDE	VERDE	ROJO	AZUL	VERDE
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VERDE	AZUL	ROJO	ROJO	AZUL
ROJO	ROJO	VERDE	AZUL	VERDE
AZUL	VERDE	AZUL	VERDE	ROJO
ROJO	AZUL	VERDE	AZUL	VERDE
AZUL	VERDE	ROJO	VERDE	ROJO
VERDE	ROJO	AZUL	ROJO	AZUL
AZUL	VERDE	VERDE	AZUL	VERDE
VERDE	ROJO	AZUL	ROJO	ROJO
ROJO	AZUL	ROJO	VERDE	AZUL
VERDE	ROJO	AZUL	ROJO	VERDE
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ROJO	VERDE	AZUL	ROJO	VERDE
VERDE	ROJO	VERDE	AZUL	AZUL
ROJO	AZUL	ROJO	VERDE	ROJO
VERDE	ROJO	VERDE	AZUL	VERDE


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ROJO	AZUL	VERDE	ROJO	AZUL
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VERDE	AZUL	ROJO	ROJO	AZUL
ROJO	ROJO	VERDE	AZUL	VERDE
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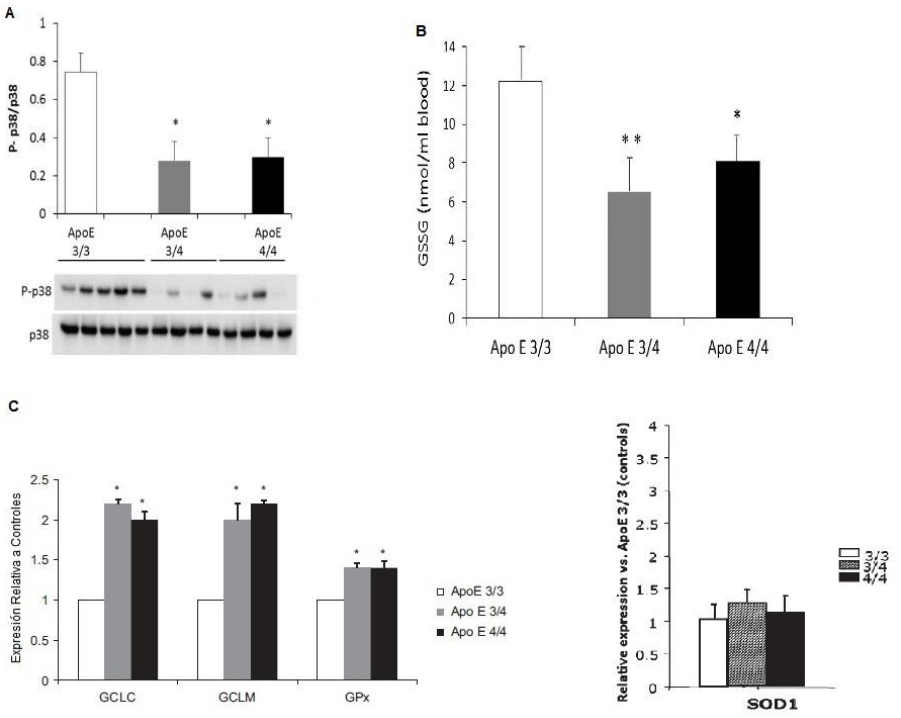
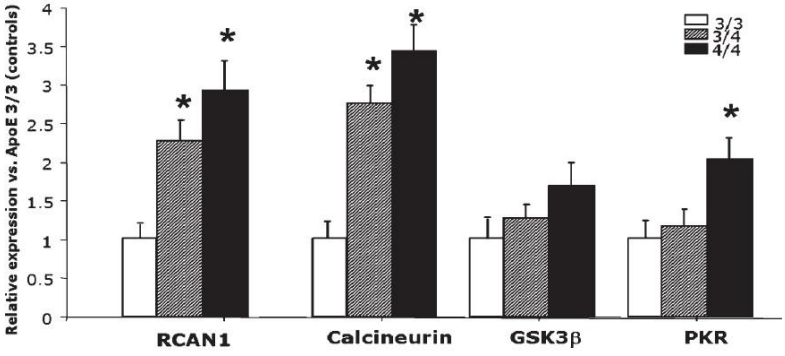
ROJO	AZUL	VERDE	AZUL	VERDE
AZUL	VERDE	ROJO	VERDE	ROJO
VERDE	ROJO	AZUL	ROJO	AZUL
AZUL	VERDE	VERDE	AZUL	VERDE
VERDE	ROJO	AZUL	ROJO	ROJO
ROJO	AZUL	ROJO	VERDE	AZUL
VERDE	ROJO	AZUL	ROJO	VERDE
AZUL	AZUL	ROJO	VERDE	ROJO
ROJO	VERDE	VERDE	AZUL	AZUL
AZUL	AZUL	ROJO	VERDE	ROJO
ROJO	VERDE	AZUL	ROJO	VERDE
VERDE	ROJO	VERDE	AZUL	AZUL
ROJO	AZUL	ROJO	VERDE	ROJO
VERDE	ROJO	VERDE	AZUL	VERDE

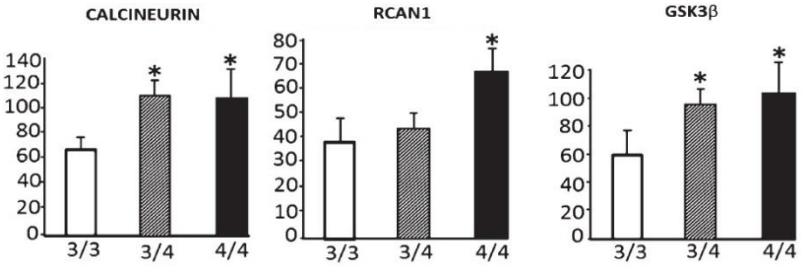
8.5 ANNEX 5 – Results from the 2008 Study



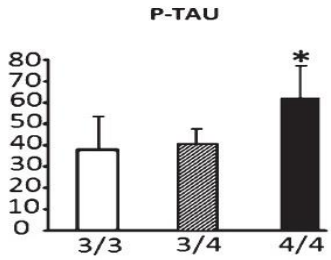
Reductive stress in *APOE4* carriers shown as lower p-p38/p38 ratio (A), lower levels of GSSG in whole blood (B), and increased expression of antioxidant enzymes GCLC, GCLM and GPx1 and normal levels of SOD1 (C) in 3/4 and 4/4 carriers relative to controls.



Gene expression measured by PCR. Cellular stress shown as increased expression of the enzymes RCAN1 and calcineurin in both heterozygous and homozygous *APOE4* carriers and increased PKR only in homozygous carriers.



Levels of enzymes measured by WB. Cellular stress shown by the increase protein expression of calcineurin and GSK3β in both carrier groups and increased RCAN1 in homozygous carriers.



Levels of enzymes measured by WB. Increased levels of p-tau 231 in homozygous carriers.

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