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TRANSLATIONAL STUDIES ON ALZHEIMER'S DISEASE: A FOCUS ON CHOLESTEROL METABOLISM, THIOREDOXIN-80 AND MICROGLIA FUNCTION

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Translational studies on Alzheimer's Disease: A focus on cholesterol metabolism, Thioredoxin-80 and microglia function

THESIS FOR DOCTORAL DEGREE (PH.D.)

By

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ABSTRACT

Disturbances in cholesterol metabolism and inflammatory processes may have a negative impact on brain health. These two aspects are considered to be signs of early changes in Alzheimer's Disease (AD). However, clear molecular links between them as well as mechanisms for their early detection are missing in the context of neurodegeneration and AD. 27-hydroxycholesterol (27-OHC), a cholesterol metabolite and Thioredoxin-80 (Trx80), an antioxidant-derived peptide with immune modulatory properties have been shown to be altered in the brain of AD patients. Thus, this thesis aims to investigate the involvement of 27-OHC and Trx80 in AD.

In **Papers I**, **II** and **IV**, we investigated the effect of alterations in cholesterol metabolism and inflammation during aging in relation to cognition and different risk factors for AD. In **Paper I** we measured 27-OHC levels in serum samples of older adults undergoing a two-year multimodal lifestyle/vascular intervention. At baseline, higher 27-OHC correlated with lower memory scores, total gray matter and hippocampal volume. After the two-year intervention trial, 27-OHC levels decreased in the intervention group. This reduction was positively correlated with improved memory scores among the intervention group. In **Paper II**, Trx80 was measured in serum samples in the same individuals as the previous study and in patients with varying degrees of dementia. We found that serum Trx80 levels were increased in AD cases and were positively correlated with older age and associated with the presence of ApoE4 genotype, indicating that serum Trx80 levels are sensitive to two main risk factors of AD.

Previous studies in our lab showed that Trx80 can prevent amyloid-beta (Aβ) aggregation and inhibit Aβ toxic effects in cell cultures. In view of these findings, in **Papers III** and **IV** we investigated whether Trx80 could have a similar effect on Aβ in *in vivo* models of amyloid pathology by inducing specific activation responses in brain cells. In **Paper III**, we further explored the anti-amyloidogenic properties of Trx80 *in vivo* in *Drosophila melanogaster* models. Trx80 expression in Drosophila brain prevented Aβ42 accumulation and rescued the lifespan reduction and locomotor impairments observed in Aβ42-expressing flies by enhancing the autophagy-lysosomal pathway. In **Paper IV**, we aimed to further clarify the biological function of Trx80 and its regulation in the brain both under healthy and AD-relevant pathological conditions. We show that neurons are the main source of Trx80 in the

brain, and that its levels increase during normal aging, oxidative-stress conditions and in a mouse model of amyloid pathology. RNA-sequencing analysis revealed that Trx80 induces microglia activation into a phenotype compatible with type-I interferon response microglia.

These findings highlight the involvement of cholesterol metabolism and inflammation, related to changes in 27-OHC and Trx80, in AD pathogenesis. 27-OHC and Trx80 should be further investigated not only as potential biomarkers for AD, but also as part of therapeutic and preventive strategies in AD.

PROIEKTUAREN AZALPEN LABURRA

Bizi itxaropena luzatzearen ondorioz zahartzaroarekin lotutako osasun-arazoak areagotu egiten dira, eta horietan nabarmenenetako bat Alzheimer gaitza da. Izatez, adineko pertsonen artean gaixotasun neurodegeneratiborik ugariena da, eta gaur egun ez dago sendabiderik ezta tratamendu eraginkorrik ere.

Fisiopatologikoki, Alzheimer pazienteen laginetan bi erakusle patologiko azal daitezke nagusiki: amiloide plakak eta bilduma neurofibrilarioak. Lehenengoak garuneko neuronen inguruan sortzen dira eta beta-amiloide proteinez osaturik daude. Bigarrenak, aldiz, neuronen barnean metatzen diren egitura-proteina akastunez osaturik daude. Amiloide plaka bezala, hauek ere disolbaezina den masa metatu bat eratzen dute eta biek, Alzheimer gaixotasunaren sintoma klasikoak sortzeko gai dira; adibidez memoria galera, depresioa eta antsietatea. Hala ere, lesio hauek gaixotasunaren lehen sintomak baino hainbat urte lehenago agertzen dira eta beraz, sintomen agerpena gaixotasuna oso garatua dagoenaren seinaletzat jotzen da. Hau dela eta, Alzheimerrari aurre egiteko edo gaixotasuna ahalik eta luzeen ekiditeko, diagnostiko goiztiarra eta zehatza garatzea premiazkoa da.

Horretarako lehenik eta behin Alzheimerraren garapena baldintzatzen dituzten arrisku-faktoreak identifikatu eta aztertu behar dira. Alzheimerra gaixotasun konplexua da eta faktore anitzek eragiten dute harengan. Aurretiaz aipatutako zahartzeaz gain, Alzheimerra oinarri genetiko eta ez-genetikoz osatuta dagoela uste da. Hauetariko hainbat faktore ezagunak dira baina haien arteko erlazio molekularrak ez dira oraindik argitu.

Ikerketa proiektu honetan, kolesterol metabolismoaren desoreka eta hantura edo inflamazio prozesuek Alzheimerrarekiko duten erlazioa aztertu dugu. Berez, garuna kolesterol-metabolismo eta inflamazio-prozesuetan gerta daitezkeen aldaketekiko oso sentibera da eta Alzheimer garapenaren hasiera-pauso adierazgarritzat ditugu. Zehazki, proiektu honetan 27-hidroxikolesterola (27-OHK), kolesterol-metabolito bat eta Thioredoxin-80 (Trx80), erantzun immunologikoak erregulatzen dituen proteina antioxidante-eratorri bat ikertu ditugu, bi molekula hauen ugaritasunek aldaketa garrantzitsuak pairatzen baitituzte Alzheimer gaixoen garunean. Honela, tesi honek 27-OHK eta Trx80ren harreman eta parte hartzea ikertzea izan du helburu Alzheimer gaixotasunaren testuinguruan.

Ikerketa ugarik kolesterolari Alzheimer gaitzean parte-hartzaile garrantzitsua izatea leporatu diote. Nahiz eta kolesterol maila altuak hainbat gaixotasun neurodegeneratiboekin erlazionatuak egon, kolesterolak ezin du garuna babesten duen barrera hematoentzefalikoa gurutzatu, beraz, zelan baldintzatu dezake kolesterolak garuna?

Bere forma oxidatura eraldatuz, 27-OHK modura, kolesterolak, posible du barrera hematoentzefalikoa gurutzatzea eta garuneko gotorlekura iristea. Hainbat ikerketek garuneko zelula desberdinengan 27-OHKak dituen efektuak aztertu dituzte eta kolesterol bitartekaria baino gehiago dela ondorioztatu dute. Izan ere, 27-OHKa gain-ekoizten duten xagu transgenikoekin egindako hainbat ikerketek helarazi dutenez, xagu hauek ahalmen kognitibo urritua dute eta hau hainbat arrazoirengatik izan daiteke. Izan ere, animalia hauen neuronek glukosa barneratzeko ahalmen mugatua ez ezik, haien adarkatze ahalmena eta sinapsi kopurua murriztuak ere badituzte. Honetaz gain, 27-OHKa Alzheimer gaixoen garunean metatzen dela ere egiaztatu dute. Honek guztiak 27-OHKa eta Alzheimerraren arteko harreman estua iradokitzen du, baina orain arte metabolito hau ez da Alzheimerraren prebentzio proiektuetan ikertu.

I. artikuluan, 27-OHKak funtzio kognitiboan duen eragina izan dugu aztergai 2 urtez bizimodu eta elikadura osasuntsua eraman duten pertsona nagusietan. Honetarako, odol analisiak, test kognitiboak eta eskaner bidezko garunaren irudiak egin zitzaizkien pazienteei ikerketa hasi baino lehen eta bi urteren buruan. Hasiera puntuan, harreman estua zegoela ikusi genuen odoleko 27-OHK maila altuaren eta memoria-testaren emaitza baxuen artean. Era berean, 27-OHK maila altuak garuneko hainbat atalen bolumen murriztuekin bat zetozela ere egiaztatu genuen. 2 urte beranduago, bizimodu eta elikadura osasuntsua jaso zuten nagusiek aldiz, odoleko 27-OHK maila murriztea lortu zuten, eta beherapen hau memoria test emaitzen hobekuntzarekin bat zetorrela ohartu ginen. Aldiz, efektu hau ez zen kontrol taldean eman. Emaitza hauek modu bateratuan aztertuz bi ondorio nagusi atera ditzazkegu. Alde batetik, odoleko 27-OHKak garunean ematen diren aldaketa neurodegeneratiboen erreportari izateko potentziala duen molekula dela ondorioztatu dugu. Bestalde, odoleko 27-OHKa bizimodu osasuntsu baten bidez kontrolatu eta maila normaletara jaitsi daitekela eta berarekin datorren galera kognitiboa berreskuratu daitekeela berretsi dugu.

Gainera, proiektu honetan, Trx80 proteina Alzheimerraren testuinguruan ere ikertu dugu. Proteina hau erreakzio immunologikoak eta inflamazioa sortzeko ahalmena duela ikusi da eta gaixotasun kroniko askotan oso ugaria dela baieztatu da. Nahiz eta Alzheimer pazienteen garunetan inflamazio prozesu kroniko eta sakona gertatu, Trx80 proteina oso urria da bertan. Hau dela eta, **II. artikukuan** Trx80 biomarkatzaile modura erabili daitekeen ikertzeko asmoz, proteina hau Alzheimerra garatzeko arriskua duten pertsonetan eta Alzheimer dementzia maila desberdinak dituzten pazienteen odolean neurtu genuen. Odoleko Trx80 mailak adinarekin gora egiten duela eta are ugariagoa dela Alzheimer gaixoetan erdietsi dugu. ApoE4 proteinaren ekoizpenak Alzheimerra garatzeko probabilitatea hainbat aldiz areagotzen du ApoE4 ekoizten ez duten pertsonekin alderatuz eta horregatik Alzheimer arrisku faktore genetiko argiena da. Guk azterturiko pertsona taldean, ApoE4 proteina ekoizten duten pertsonek Trx80 gehiago dutela ere ikusi dugu. Honek guztiak ondorioztatzen duenez, odolean neurtutako Trx80 sentibera da ezagutzen diren Alzheimer arrisku faktore nagusiekiko eta Alzheimer biomarkatzaile izateko potentziala izan dezakeen proteina dela uste dugu.

Hala ere, Trx80k garunean duen funtzio biologikoa ez da ezaguna. *In vitro* eginiko ikerketei esker, jakina da Trx80k gaitasuna duela beta-amiloide proteinak zelulengan dituen efektu toxikoak ekiditeko. Aurkikuntza hau abiapuntutzat hartuta, **III. eta IV. artikuluetan** Trx80k beta-amiloide proteinarengan duen efektua *in vivo*, hau da, zelulak baino komplexuagoak diren organismoetan aztertzea izan dugu helburu. Honetarako, **III. artikuluan** giza-jatorridun Trx80 eta beta-amiloidea garunean gain-ezkoizten dituzten euli transgenikoak erabili ditugu, alde batetik euliak genetikoki eraldatzea eta haiekin lan egitea beste edozein animaliarekin egitea baino errazagoa delako, eta bestalde are garrantzitsuagoa, gizakietan gaixotasun sortzaile diren geneen %70ek bere ortologoa dutelako eulietan.

Beta-amiloidea gain-ezkoizten dituzten euli hauek aztertzean, biziraupen laburragoa eta mugimendu ahalmen mugatuagoa dutela ikusi dugu euli normalekin alderatuz. Aldiz, beta-amiloidearekin batera Trx80 ekoizten dituzten euliek, euli arrunten biziraupen eta mugimendu gaitasun antzekoak dituztela berretsi dugu eta beraz, Trx80 organismo komplexuetan ere funtzionalki beta-amiloidearen efektu toxikoetatik babesteko ahalmena duela ondoriozta daiteke. Trx80ak beta-amiloidearen efektu toxikoak ze mekanismo molekularraren bidez ezeztatzen dituen ikertzeko, euli hauen garunen azterketa biokimikoa burutu dugu. Azterketaren

arabera, bi proteinak ekoizten dituzten euliek, garunean metatutako beta-amiloide kopuru txikiagoa dutela ikusi dugu, hots, Trx80ak beta-amiloidearen degradazioan parte hartu dezakeela ondorioztatu dugu.

IV. artikuluan, Trx80aren funtzio eta erregulazio biologikoa hobeto zehaztea eta aztertzea izan dugu helburu. Honetarako, lehenik eta behin garuneko zein zelula motak ekoizten duen argitu beharra zegoen. Garuneko zelula desberdinen azterketa biokimikoa egin eta gero, neuronen, astrozito eta mikrogliaren artean, neuronak direla Trx80 ekoizle nagusienak baieztatu dugu. Ondoren, Trx80a erreakzio immunologikoekin harreman estua duela jakina denez, Trx80aren efektua garuneko zelula immuneetan, hau da, mikroglian aztertu dugu. Zelula hauek, Trx80aren presentzian aktibazio immunologiko berezi bat pairatzen dutela ikusi dugu, hots, bakterio edo birus infekzioetan aktibatzen dituzten bidezidor metabolikoak aktibatzen dituzte Trx80aren ondorioz. Hala ere, mikrogliaren aktibazio mota honek garunean zein-nolako efektuak dituen ikertu beharra dago oraindik.

Gauzak honela, tesi honetan Alzheimer gaixotasunaren eta hainbat arrisku faktoreen arteko harremana aztertu da. Hauen artean, 27-OHK kolesterol metabolitoak eta Trx80 proteina immunomodulatzaileak Alzheimer gaitzan duten parte hartzea ikertu da. Molekula hauen potentzial biomarkatzailea ez ezik, garunean dituzten efektuak ere aztertu ditugu. Aurkeztutako aurkikuntzek bi molekula hauek sakonago ikertzeko beharra azpimarratzen dute eta etorkizunean estrategia terapeutiko eta prebentiboetan kontutan izateko moduko molekulak direla uste dugu Trx80 eta 27-OHKa. Oraindik ere, aurretik bide luzea daukagun arren, eskuartean ditugun proiektuek Alzheimer gaitzaren fisiopatologia hobe ulertzeko eta bai prebentzioa eta terapia berriak proposatzeko ahalmena dutela uste dugu, beti ere gaitz honen inguruan dauden zoritxarrak gutxitzeko edo desagerrarazteko.

LIST OF SCIENTIFIC PAPERS

This doctoral thesis is based on the following original papers and manuscripts, referred to in the text by Roman numerals.

I. Anna Sandebring-Matton, **Julen Goikolea**, Ingemar Björkhem, Laura Paternain, Nina Kemppainen, Tiina Laatikainen, Tiia Ngandu, Juha Rinne, Hilkka Soininen, Angel Cedazo-Minguez, Alina Solomon and Miia Kivipelto.
27-Hydroxycholesterol, cognition, and brain imaging markers in the FINGER randomized controlled trial.
Alzheimer's Research and Therapy, 2021, 13:56.

II. **Julen Goikolea**, Gorka Gerenu, Makrina Daniilidou, Francesca Mangialasche, Patrizia Mecocci, Tiia Ngandu, Juha Rinne, Alina Solomon, Miia Kivipelto, Angel Cedazo-Minguez, Anna Sandebring-Matton, Silvia Maioli**.**
Serum Thioredoxin-80 is associated with age, ApoE4 and neuropathological biomarkers in Alzheimer's Disease: A potential early sign of AD.
Manuscript. *Submitted*.

III. Gorka Gerenu, Torbjörn Persson, **Julen Goikolea**, Javier Calvo-Garrido, Raúl Loera-Valencia, Philipp Pottmeier, Cesar Santiago, Helen Poska, Jenny Presto and Angel Cedazo-Minguez.
Thioredoxin-80 protects against amyloid-beta pathology through autophagic-lysosomal pathway regulation.
Molecular Psychiatry, 2019, 26(4):1410-1423.

IV. **Julen Goikolea**, Jean-Pierre Roussarie, Gorka Gerenu, Maria Latorre, Raul Loera-Valencia, Angel Cedazo-Minguez, Patricia Rodriguez-Rodriguez and Silvia Maioli.
Neuron-derived Thioredoxin-80: a novel regulator of type-I interferon response in microglia of relevance to Alzheimer's disease.
Manuscript.

OTHER PUBLICATIONS BY THE AUTHOR NOT INCLUDED IN THE THESIS

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Hypercholesterolemia and 27-Hydroxycholesterol Increase S100A8 and RAGE Expression in the Brain: A Link Between Cholesterol, Alarmins, and Neurodegeneration.
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II. David Guerrero-Gómez, José Antonio Mora-Lorca, Beatriz Sáenz-Narciso, Francisco José Naranjo-Galindo, Fernando Muñoz-Lobato, Cristina Parrado-Fernández, **Julen Goikolea**, Ángel Cedazo-Minguez, Christopher D Link, Christian Neri, María Dolores Sequedo, Rafael P Vázquez-Manrique, Elena Fernández-Suárez, Veit Goder, Roser Pané, Elisa Cabiscol, Peter Askjaer, Juan Cabello, Antonio Miranda-Vizuete.
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Cell Death Differentiation. 2019 Sep;26(9):1545-1565.

III. Raul Loera, **Julen Goikolea**, Cristina Parrado, Paula Merino, Silvia Maioli.
Alteration in cholesterol metabolism as risk factor for developing Alzheimer's disease: Evidence of novel targets for treatment.
Journal of Steroid Biochemistry and Molecular Biology. 2019 Jun;190:104-114. Review.

CONTENTS

LIST OF ABBREVIATIONS

AD – Alzheimer's Disease

EOAD – Early Onset Alzheimer's Disease

LOAD – Late Onset Alzheimer's Disease

NFT – Neurofibrillary Tangles

Aβ – Amyloid-Beta

APP – Amyloid Precursor Protein

SCI – Subjective Cognitive Impairment

MCI – Mild Cognitive Impairment

CSF – Cerebrospinal Fluid

GWAS – Genome Wide Association Studies

APOE – Apolipoprotein E

CLU – Clusterin

ABCA7 – ATP Binding Cassette Subfamily A Member 7

Trem2 – Triggering Receptor Expressed On Myeloid Cells 2

CR1 – Complement receptor type 1

PICALM – Phosphatidylinositol Binding Clathrin Assembly Protein

BIN1 – Bridging Integrator 1

BBB – Blood Brain Barrier

7−HOCA – 7α-hydroxy-3-oxo-4-cholestenoic acid

CYP7B1 – Oxysterol 7α-hydroxylase

Cyp27A1 – Sterol 27-hydroxylase

27-OHC – 27-hydroxycholesterol

24S-OH – 24S-hydroxycholesterol

CYP46A1 – cholesterol 24-hydroxylase

HSD3B7 – 3β-hydroxy-C27-steroid dehydrogenase/isomerase

Trx1 – Thioredoxin-1

Trx80 – Thioredoxin-80

RXRγ – Retinoid Receptor gamma

RAS – Renin-Angiotensin System

AngIV – Angiotensin IV

FDG-PET – 2-[18F] fluoro-2-deoxy-d-glucose positron emission tomography

LXR – Liver X Receptor

ARC – Activity regulated cytoskeleton associated protein

PSD95 – Post-Synaptic Protein 95

GLUT4 – Glucose Transporter Type 4

TNF-α – Tumor Necrosis Factor- α

IL-1β – Interleukin-1β

IL-6 – Interleukin-6

RAGE – Receptor for Advanced Glycation End Product

ROS – Reactive Oxygen Species

PI3K – Phosphoinositide 3-Kinase

ERK – Extracellular Signal-Regulated Kinase

Nrf2 – Nuclear Factor Erythroid 2 p45-Related Factor 2

HO-1 – Heme Oxygenase-1

$OH^{\bullet}$ – Hydroxyl radical

$O2^{\bullet -}$ – Superoxide anion

H_2O_2 – Hydrogen peroxide

Ca^{2+} – Calcium cation

HNE – 4-hydroxy-2, 3-nonenal

Cu^{2+} – Cupper cation

Zn^{2+} – Zinc cation

Cys – Cysteine

TrxR – Thioredoxin Reductase

NAPDH – Nicotinamide adenine dinucleotide phosphate

ARE – Antioxidant Response Element

CRE – cAMP response element

Prx – Peroxiredoxin

sMaf – small musculoaponeurotic fibrosarcoma

ASK-1 – Apoptosis Signal-regulating Kinase-1

NLRP3 – NOD-, LRR- and pyrin domain-containing protein 3

NF-κB – Nuclear Factor kappa-light-chain-enhancer of activated B cells

ERα – Estrogen Receptor-α

HIF-1 – Hypoxia Inducible Factor

PTEN – Phosphatase and Tensin homolog

ECEF – Eosinophil Cytotoxicity-Enhancing Factor

IFN-γ – Interferon- γ

PMN – Polymorphonuclear cells

Icam-1– Intercellular Adhesion Molecule 1

CD86 – Cluster of Differentiation 86

CD40 – Culster of Differentiation 40

IL-10 – Interleukin-10

RA – Rheumatoid arthritis

CCL1 – C-C motif chemokine ligand 1

CXCL1 –C-X-C motif chemokine ligand 1

ADAM10 – A Disintegrin and metalloproteinase domain-containing protein 10

NO – Nitric Oxide

FAD – Flavin adenine dinucleotide

DAMPs – Damage-Associated Molecular Patters

PRR – Pattern Recognition Receptor

HMGB1 – High-Mobility Group Box 1

CX3CL1 – Chemokine (C-X3-C motif) ligand 1

DAM – Disease-Associated Microglia

TYROBP – TYRO protein tyrosine kinase-Binding Protein

MHC class II – Major Histocompatibility Complex class II

FINGER – Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability

CERAD – Consortium to Establish a Registry for Alzheimer's Disease

CAIDE – Cardiovascular Risk Factors, Aging and Dementia

MMSE – Mini-Mental State Examination

PMI – *post mortem* interval time

UAS – Upstream Activating Sequence

ELISA – Enzyme-linked immunosorbent assay

AMPKα – AMP-activated protein kinase alpha

CSFR1 – Colony-Ftimulating factor 1 Receptor

PMA – Phorbol 12-Myristate 13-Acetate

ARC – Activity regulated cytoskeleton associated protein

RUNX – Runt-related transcription factor

1 INTRODUCTION

1.1 DEMENTIA AND AD

Dementia is a term that encompasses several diseases characterized by a deterioration in cognitive skills sufficient to cause a reduction in the ability to perform everyday activities (1). As of September 2021, around 55 million people are estimated to suffer from dementia in the world and this number is expected to rise to 78 million in 2030, as the worlwide population ages (2). Alzheimer's Disease (AD), the most common form of dementia, accounts for 60% of all dementia cases, followed by vascular dementia (30-15%) (3), frontal-temporal dementia (5.4%), Lewy body dementia (2.5%) and Parkinson's Disease associated dementia (1%) (1, 4). However, autopsy-verified studies show that mixed-dementias, including/presenting both vascular and neurodegenerative AD pathology, may account for the majority of dementia cases (5, 6).

AD is an irreversible and incurable neurodegenerative disorder that chronically impairs several brain functions. Loss of memory is a frequent early symptom that can be followed by a long list of other symptoms as the disease progresses, such as disorientation, depression or anxiety (7). Life expectancy after being diagnosed with AD is approximately 10 years. AD is generally divided into early-onset AD (EOAD) and late-onset AD (LOAD) (8). EOAD takes place before the age of 65 and accounts for less than 5% of all AD cases (9, 10). Conversely, LOAD is classified by an onset after the age of 65 and comprises most AD cases (10, 11). Both, EOAD and LOAD can be of familial or sporadic origin, where most patients suffering from familial AD will carry autosomal dominant inherited mutations in the *APP* (Amyloid precursor protein), *PSEN1* (presenilin1) and/or *PSEN2* genes (9, 10, 12). Sporadic LOAD is suggested to be multifactorial, with several environmental and genetic factors contributing to the onset and development of the disease (8, 13, 14).

At a molecular level, AD is characterized by two main pathological lesions: extracellular aggregates of amyloid–β (Aβ) protein, known as senile plaques, and intracellular accumulation of neurofibrillary tangles (NFTs) of hyperphosphorylated microtubule-associated tau proteins in neurons (15). These protein accumulations are frequently observed together with synaptic loss, neuroinflammation and neuronal death.

Aβ has long been considered to be the driving force in the development of AD. Under pathological conditions, APP is preferentially cleaved by β- and γ-secretases leading to excessive amounts of the hydrophobic and aggregation prone Aβ peptide, which results in its accumulation and inadequate clearing (6). Aβ deposits are commonly found extracellularly, in the form of senile plaques but they can also be present inside neurons, where they contribute to synaptic dysfunction and neurodegeneration. Morphologically, extracellular amyloid plaques are classified as dense core and diffuse plaques. Dense core plaques are dense reticular compact amyloid accumulations that contain dystrophic neurites, reactive microglia and astrocytes and are generally associated with synaptic loss and neuronal death (16). Conversely, diffuse plaques consist of degenerating neurons with tau filaments and are more commonly found in brains of healthy old individuals (17).

Under pathological conditions, the microtubule associated protein tau gets aberrantly phosphorylated, causing the disassembly of microtubules and the aggregation into paired helical filaments that leads to the disruption of axonal transport and dendrite breakdown (18, 19). The progression of NFTs spread is more predictable than amyloid plaques and the density and distribution of NFTs have been observed to correlate better with clinical features of dementia (6).

Other studies pointed out that defective clearance of Aβ and tau underlie most of the sporadic AD cases (15). Several proteases have been shown to break down Aβ peptides (20), but new strong evidence shows that large part of the Aβ turnover in physiological conditions relies on bulk flow via the perivascular circulation and the glymphatic system in the brain and any disruption of this system could contribute to AD (21).

Another important cellular mechanism for clearing dysfunctional protein aggregates is macro-autophagy (also known as autophagy) and it is believed to be a major player in Aβ clearance (22). Conversely, defects in autophagy, in the form of accumulation of immature autophagic vesicles in dystrophic neurites or lysosomal deficits are common pathological events in AD brains (23). The fact that autophagosomes seem to accumulate in AD brains does not necessarily indicate that autophagy is upregulated (24). In fact, the expression of beclin-1, an essential protein for autophagy initiation, is decreased in AD and various attempts to activate beclin-1 levels lead to induction of autophagic activity, resulting in reduced

extracellular and intracellular levels of amyloid pathology and rescue of cognitive decline in AD mouse models (24, 25). However, it is still unknown whether dysfunctional autophagy is a consequence or a cause of AD.

The first pathophysiological signs of AD pathology start years and even decades before the first clinical symptoms appear (26). Older adults can often be diagnosed with subjective cognitive impairment (SCI), a common diagnosis for initial complaints regarding memory problems that are associated with a higher risk to develop dementia (27). Neuro-imaging studies have shown that SCI patients have smaller hippocampal volume than healthy controls (28). Moreover, the biomarker profile from cerebrospinal fluid (CSF) of SCI patients has shown more similarities to the AD profile than to the one from healthy individuals (29). Mild cognitive impairment (MCI) is a prodromal dementia state and it has been considered to be an intermediate state between SCI and AD. MCI patients display mild memory problems without signficant impairment of their daily activities. The conversion rate from MCI to AD is estimated to be between 10 to 15% per year (30). Taking this into consideration, understanding and identifying which factors trigger the early stages of cognitive deterioration might be fundamental in designing effective treatments.

1.2 RISK FACTORS FOR AD

Aging is the most significant risk factor to develop AD (1). However, AD is a complex and multifactorial disorder that results from the combination of environmental as well as genetic risk factors (11, 31).

From studies performed with twins, it was estimated that the heritability of LOAD could be up to 70% (11). To date, over 30 AD risk loci have been identified via Genome Wide Association Studies (GWAS) (32), highlighting the relevance of genetic susceptibility to AD. Some of the most relevant genetic polymorphisms are found in genes related to cholesterol metabolism (*APOE*, *CLU* and *ABCA7*), endosomal vesicle turn-over (*PICALM* and *BIN1*) and innate immune system (*TREM2*, *CLU* and *CR1*) and are considered important contributors to the disease (32). Among all of them, the strongest genetic risk factor associated with LOAD is the presence of the ε4 allele of the cholesterol transporter Apolipoprotein E (ApoE) (33). ApoE has three isoforms (ε2, ε3 and ε4), each of them conferring a varying level of AD risk, where ε4 involves the highest risk (34). It is estimated that carrying one copy of *APOE4* increases the risk to develop AD threefold, whereas being

homozygous for *APOE4* increases the risk up to fifteenfold in comparison to those carrying two copies of *APOE3* (33, 35, 36). Moreover, several studies have shown that the presence of ApoE4, in combination with risk factors of environmental origin including diabetes (37), hypertension (38), obesity and hypercholesterolemia (39), sedentary life and alcohol consumption (40), elevates both the risk and the severity of the disease. In fact, it has been reported that the combination of ApoE4 isoform with a high fat diet induces a greater memory impairment in mice than the one observed in ApoE4 mice fed with normal diet (41). Importantly, several environmental factors are modifiable and are therefore potential therapeutic and preventive targets to delay AD onset (42-44).

1.3 CHOLESTEROL METABOLISM, OXYSTEROLS AND AD

Several studies suggest a relationship between increased risk to develop AD and high levels of cholesterol in the periphery. This relationship between plasma cholesterol and AD has been further supported by *in vivo* studies where AD mouse models fed with diets with high fat content exacerbated AD-like neuropathological features (45). Moreover, several epidemiological studies on cholesterol levels and AD concluded that chronic hypercholesterolemia in mid-life sustained over the years is an important driver of AD development (46-48).

Despite the evidence demonstrating the impact of blood cholesterol in the development of AD, both cholesterol and the lipoproteins responsible for its transport are unable to cross the blood brain barrier (BBB) (49). This implies that brain cholesterol levels are independent of peripheral cholesterol, what suggests that other factors affect the interplay between AD and excessive peripheral cholesterol (50, 51). Another example supporting the notion of two independent pools of cholesterol is the fact that high cholesterol diets increase blood cholesterol levels in different animal models, while total brain cholesterol remains unchanged. Nevertheless, high fat diets increase Aβ accumulation and lead to cognitive impairment (52). Importantly, these diets also increase the levels of oxidized cholesterol metabolites known as oxysterols, in some cases up to tenfold. Oxysterols, unlike cholesterol, can cross the BBB and reach the brain (53). Therefore, oxysterols could represent the missing link between brain and blood cholesterol levels but also be responsible for the detrimental effects of high fat diets on cognition observed in several animal models (54).

More than 20% of total cholesterol in the human body is found in the brain and it is regarded as an essential component for several neurobiological processes, such as synapse, myelin formation, cell membrane stability or cell homeostasis, among many others (55-57). Brain cholesterol is synthetized *de novo* from acetyl-CoA mainly by astrocytes but also by oligodendrocytes (58). Neurons uptake astrocyte-produced ApoE-bound cholesterol in a receptor-mediated manner (59). Conversely, this system is also employed as a cholesterol secretory mechanism, yielding around 2 mg of ApoE bound cholesterol daily into the CSF (57). However, conversion of cholesterol into 24S-hydroxycholesterol (24S-OH) is the main cholesterol elimination mechanism in the brain. 24S-OH is mainly produced in neurons, since they express cholesterol 24-hydroxylase (CYP46A1), the enzyme responsible for the conversion of cholesterol into 24S-OH (60). This enzymatic mechanism is estimated to convert around 6-12 mg/day of cholesterol into 24S-OH, which crosses the BBB outflowing to the periphery (56). The outflow of 24S-OH is counterbalanced by the inflow of 27-hydroxycholesterol (27-OHC) into the brain (61).

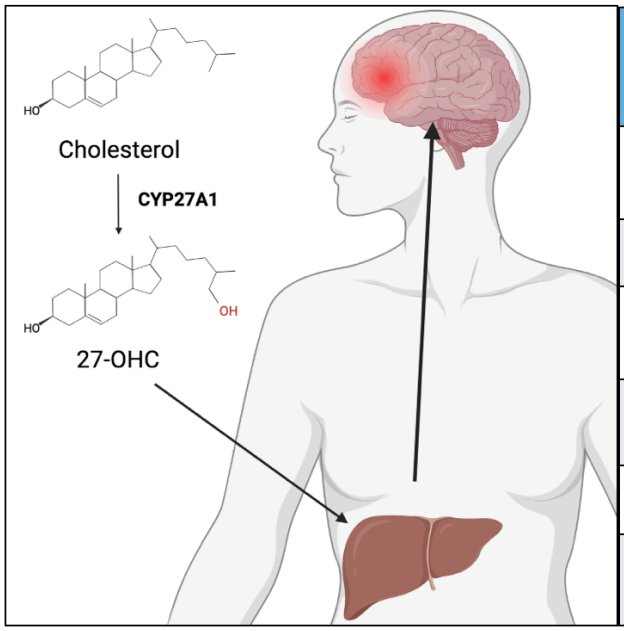
Brain cholesterol homeostasis is essential for normal brain development and function. This homeostasis is maintained by a thoroughly regulated balance between the excretion of oxysterols into the circulation and brain cholesterol biosynthesis. Any disturbance of this balance constitutes a risk factor for AD development (54). Recent evidence shows that oxysterols do not only play a role as intermediaries of cholesterol metabolism but they can also function as signaling molecules in the brain. Both oxysterols, 24S-OH and 27-OHC have been suggested to modulate brain cholesterol synthesis: 24S-OH depletion promotes cholesterol synthesis, possibly as a compensatory mechanism, and 27-OHC has shown inhibitory effects on cholesterol biosynthesis in the brain (62).

1.3.1 27-HYDROXYCHOLESTEROL AND COGNITION

27-OHC is synthetized by the enzyme sterol 27-hydroxylase (CYP27A1), located in the mitochondria and expressed by most cell types in the body but mainly in the liver (54). Brain CYP27A1 levels are very low and thus, small quantities of 27-OHC are present in the brain (63). Since 27-OHC can cross the BBB, there is a net influx of this oxysterol into the brain where its levels are kept low due to efficient metabolism (53, 64). 27-OHC is metabolized by Cyp27A1, oxysterol 7α-hydroxylase (CYP7B1) and 3β-hydroxy-C27-steroid dehydrogenase/isomerase (HSD3B7) that convert 27-

OHC into 7β-hydroxy-3-oxo-4-cholestenoic acid (7-HOCA). This oxysterol is mainly excreted from the brain into the peripheral circulation but also has signaling functions in the brain (65, 66).

Several studies have suggested that high 27-OHC levels may contribute to the cognitive deficits observed in AD patients with hypercholesterolemia, where high peripheral cholesterol levels are associated with elevated influx of 27-OHC into the brain (61) (**Figure 1**). Postmortem AD brain and CSF analysis have shown lower levels of 24S-OH and elevated levels of 27-OHC among several oxysterols. Accordingly, the levels of CYP46A1 were markedly reduced, whereas CYP27A1 levels were found to be increased (67).



Non-pathological conditions	Pathological conditions (altered cholesterol metabolism and risk for AD)
27-OHC is produced by the activity of Cyp27A1 mostly in the liver	Increased levels of 27-OHC in the brain, CSF and Plasma
About 5 mg/day of 27-OHC flow into the brain	Increased Cyp27A1 levels in the brain
27-OHC is converted into 7-HOCA by the activity of Cyp27A1 and Cyp7B1	27-OHC inhibits cholesterol biosynthesis in the brain
	Reduced Cyp7B1 levels and activity in neurons
	27-OHC reduces brain glucose uptake
	27-OHC increases inflammation and oxidative stress

***Figure 1**. 27-OHC metabolism in non-pathological and pathological conditions when cholesterol metabolism is altered. **Left)** Schematic representation of 27-OHC production mainly in the liver by the activity of the enzyme Cyp27A1 from cholesterol. This oxysterol travels through the peripheral circulation and crosses the BBB by diffusion, reaching the brain. **Right)** 27−OHC in the brain is converted into 7−HOCA by the enzymes CYP7B1, CYP27A1, and HSD3B7. 7−HOCA diffuses through the BBB into the peripheral circulation where it is degraded. In the case of altered cholesterol metabolism, excessive levels of 27−OHC may be present in the periphery. As a result, high amounts of 27−OHC would cross the BBB and reach the brain where it accumulates. High levels of 27-OHC can promote oxidative stress and activate inflammatory responses in the brain. Moreover, 27−OHC inhibits cholesterol biosynthesis. In AD the levels and activity of CYP7B1 enzymes appear to be reduced producing lower levels of 7−HOCA.*

The study of 27-OHC in different animal models has established important associations between 27-OHC, deterioration of cognitive functions and AD (61, 63, 68-71). Elevated 27-OHC levels were also reported on aged mice expressing the Swedish Alzheimer mutation APP751 (65). Furthermore, mice overexpressing the enzyme CYP27A1 and therefore, producing higher levels of 27-OHC, develop cognitive impairment and show reduced expression of Activity regulated cytoskeleton associated protein (ARC), a protein involved in long-term memory formation (70). Conversely, CYP27A1 knock out mice showed amelioration of memory deficits and restored ARC levels in response to high cholesterol diet when compared to wild-type controls (61, 63). This evidence points towards 27-OHC as an important driver of the cognitive impairment caused by dietary cholesterol. Previous work from our group investigated the possible mechanisms behind the deleterious effects of 27-OHC on cognitive function (71). This study determined the involvement of Retinoid Receptor gamma (RXRγ) in the synaptic disruption caused by 27-OHC, inducing a significant decrease in Post-Synaptic Protein 95 (PSD95) mRNA and protein levels both *in vitro* and *in vivo*.

Besides the association between elevated 27-OHC levels and cognitive impairment in animal models, many *in vitro* studies explored the possibility of a direct causal relationship between 27-OHC and AD pathology. 27-OHC treatment resulted in increased production of Aβ and hyperphosphorylated tau protein in cultured cells (72). 27-OHC treatments on neuroblastoma cells resulted in endoplasmic reticulum stress as well as a reduction in the levels of leptin, whose signaling dysregulation is considered another feature of AD (73). Loss of BBB integrity is considered to be an early biomarker of cognitive dysfunction (74). 27-OHC via Liver X Receptor (LXR) activation, has the potential to affect BBB composition by altering the expression of cholesterol transporters in endothelial cells and pericytes surrounding the capillary vessels of the brain (75).

Alterations in glucose metabolism are a common feature of AD and other neurodegenerative diseases (76). AD patients display an overall reduction in glucose uptake in certain brain areas as reported by 2-[18F] fluoro-2-deoxy-d-glucose positron emission tomography (FDG-PET)(77, 78). A previous study from our group explored the effects of 27-OHC on glucose metabolism and found that 27-OHC affects the activity of the brain Renin-Angiotensin System (RAS) (68). The role of this system, via its downstream peptide Angiotensin IV (AngIV), has been associated

with memory and learning function and with the processing of sensory information (79). 27-OHC treatment increased the levels and activity of insulin-regulated aminopeptidase (IRAP), which is the receptor of AngIV and an inhibitor of glucose transporter GLUT4 (68). CYP27A1 overexpressing mice displayed reduced levels of GLUT4 and reduced glucose uptake in the brain. These results indicate that 27-OHC can affect glucose metabolism by modulating RAS signaling and can consequently contribute to worsening cognition.

27-OHC has been associated with cardiovascular diseases. Patients suffering from atherosclerosis often have elevated levels of 27-OHC in the blood and it is the most common oxysterol present in atherosclerotic lesions (80). Additionally, 27-OHC accumulation has been shown to correlate with the number of macrophages present at the lesion. A recent study noted that 27-OHC induces a pro-inflammatory response, measured by the release of TNF-α, IL-1β and IL-6, leading to atherosclerotic plaque instability (81).

Excess of 27-OHC has also been linked to neuroinflammation by triggering an alarmin response. Alarmins are endogenous molecules that, when released due to tissue damage, promote inflammatory responses (82). In AD, several alarmins have been found to be altered, among them S100 calcium-binding proteins (83). In fact, mice overexpressing amyloid precursor protein, the precursor of Aβ, accumulate S100A8 aggregates in the brain even before Aβ plaque formation (69). A recent paper from our group showed that excessive 27-OHC levels induce an increase in S100A8 and its receptor, the receptor for advanced glycation end-product (RAGE) in neurons and astrocytes (84). This study also showed that the upregulation of RAGE elicited by 27-OHC was mediated by RXRγ.

27-OHC not only promotes pro-inflammatory responses but also the activation of cell survival pathways that appear to be modulated by reactive oxygen species (ROS) (85). *In vitro*, 27-OHC treatments induce the expression of nuclear factor erythroid 2 p45-related factor 2 (Nrf2) through extracellular signal-regulated kinase (ERK) and the phosphoinositide 3-kinase (PI3K)/Akt pathways. Nrf2 is a transcription factor for many antioxidant proteins including heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxireductase, that have also been found to be elevated upon 27-OHC treatment (86).

1.4 OXIDATIVE STRESS

Oxidative stress is a phenomenon caused by an imbalance between the production of reactive oxygen species and the ability of a biological system to detoxify and counteract the effects of these reactive products (87). Most ROS are small sized, short-lived, and highly reactive molecules. They can be found as free radicals derived from oxygen such as hydroxyl radical ($OH^{\bullet}$) and superoxide anion ($O2^{\bullet-}$), or non-radical molecules like hydrogen peroxide (H_2O_2) (88).

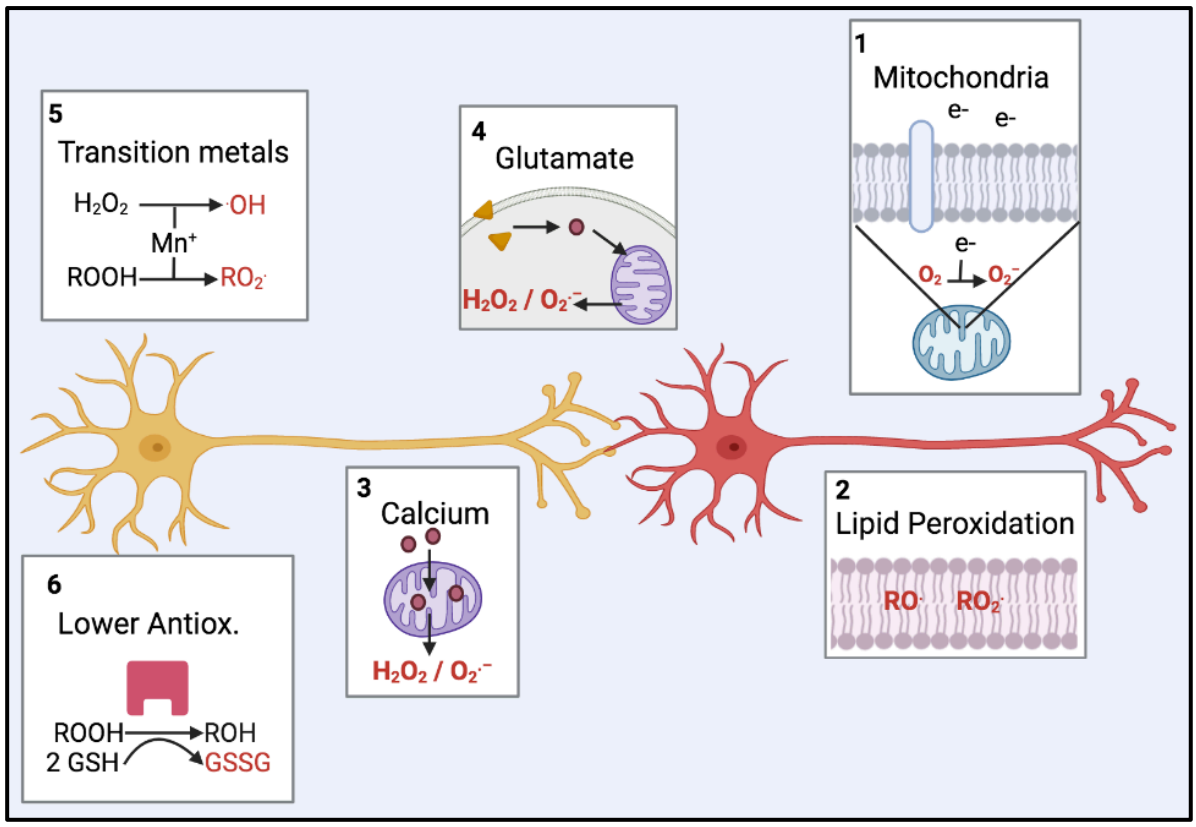


***Figure 2**. Mechanisms of brain susceptibility to oxidative stress. **1)** High oxygen utilization in mitochondria. Depicts O_2^- generation inside mitochondria. **2)** Lipid peroxidation. Depicts $RO^{\cdot}$ and $RO_2^{\cdot}$ within neuronal membranes. **3)** Calcium. Depicts mitochondrial Ca^{2+} overload induced ROS generation. **4)** Glutamate. Depicts glutamate induced Ca^{2+} release inducing ROS. **5)** Redox active transition metals. Depicts manganese ion catalyzing an oxidative reaction. **6)** Lower antioxidant defense. Depicts reduction of antioxidant reactions due to low GSH concentration.*

In physiological conditions, adequate amounts of ROS are produced intracellularly as by-products of oxygen metabolism. Indeed, mitochondria are the main source of ROS in the cell (89, 90), specially $O2^{\bullet-}$, which is produced as a consequence of the mitochondrial electron transport chain activity (89). ROS levels are tightly regulated by the cellular antioxidant system and they play important physiological roles in cell signaling processes such as activation of transcription factors, apoptosis (91),

immunity (92) and cellular differentiation among others (90). When the delicate redox homeostasis breaks, oxidative stress prevails (93). In these oxidative stress conditions, excessive intracellular amounts of ROS interact with macromolecules in the cell, altering their composition and thus their functions (93). This event triggers the oxidation of proteins (particularly aromatic or cysteine residues), lipids and nucleic acids which causes their malfunction.

The brain is especially sensitive to oxidative stress for various reasons (**Figure 2**):

- It is highly dependent on oxygen, requiring 20% of the total oxygen consumed by the body (94). This oxygen is used to produce ATP by oxidative phosphorylation, that helps to maintain ionic gradients and support synaptic activity, among other purposes (95).
- Neuronal membranes as well as myelin sheets wrapped around axons contain high amounts of lipids sensitive to lipid peroxidation. It has been shown that peroxidation of the double bonds in neuronal polyunsaturated lipids results in the generation of lipid by-products such as, 4-hydroxy-2, 3-nonenal (HNE), malondialdehyde and F2-isoprostanes (96, 97). These by-products are biochemically reactive molecules that stimulate tau phosphorylation.
- Neuronal action potentials cause calcium (Ca^{2+}) flux in the presynaptic terminals, triggering the exocytosis of neurotransmitter vesicles (98). Increasing evidence suggests a mutual interplay between ROS and Ca^{2+} for cellular signaling purposes (99). However, the reliance on calcium signaling can be a cause of oxidative stress.
- Excitatory neurotransmitters can be a source of ROS when they are found in excessive amounts, causing excitotoxicity (100). Glutamate excitotoxicity leads to cell death by inducing a Ca^{2+} overload that is followed by mitochondrial ROS release, as seen in apoptotic and necrotic cells (101). Necrotic cells can, in turn, amplify excitotoxicity by further elevating the levels of extracellular glutamate (88).
- The brain is particularly enriched in redox active metal ions such as Fe^{2+} and Cu^{+} (98). These metals play an essential role as cofactors for many enzymatic activities and neurotransmission, including myelination, synaptic plasticity and synthesis of neurotransmitters (102). However, the loss of Fe^{2+} and Cu^{+} homeostasis can produce ROS via Fenton-like reactions (102). In AD brains, neuronal levels of Cu^{2+} and Zn^{2+} can reach up to three times their physiological

levels (103, 104). These cations bind N-terminal ends of Aβ peptides and undergo continuous redox reactions, generating greater amounts of ROS (105).

To counteract all the above mentioned causes of oxidative stress, the brain has modest antioxidant defenses relative to other organs in the body, such as the liver, making the brain more susceptible to disrupted redox homeostasis (106). This increased vulnerability of the brain supports the theory that oxidative stress could potentially play a central role in AD development.

1.4.1 THIOREDOXIN-1

The thioredoxin family of proteins comprises one of the main antioxidant systems in the body. The members of this protein family are characterized by a common structural motif known as the thioredoxin fold, present in several proteins from different classes. Protein disulfide isomerases, glutathione transferases and oxidoreductases like thioredoxin and glutaredoxins, contain a thioredoxin fold (107). This structural motif consists of four-stranded β-sheets and three surrounding α-helices. In addition to the thioredoxin fold, thioredoxin-1 (Trx1) has an extra β-sheet and α-helix at the N-terminus (108) accounting for 105 amino-acid residues in total (109).

Three main thioredoxin proteins with oxidoreductase activity are expressed in humans: Trx1, Trx2 and SpTrx. Trx1 is a cytosolic protein that under certain oxidative stimuli translocates to the nucleus or is secreted to the extracellular space. Trx2 is mainly present in mitochondria and SpTrx is predominantly expressed in spermatozoa. All thioredoxin family members contain a redox-active site, highly conserved throughout evolution, that consists of the following amino-acid sequence: –Cysteine-Glycine-Proline-Cysteine–. Trx1 contains five cysteine residues: two of them (Cys^{32}, and Cys^{35}) are part of the catalytic site and the other three (Cys^{62}, Csy^{69} and Cys^{73}) are available for physiological modulation. Cys^{73} is located on a hydrophobic part of the protein, and it is able to form an homodimer by an intermolecular disulfide bond. Cys^{73}–Cys^{73} homodimerization suppresses the activity of Trx1 since the dimerization hides the catalytic site of the enzyme (110). Thus, the Trx1 monomer-dimer balance regulates the catalytic activity of Trx1.

The thioredoxin system consists of Trx1, thioredoxin reductase (TrxR) and NADPH. Trx1 functions as a reductase via the dithiol/disulfide exchange reaction on oxidized

protein substrates that most of the times contain disulfide bonds (111). Due to this reaction, Trx1 becomes oxidized and needs to be reduced in order to regain antioxidant function. This reaction is catalyzed by TrxR, which receives electrons from NADPH, using FAD as a cofactor (112).

The promoter region of the gene that codifies for Trx-1 (*TXN*) contains a series of stress-response elements, including antioxidant response element (ARE) and cAMP response element (CRE) (111). Under homeostatic conditions, Nrf2 is located in the cytosol, where it forms a complex with two molecules of Kelch-like ECH-associated protein 1 (Keap1), which act as negative regulators of Nrf2 (113). In response to oxidative stress, Keap1 becomes oxidized, resulting in Keap1 inactivation and Nrf2 stabilization and translocation to the nucleus where it heterodimerizes with small musculoaponeurotic fibrosarcoma (sMaf) proteins, and binds to the ARE element to activate the transcription of its target genes such as Trx1 (114).

Many studies show the involvement of Trx1 in redox-associated cellular processes such as apoptosis and inflammation. Trx1 can bind apoptosis signal-regulating kinase-1 (ASK-1) to inhibit apoptotic signaling (111). ASK1 is bound to Trx1 in reduced conditions but when Trx1 becomes oxidized, ASK1 dissociates from Trx1 and interacts with TRAF2/6, inducing apoptotic signaling (115). Regarding inflammation, Trx1 exerts ambiguous functions. On the one hand, Trx-1 might induce atheroprotective effects by promoting an anti-inflammatory response in macrophages (116). On the other hand, Trx1 enables nuclear NF-κB DNA-binding and thereby facilitates pro-inflammatory responses in monocytes and dendritic cells (117). In addition, Trx-1 plays an important role in NLRP3 inflammasome activation and IL- 1β production in macrophages (111, 118). Furthermore, a large variety of proteins containing redox-sensitive cysteines, such as estrogen receptor-α (ERα) (119), Hypoxia Inducible Factor-1 (HIF-1)(120), phosphatase and tensin homolog (PTEN) (121) and glucocorticoid receptor (122) interact with Trx1 regulating their activity in this way.

Trx1 is ubiquitously expressed in different tissues and cell-types. However, Trx1 expression in the brain is lower compared to other organs (123). This could potentially explain why the Trx1 system is sensitive to disturbances in neurodegenerative conditions. Several studies have investigated the levels of Trx1 in AD brains with contradictory results. Early studies analyzing Trx1 levels in

hippocampus, amygdala, and temporal lobe brain regions in human AD postmortem samples, found a significant reduction in AD compared to healthy subjects. The same study also showed an increased activity of TrxR in AD brains (124). Previous results from our lab showed similar results: Trx1 levels were reduced in neurons from the frontal cortex and hippocampus of AD postmortem samples as determined by immunohistochemistry (125). However, another study reported no differences in overall Trx1 levels on AD brain hippocampal sections. The authors showed that, the localization of the protein changed, increasing in cytosol and decreasing in nucleus of AD compared to controls (126). Several experimental studies investigated the role of Trx1 in AD pathogenesis. Trx1 has been associated with protection against Aβ induced toxicity. Both Trx1 overexpression in a neuroblastoma cell-line as well as Trx1 treatment of rat neuron primary cultures, showed a protective effect on Aβ-induced cell death (124, 125).

1.4.2 THIOREDOXIN-80

The last 24 amino-acids of the C-terminus of Trx1 can be cleaved to generate a 80-84 amino acid long peptide known as Thioredoxin-80 (Trx80)(127). This protein has a similar conformation to Trx1 but due to the cleavage, it lacks one α-helix and one β-strand motif (128). This structural change has different effects on the features and function of Trx80. Firstly, Trx80 lacks oxidoreductase capacity and it is not a substrate of TrxR, however, it can be reduced by Trx1 (127). Secondly, unlike Trx1, which is mainly found as a monomer under physiological conditions, Trx80 forms stable dimers and higher molecular aggregates. The molecular weight of monomeric Trx80 is around 10 KDa, as reported by several authors (129, 130). However, when measuring Trx80 in brain homogenates, it is predominantly found as a 30 KDa protein and sometimes it forms further molecular aggregates of 60 to 80 KDa (131). It is likely that the driving force behind Trx80 tendency to aggregation is the hydrophobic interactions taking place when the inner hydrophobic surface area is exposed upon the C-terminal cleavage of Trx1 (128).

Trx80 has been mainly studied in the periphery, where it was initially characterized as an eosinophil cytotoxicity-enhancing factor (ECEF) produced by monocytes isolated from peripheral blood mononuclear cells (132). However, Trx80 is not exclusively produced by monocytes, other immune cells such as T and B cells also produce it albeit in much lower quantities (127). Other tissues and non-immune cell-

types such as synoviocytes have been reported to produce it as well (133). Similarly to Trx1, Trx80 is also present in blood serum but there is no correlation between Trx80 and Trx1 levels (128, 134). Our group reported detectable levels of Trx80 in human CSF and that it is produced in the brain by neurons and astrocytes (131). In neurons, Trx80 is localized in soma and neurites whereas in monocytes, it is found on the cell surface, facing the extracellular environment. Trx80 can be secreted outside of the cell, however, the mechanism is still unknown since it lacks a signal peptide (133).

In the periphery, Trx80 triggers innate immunity by inducing the proliferation and differentiation of human monocytes as shown in **Figure 3** (135, 136). Specifically, it induces the upregulation of cell surface pathogen recognition receptors, such as CD14 (a receptor that upon activation, promotes the phagocytosis of microbes, among other functions) and stimulates the production of interferon- γ (IFN-γ) (130).

In addition, Trx80 induces the upregulation of molecules that are essential for T-cell activation and function, (for example CD86, CD54, CD40) and acts as a chemoattractant signal for monocytes, T cells and polymorphonuclear cells (PMNs) (137). Importantly, cysteine mutations of the redox-active site did not affect Trx80's biological effects, suggesting that the disulfide-reductase activity is not required for the biological effects described above (135). Treatment of CD14+ monocytes with Trx80 stimulates the release of several pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8 and anti-inflammatory cytokine IL-10 (138). IL-10 has been reported to play an important role in the regulation of the inflammatory response elicited by monocytes and dendritic cells, by limiting their chemokine secretion and regulating their antigen-presenting capacity. Moreover, IL-10 promotes the development of regulatory T cells and has been reported to suppress pro-inflammatory responses of T helper 17 cells (139). Thus, Trx80's effect on CD14+ monocytes may represent an auto-regulatory inflammatory mechanism (138).

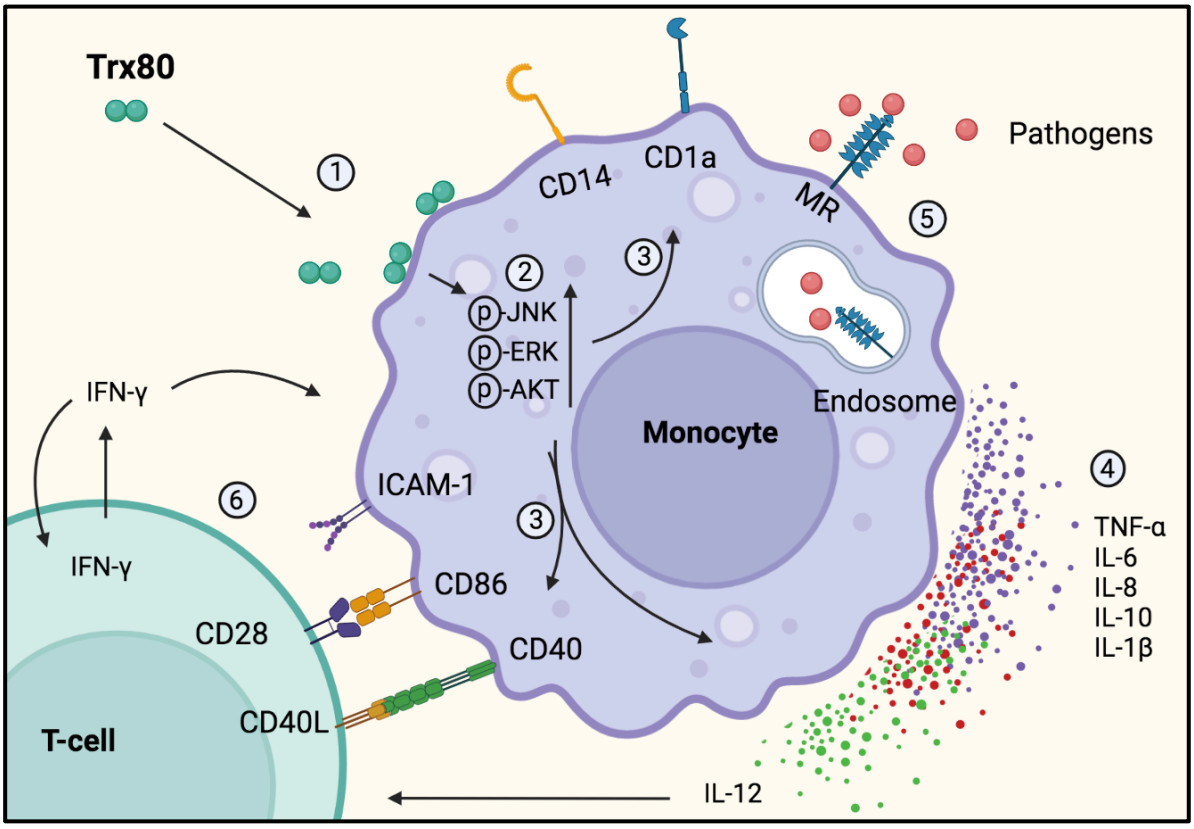


***Figure 3**. Trx80's effect on the interplay between monocytes and T-cells. **1)** Trx80 presumably binds to a receptor localized to the cell surface of monocytes and macrophages. **2)** Trx80 induces the activation of intracellular signaling of ERK, AKT and JNK pathways. **3)** Trx80 enhances the expression of cell surface receptors for T-cell activation (Icam-1, CD86 and CD40) as well as receptors for pathogen recognition (CD14, Cd1a and Mannose receptor (MR)). **4)** Trx80 induces the production and secretion of cytokines (TNF-α, IL-6, IL-8 IL-10, IL-1β). **5)** Trx80 induces the internalization and elimination of pathogens. **6)** Trx80 stimulates the secretion of IL-12 from monocytes and IL-12 enhances T-cell cytotoxicity as well as the secretion of IFN-γ in synergy with IL-2.*

Another study described the ability of Trx80 to interact with the complement system, a group of plasma proteins that represent the primary defense mechanism of the innate immune system. This study reported that, in contrast to Trx1, that has an inhibitory effect on complement activation, Trx80 can bind to peptides known to induce the activation of the complement system via the classical and alternative pathways (140). For example, Trx80 binds to C1q and propertin, which initiates the complement activation cascade and production of the anaphylatoxin C5a, resulting in elevated neutrophil chemotaxis. However, Trx80 failed to induce a full complement cascade activation, since its action on late stage complement activation is weak. In addition, the inflammatory potential of Trx80 was limited by the binding to C4b and factor H, which are inhibitors of the complement system (140).

In agreement with its function as a regulator of immunity and inflammation, Trx80 seems to be associated with chronic inflammatory disease, such as Rheumatoid arthritis (RA) and Atherosclerosis. Elevated Trx80 levels have been reported in synoviocytes from RA patients (133). This study also reported that in vitro stimulation of RA synoviocytes with pro-inflammatory molecules such as IL-1β and TNF-α induces increased Trx80 production. However, the treatment of RA synoviocytes with an oxidative stimulus like H_2O_2, despite inducing an increase in Trx1 levels, did not have any effect on Trx80 levels, indicating that Trx1 increase is not sufficient to produce higher Trx80 levels. On the other hand, Trx80 seemed to colocalize with reactive macrophages in atherosclerotic lesions together with TNF-α (141). The presence of TNF-α in atherosclerotic lesions has been associated with a stronger inflammatory response elicited by activated macrophages and perpetuates those lesions (134).

Our group reported that Trx1 can be cleaved by the α-secretase activity of ADAM 10/17 to generate Trx80 and the treatment with phorbol 12-myristate 13-acetate (PMA) increases intracellular and extracellular levels of Trx80 (129, 131). Importantly, α-secretase activity is decreased in cases of AD, where lower levels of Trx1 have been reported (142, 143). Consequently, Trx80 levels were significantly reduced in AD brains, even in those regions with heavy amyloid-beta plaques deposition and consequent pro-inflammatory activity (144, 145). Similarly, Trx80 levels were also strongly reduced in CSF samples from AD and mild-cognitive impairment (MCI) patients in comparison to controls (131). Interestingly, *in vitro* experiments showed that Trx80 prevents Aβ aggregation and inhibits its toxic effects (131). However, the biological function of this peptide in the brain is still unknown.

1.5 NEUROINFLAMMATION

Neuroinflammation is referred to as an inflammatory response within the Central Nervous System (CNS). This response can be elicited by diverse pathological insults like trauma, ischemia, exposure to toxins and infections (146). The hallmark of this process is the production of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-18 and TNF, chemokines, including C-C motif chemokine ligand 1 (CCL1), CCL5 and C-X-C motif chemokine ligand 1 (CXCL1), small-molecule messengers, for instance prostaglandins and nitric oxide (NO) (147) as well as ROS generated by the innate immune system of the CNS. The cells commonly involved in this process are

microglia and astrocytes, but infiltrating blood cells can contribute to neuroinflammation as well, especially when the BBB is damaged (147).

Accumulating evidence shows that together with the AD pathological hallmarks, Aβ plaques and NFTs, neuroinflammation contributes greatly to AD pathogenesis (148). Acute inflammation is a tightly controlled defense mechanism against infections and injury, maintained by a balance between pro- and anti-inflammatory factors. However, in AD, the sustained and elevated inflammatory response due to the accumulation of aberrant protein aggregates and excessive cytokine release can lead to chronic inflammation, which may result in cumulative damage (149). Chronic neuroinflammation is not only present in AD, other neurodegenerative disorders such as Parkinson's disease (150), multiple sclerosis (151) and amyotrophic lateral sclerosis (152) also display elevated levels of inflammation. Increasing evidence shows that inflammatory processes occur prior to neurodegeneration (148, 153, 154). In fact, epidemiological studies have reported that inflammatory proteins are already present in CSF at early stages of AD, suggesting that they might contribute to, rather than be a consequence of, the formation of Aβ and NFT pathologies (154).

1.5.1 THE ROLE OF MICROGLIA IN AD-RELATED NEUROINFLAMMATION

Microglia are innate immune cells that reside in the CNS. These cells, that belong to the myeloid lineage, were initially thought to seed in the CNS as any other tissue specific macrophages. However, microglial progenitors arise from primitive hematopoiesis at the early stages of embryonic development (155). They emerge from the yolk-sac, an event that is runt-related transcription factor (RUNX) and macrophage colony-stimulating factor 1 receptor (CSFR1)-dependent. At embryonic day 8.5, these myeloid precursors are transported to the embryonic brain where they are maintained in a self-renewal manner and independent of circulating monocytes (155).

Microglial activity and function is believed to adapt to every step of the CNS development and maturation by adopting different regulatory networks and it plays important roles in many developmental processes such as synapse plasticity, neuronal apoptosis, synaptic pruning, and immune surveillance (146). In a healthy brain, microglia display a ramified morphology with long processes that extend and retract to continuously survey the CNS microenvironment, sensing danger signals and directing a response towards the site of injury. Upon trauma or infection by a

pathogen, damaged or dying cells release molecules known as damage-associated molecular patterns (DAMPs) or alarmins that serve as a warning signal (156). DAMPs can be of intracellular origin, as for example heat-shock proteins, high-mobility group box 1 (HMGB1) histones, S100 proteins, and plasma proteins, like fibrinogen, among many others. When these endogenous danger signals are released to the extracellular space they are recognized by pattern-recognition receptors (PRR) expressed by immune cells, including microglia. The main function of PRRs is to recognize pathogens, cell debris and abnormal proteins, including Aβ, and to induce an adequate microglia response which generally involves the stimulation of phagocytosis and mediation of inflammatory response (156). Activated microglia aim at eliminating the source of pathogenic stimuli by activating the expression of adequate responses, including interferons and receptors for chemokines (157). These actions conform the neuroinflammatory process that is usually resolved once the stimulus is neutralized, leading to a phase of biosynthesis of active mediators that promote homeostasis and repair of the affected tissue (158). However, when the magnitude or the duration of the inflammatory process is dysregulated, it can lead to a sustained inflammatory process that contributes to the pathogenesis of neurodegenerative diseases (159).

In addition to their central role as immune cells of the brain, microglia have an active role in preserving neuronal homeostasis. In order to achieve this, microglia senses neuronal activity by expressing different neurotransmitter receptors (160), whose stimulation affects key microglial functions such as cytokine production, phagocytosis and cellular motility among others (161, 162). Conversely, several neuronal receptors are activated by microglia-derived molecules, allowing microglia a certain control over neurotransmission (160). This bidirectional communication between neurons and microglia contributes to the maintenance of brain homeostasis in roles like neurogenesis and axonal growth (163), synapse pruning (164) and circuit refinement (165) as well as modulation of synaptic activity (166).

Increasing evidence shows that this communication is dysregulated in neurodegenerative disorders (160). Under physiological conditions, CD200–CD200R and CX3CL1–CX3CR1 signaling pathways are known to maintain microglia in a homeostatic state (167). However, the expression of CD200, CD200R and CX3CR1 is reduced in AD brains, suggesting a change in physiological microglial behavior (168). Aβ treatments on astrocytes have been shown to activate NF-κB

pathway, promoting an enhanced release of complement C3, which in turn interacts with its receptor, C3aR, present in neurons and microglia (169). This interaction causes neuronal dysfunction and microglial activation (170). Conversely, the release of IL-1α, C1q and TNFα by microglia has been shown to induce a neurotoxic reactive state in astrocytes (171), suggesting that under inflammatory conditions, microglia and astrocytes can form a positive feedback loop of inflammatory response that could become dysregulated and chronic (146).

Microglia have a complex role in AD development owing to their variety of activation pathways. Transcriptional studies in AD mouse models have shown that disease progression is often accompanied by a transition from homeostatic to reactive microglial states (144). One of the best characterized reactive microglia phenotypes is the disease-associated microglia (DAM), which have been shown to downregulate homeostatic genes and upregulate genes associated with AD, including *APOE*, *TREM2* and *TYRO* protein tyrosine kinase-binding protein (*TYROBP,* Dap-12) (172). Keren-Shaul and colleagues also showed that homeostatic microglial transition to DAMs consists of two steps, an initial Trem-2 independent step followed by a Trem2-dependent step, remarking the relevance of Trem2 in AD pathology. Moreover, temporal tracking of microglial activation in an CK-p25 inducible mouse model of severe neurodegeneration identified differential gene expression clusters for early and late-stage microglia changes (173). Early changes involved the upregulation of genes related to proliferation whereas late-stage responses involved the upregulation of immune related responses typified by modules of co-regulated type-I interferon response genes and major histocompatibility complex class II (MHC class II) components, respectively. In agreement with these findings, another transcriptomic study using the App knock-in mouse model (APP^{NL-G-F}) showed that Aβ accumulation accelerated the activation of microglia into two main reactive microglia states. One of those states was enriched in AD-risk genes (*APOE*, *TREM2* and *TYROBP* among others) and MHC class II genes whereas the other overexpressed type-I interferon response genes (144). Moreover, they also noted that these microglial states were even present during normal aging.

Although complex, genetic and early functional studies suggest that microglia may directly contribute to AD pathology (174). GWAS findings have uncovered rare genetic variants that are associated with LOAD and implicate immune and microglial networks as important players in the development of AD. In fact, several of those

genes (*CR1, SPI1, TREM2, ABCA7*, *CD33,* and *INPP5D*) are expressed by microglia (175). However, the exact contribution of these cells in AD is not yet fully understood.

2 RESEARCH AIMS

The brain is sensitive to changes in cholesterol metabolism and inflammatory processes. These factors are important contributors to AD development as it is reflected in the brains of AD patients. Moreover, these processes are interconnected, and disturbances in either cholesterol metabolism or chronic inflammation can lead to alterations in the other and vice versa. However, clear molecular links between them and early detection of these changes in relation to neurodegeneration and AD are missing. 27-OHC, a cholesterol metabolite and Trx80, an antioxidant-derived peptide with immune modulatory properties have been shown to be altered in the brain of AD patients. Thus, this thesis aims to investigate the involvement of 27-OHC and Trx80 in the context of AD.

Specifically, the aims include:

- To explore the potential of serum 27-OHC and Trx80 as early biomarkers for AD and their relation to early AD changes and levels of known AD biomarkers (**Papers I, II and IV**).
- To analyze the relationship between Trx80 levels and AD risk factors, including aging, ApoE4 genotype, alteration in cholesterol metabolism and inflammation (**Papers II and IV**).
- To characterize the biological function of Trx80 and how its synthesis is regulated in the brain (**Papers II, III, and IV**).
- To investigate the role of Trx80 on AD pathology (**Papers III and IV**).

3 MATERIALS AND METHODS

This thesis has been generated by the use of several methods. Here, a short presentation of the methodologies is discussed. Nevertheless, for a more detailed description of the methods and models used please refer to the respective papers.

3.1 EXPERIMENTAL MODELS

3.1.1 Human Subjects

Animal models allow the manipulation of molecular mechanisms that help elucidating fundamental aspects of disease pathogenesis. However, findings in animals have limitations when translating or mirroring human diseases. Thus, it is important to be cautious when extrapolating results from animal studies into human diseases. For this reason and to carry out a translational approach we have utilized data from human subjects belonging to two different cohorts of patients: i) the Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability (FINGER) and ii) the GEDOC biobank study.

FINGER consisted of a two-year randomized control trial (RCT) that reported beneficial cognitive effects in an at risk older general population (42). The recruitment of patients was based on cognitive performance at slightly lower or at mean level expected for their age according to Finnish population norms for the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) (176), and six points or higher in the Cardiovascular Risk Factors, Aging and Dementia (CAIDE) risk score (177). This RCT consisted in a multimodal lifestyle and vascular intervention, including diet, physical exercise, cardiovascular management as well as cognitive training (43). In contrast, controls were giving regular health advise. Our exploratory sub-study included blood serum data from 47 FINGER trial participants (21 woman and 26 men, mean age 71 years) who underwent both MRI and PET imaging (178). Further information is available in Table 1 from **Paper I** and Table 2 from **Paper II**.

The study on serum from the GEDOC biobank cohort was performed on blood serum samples from 99 patients recruited at the Karolinska University Hospital memory clinic. The cohort consisted of 37 SCI, 31 MCI and 31 AD cases. The SCI group consisted of individuals with no memory-related deficits upon objective testing. MCI

diagnosis was previously described by Winblad at al. and defined upon the following three criteria (179):

- Patients do not exhibit signs of dementia, but their cognitive abilities are not considered normal either. These individuals do not fullfil the criteria for a diagnosis of dementia according to the Diagnostic and Statistical Manual of Mental disorders, fourth edition (DSM-IV).
- These individuals experience cognitive decline assessed either subjectively by the patients and/or an informant and additionally exhibiting significant impairment on objective cognitive tasks or a decline in performance overtime.
- The patient has intact or minimal loss of basic daily living activities.

Finally, AD patients were diagnosed as such if they met the DSM-IV criteria for AD diagnosis (180). Additionally, the patients were further examined based on a comprehensive clinical protocol including detailed psychological and clinical tests, Mini-Mental State Examination (MMSE), blood tests and CSF analysis of (total-tau, phospho-tau and Aβ42), further described in Table 1 of **Paper II**.

Postmortem brain tissue provides valuable information when studying human neurodegenerative disease like AD. However, many factors can affect the results obtained from this material such as *postmortem* time interval (PMI), age, sex, temperature of storage and tissue processing procedure. Therefore, whenever it is possible, it is important to have age and sex matched cohorts and with a minimal PMI difference between diagnostic groups. *Postmortem* human brain samples in **Paper II** were obtained from the CIEN foundation tissue bank for neurological research (BT-CIIN) in Spain. Information including age, sex, ApoE4 genotype, postmortem interval and dementia diagnosis from the tissue donor is displayed in supplementary table 1 of **Paper II**.

3.1.2 *In vivo* models

3.1.2.1 Mouse models

The mouse models used in **Paper IV** are based on the C57BL/6J background strain. This strain is widely used for a general multipurpose model as well as for the development of transgenic/knock-in models. For our purposes male and female mice with age and sex matched control mice were used. All the animals were housed on

a 12h light-dark cycle with controlled temperature and humidity. Normal chow feed was provided ad libitum.

3.1.2.1.1 CYP27A1-overexpressing mouse model (Cyp27Tg)

Cyp27A1-overexpressing mouse is a useful model to investigate the *in vivo* effects of elevated levels of 27-OHC. Another interesting aspect of this model is that it does not show significant differences in total plasma cholesterol, phospholipid and triglyceride levels between transgenic animals and wild-type controls. However, Cyp27Tg mice had a three to five-fold increase of 27-OHC in blood in comparison to control littermates. In **Paper IV**, 22-month-old male Cyp27Tg mice and age matched wild-type control mice were used.

3.1.2.1.2 APP^{NL-G-F} knock-in mice

Since the discovery of the familial form of AD caused by mutations in the *APP* and *PSEN* genes, several mouse models have been generated to overproduce APP and induce Aβ pathology. However, overexpression of several mutations can cause artificial phenotypes in mice that can significantly differ from the human AD pathology. To bypass such issue, Saito and colleagues, generated a mouse model that expresses *APP* containing a humanized Aβ sequence with three mutations, the Swedish (NL), the Iberian (F) and the Arctic (G). In contrast to *APP* overexpressing animal models, APP^{NL-G-F} mice produce endogenous levels of APP. In this mouse model Aβ deposition starts between the second and third months of age and the first signs of cognitive impairment are detected at six months of age. Since APP^{NL-G-F} mice do not exhibit NFTs or neuronal loss, it has been suggested that this mouse model should be considered as an early AD model (144). Importantly, APP^{NL-G-F} mice develop glial activation and reactivity, and this model has been previously used to study transcriptomic changes in microglia in the context of Aβ pathology. In **Paper IV**, 3 and 10-month-old female APP^{NL-G-F} mice and age and sex matched wild-type (WT) mice cortical samples were used. Additionally, we used transcriptomic data from microglia extracted from 12-month-old APP^{NL-G-F} mice.

3.1.2.2 Drosophila Melanogaster

In **Paper III**, a transgenic Drosophila model was used to investigate the effect of Trx80 on Aβ related neurotoxicity. This experimental model provides strong advantages for research. It is inexpensive to maintain and easy to handle. It allows

fast production of new genotype due to its short generation time and it ensures a significant number of biological replicates to perform experiments. The *Drosophila Melanogaster* genome has around 17,000 genes, of which many are analogs to human genes (181). This makes it a simple but useful model for studying human disease. Drosophila files contain four chromosomes including the sex chromosome. To keep track of the fly crossings, balancer chromosome fly lines are used. The balancer carries a physical trait/marker to distinguish genotypes without genetic screening. Homozygous flies for the balancer are not viable, ensuring that flies carrying the balancer also carry the transgene of interest. Transgenes are expressed by the GAL4/UAS system. In brief, this system uses an upstream activating sequence (UAS) that is inserted upstream of the gene of interest (182). This sequence has a GAL4 binding site that regulates the expression of the gene of interest. The crossing of a fly line containing the gene of interest and the UAS sequence with another fly line containing the GAL4 driver with a tissue specific expression results in the desired gene expression in selected tissues (183). We used a driver line called ElavC155 that expresses the transgenes on neurons (184). In our project we overexpressed human proteins that are absent in normal flies, which must be taken into account when interpreting the results.

3.1.3 Cell cultures

3.1.3.1 SH-SY5Y neuroblastoma cell line

The cellular work shown in **Paper III** has been carried out in a human neuroblastoma cell line (SH-SY5Y). This model is commonly used to study cellular mechanisms that allows genetic manipulation either though overexpression or silencing of genes. In addition, its fast replication rate allows for the attainment of a large number of cells while no ethical permit is needed. Nevertheless, it is an immortalized cancer cell line. Brain neurons are post-mitotic whereas neuroblastoma cells are not. This implies significant metabolic differences with normal brain neurons. Hence, the data obtained with these cells should be carefully interpreted, avoiding any direct extrapolation to what occurs in the human brain.

3.1.3.2 Mouse primary neuronal, astrocyte and microglia cultures

Mouse primary neuronal, astrocyte and microglia cultures are prepared from mouse embryos. These cells allow a better understanding of the biological and molecular processes in the brain in a more reliable way than neuroblastoma cells. The

enrichment of either neurons or glia to ensure a healthy culture hampers the study of the communication between different cell-types. Moreover, their use allows genetic manipulation, which we have employed in this thesis by using small interfering RNAs (siRNAs).

3.2 ANALYTICAL TECHNIQUES

3.2.1 Western blot

Western-blot technique was used to analyze Trx80 and Trx1 protein levels, as well as autophagic proteins. This technique detects a specific protein in a given sample, but it is a semi-quantitative method, meaning this technique does not deliver a real value of what is measured but rather a value that has to be related to others. Western blot involves the separation of proteins according to their molecular weight by gel electrophoresis, transfer to a nitrocellulose membrane and antibody probing. Fluorescence-labelled secondary antibodies were used for antibody detection. Signal visualization was done using the Odyssey CLx Imaging System (LI-COR) and the signal obtained by proteins of interest was quantified using NIH ImageJ software. Signal intensities of proteins of interest were normalized against GAPDH, β-Actin or Tubulin signal of the same sample, since these proteins are abundantly expressed and distributed in most cell-types and provide a control for even protein-loading of the gel. It must be considered that Western-blot technique can give false positive results, due to lack of antibody binding specificity. Proteins of different sizes, or proteins that tend to polymerize require the use of different protocols to achieve a proper detection.

3.2.2 Immunofluorescence

Immunofluorescence was performed in **Paper III** to investigate the effect of Trx80 in the degradation of Aβ in Drosophila fly brains and in **Paper IV** to analyze the interferon response *in vitro* in mouse primary microglia cultures and *in vivo* in 22-month-old Cyp27Tg cortical samples. Images were acquired with Zeiss 710 confocal microscope and Leica epifluorescence microscope. Comparison of signal intensities provides semi-quantitative data that need to be compared to other signals. Thus, sample preparation including fixation and sample sectioning are fundamental factors that can greatly influence the staining intensity. When performing

immunofluorescence analysis, staining protocol and imaging consistency between samples must be maintained.

3.2.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a technique that relies on antibodies to detect a target antigen using specific antibody-antigen interactions. The antigen or protein of interest of a sample is immobilized to a solid surface using a capture antibody immobilized on the surface. The antigen is then complexed to a detection antibody conjugated with a molecule suitable for detection such as an enzyme. Unlike the previously mentioned techniques, this is a quantitative method able to determine the concentration of the protein of interest in a given sample. ELISA was used in **Paper II** to measure Trx80 concentration in blood serum samples of patients and in **Paper III** to measure Aβ42 concentration in the brain of the flies and to determine the amount of different Aβ aggregation species.

3.2.4 RNA-sequencing analysis

RNA sequencing is a technique that reveals the presence and quantity of RNA in a biological sample at a given moment. This technique provides a detailed overview analysis of the highly dynamic cell-transcriptome. To perform the analysis, the RNA is first isolated from the rest of the biological tissue, including the genomic DNA. After controlling for RNA quality following the purification and ensuring the amount to meet the standards for the procedure, RNA is inversely transcribed to complementary-DNA (cDNA) for amplification purposes. Once cDNAs are purified, up to 200 ng of cDNA are further used for cDNA libraries preparation. After the cDNA libraries are prepared, they are multiplexed and finally sequenced.

3.3 ETHICAL CONSIDERATIONS

Any research being conducted on human subjects or animals must be examined and approved by an ethical board. In this thesis, the use of humans and animals was approved by the regional ethical committee, and the reference approval number are detailed below:

FINGER cohort ref no.: 2020-07058.

GEDOC 1 cohort ref no.: 2020-06484.

GEDOC 2 cohort ref no.: 2019-06056.

Mouse studies (the use of brains as well as neuron and microglia primary cultures) ref no: 4884-2019.

With regards to the use of mice and mouse primary cultures we have performed the necessary minimum amount of experiments for statistical significance, in compliance with the reduce aspect of the 3R principles of animal research. The use of laboratory animals in **Paper IV** was done according to the Karolinska Institutet guidelines and Swedish National guidelines. Moreover, all the patients involved in these studies granted their consent.

4 RESULTS AND DISCUSSION

4.1 EARLY SIGNS OF COGNITIVE DETERIORATION AND AD

In the brain of AD patients, abnormal accumulation of Aβ and tau and the detrimental processes that they trigger begin years before the onset of AD symptoms. By the time AD is clinically diagnosed, synaptic and metabolic changes in neurons have already occurred in several brain areas as first signs of neurodegeneration (185). Aging, along with changes in brain cholesterol metabolism and neuroinflammation, are known factors that greatly influence both the risk for AD and its progression (**Papers I, II and IV**). Therefore, we explored whether molecular targets of cholesterol metabolism and inflammation such as 27-OHC and Trx80 could have potential as early and reliable diagnostic markers of AD.

4.1.1 FINGER randomized controlled trial modulates serum 27-OHC levels and its effects on cognition

To study early changes occurring before the onset of AD symptoms and the possibility to prevent them in relation to cholesterol metabolism, we measured 27-OHC levels in serum samples from patients undergoing the FINGER randomized control trial. This cohort is represented by older adults at risk for AD who reported beneficial effects on cognition after a two-year multimodal lifestyle/vascular intervention (43). Here, we investigated the effect of this preventive intervention on 27-OHC levels, and its relation to AD neuroimaging markers and cognition (**Paper I**).

At baseline, elevated 27-OHC levels correlated with lower cognition and memory scores. In connection with this, higher 27-OHC levels correlated with lower total hippocampal volume and total gray matter. Noteworthy, these correlations between 27-OHC levels, MRI measures and cognition were not affected by total cholesterol levels in serum, suggesting that 27-OHC acts independently of cholesterol on brain function.

After the two-year intervention trial, 27-OHC levels increased in the control group but decreased in the intervention group, although the difference was not significant between the groups. Interestingly, the intervention reduced 27-OHC levels particularly in individuals with the highest 27-OHC levels measured at baseline. Furthermore, the reduction in 27-OHC positively correlated with improved memory

scores among the intervention group, strengthening the relationship between 27-OHC and cognitive function.

The findings from this study are relevant when considering that in the entire FINGER trial there were no intervention benefits on cholesterol levels (43). This suggests that 27-OHC might be a more sensitive biomarker than cholesterol when reporting the benefits of lifestyle/cardiovascular interventions on cholesterol metabolism. Moreover, these data highlight the role of 27-OHC as a possible intermediary of the negative effects of cardiovascular risk factors in the brain. In fact, patients suffering from atherosclerosis often have elevated levels of 27-OHC in the blood and it is the most abundant oxysterol present in atherosclerotic lesions (80). Previous studies from our group reported that high 27-OHC levels result in the over-activation of the brain RAS which, among other functions, regulates blood pressure in the brain (68, 70, 186) suggesting that 27-OHC might be involved in hypertension. Finally, the intervention resulted more beneficial for younger participants as their 27-OHC reduction was significant compared to older individuals, indicating that age but not sex or lipid-lowering medication had a significant impact on the FINGER intervention effect on 27-OHC levels.

4.1.2 Serum Thioredoxin-80 is associated with AD risk factors

In healthy conditions, the brain has its own mechanisms to remove misfolded and aggregated proteins, including Aβ. In fact, the defective clearance of Aβ and tau is believed to underlie most of the sporadic AD cases (15). Previous studies showed that Trx80 can prevent Aβ aggregation and inhibit Aβ toxic effects in cell cultures. Nevertheless, Trx80 is completely depleted in brains and CSF from AD patients (131). In view of this evidence linking Trx80 with AD pathology, we investigated the possible association of serum Trx80 levels with several known AD risk factors and AD biomarkers (**Paper II**).

Trx80 was measured in serum samples from two different cohorts: The GEDOC cohort, consisting of participants diagnosed with SCI, MCI and AD and participants from FINGER study that are older but cognitively healthy individuals. We found that serum Trx80 levels were significantly increased in AD and positively correlated with old age. Moreover, ApoE4-carriers had higher serum Trx80 levels than non-carriers both in the FINGER and GEDOC cohorts.

Interestingly, contrary to what it is observed in serum, Trx80 levels in the brain are decreased in ApoE4-AD compared to ApoE3-AD brain samples. As Trx80 shows neuroprotective and anti-amyloid properties an increased depletion of Trx80 could potentially contribute to the increased pathogenesis and faster progression of AD in ApoE4 carriers (187, 188). This relationship enabled us a new research path that we set out to investigate and that is featured in the subsequent chapter.

4.1.3 Brain Trx80 levels are associated with aging and altered cholesterol metabolism and decrease in AD-relevant conditions

As shown in **Paper II**, Trx80 levels increase in serum while they decrease in the brain of AD patients. To further investigate whether the presence of AD risk factors such as aging, alterations in cholesterol metabolism or Aβ-pathology could alter Trx80 levels in the brain, we measured Trx80 in the brain of different mouse models (**Paper IV**).

To test whether Trx80 levels change with aging not only in the periphery but also in the brain, we analyzed Trx1 and Trx80 protein levels in cortical homogenates from wild-type mice at 2, 8 and 22 months of age. We showed that Trx80 levels increase 1.7-fold, while Trx1 levels decrease 0.5-fold over time. Interestingly, Trx1 levels decrease in a discrete manner throughout the mouse lifespan, while Trx80 dramatically increases after 8 months of age. To test whether alterations in cholesterol metabolism affected Trx80 production in the brain, we analyzed the levels of Trx80 in cortical homogenates from 22-month-old Cyp27Tg mice. Trx80 levels were higher in Cyp27Tg animals in comparison to their aged-matched wild-type littermates, while there were no changes in Trx1 levels between Cyp27Tg and controls. These results support the hypothesis that Trx80 levels are not only a direct consequence of the abundance of its precursor, but they are regulated in a more specific manner, most likely by the regulation of the activity of the enzymes responsible for Trx1 cleavage.

Finally, to test whether amyloid pathology affects Trx80 production in the brain we measured Trx80 levels on $APP^{NL\text{-}G\text{-}F}$ mice brain homogenates over time, observing higher Trx80 levels at 3 months, followed by a decrease at 10 months of age. Noteworthy, increased levels of Trx80 take place at the same age as the first Aβ depositions occur in this mouse model (189). This suggests that Trx80 production increases as an early stress response to Aβ accumulation or Aβ oligomers-induced

alterations. Indeed, previous studies in the periphery have suggested Trx80 as an early danger-response signaling molecule (138). On the other hand, despite advanced age, Trx80 levels decrease at 10 months in APP^{NL-G-F} mice. A possible explanation for this is the fact that at this age APP^{NL-G-F} mice show advanced cognitive decline accompanied by widespread Aβ depositions, gliosis, and synaptic loss, which could be translated into alterations in the levels and/or activities of the metalloproteases responsible for the cleavage of its precursor. All these pathological events are common hallmarks of AD brains and the decrease in Trx80 that is observed in old APP^{NL-G-F} mice brains resembles the decreased we reported in AD brains.

4.2 TRX80 ROLE ON AMYLOID PATHOLOGY IN THE BRAIN

Trx80 has been previously described as a pro-inflammatory cytokine secreted by immune cells in the periphery (128, 136, 137) and it prevents Aβ aggregation and inhibits Aβ toxic effects in cell cultures (131). In view of these findings, we hypothesized that Trx80 could have a similar effect on Aβ *in vivo*, by inducing specific activation responses in brain cells (**Papers III and IV**).

In **Paper III** we investigated the effects of Trx80 on Aβ toxicity *in vivo* in *Drosophila melanogaster* models. As previously reported, human Aβ42 expression in the fly brain caused a progressive deposition of this protein accompanied by a reduction in their lifespan and locomotor activity (190, 191). In contrast, Aβ42 and Trx80 co-expressing flies showed a significant reduction of Aβ42 accumulation in the brain, that was accompanied by reduced mortality and improved locomotor activity. We hypothesized that the decrease in Aβ pathology observed in the Aβ42 and Trx80 co-expressing flies was due to an induction of Aβ42 degradation. Thus, we explored whether the positive effects of Trx80 on Aβ42 pathology were regulated by autophagy, one of the main intracellular protein degradation mechanisms. We performed a PCR array of 84 autophagy-related genes on fly brain homogenates and we found that the expression of some genes such as AMP-activated protein kinase alpha (*AMPKα*), Autophagy protein 2, 4a, 4b, 8a, 8b (*Atg2, Atg4a, Atg4b, Atg8a, Atg8b*), and *PI3K59F* was significantly increased in Trx80-expressing flies compared to wild-type flies. Similar results were observed in flies co-expressing Aβ42 and Trx80 when compared to Aβ42-expressing flies. These findings were further confirmed in a human neuroblastoma cell line, where Trx80 overexpression resulted in reduced Aβ42 protein levels as well as in increased autophagic flux.

Considering that the brain itself produces Trx80, and that it can be secreted, we investigated its effects on the brain's immune cells, microglia (**Paper IV**). Indeed, analysis of RNA-seq data from Trx80-treated microglia revealed that most of the upregulated pathways are associated with inflammatory responses and processes related to viral and bacterial recognition and elimination, matching the known effects of Trx80 in the periphery. Importantly, the transcriptomic profile of Trx80-activated microglia shows significant similarities to a subset of reactive microglia described in $APP^{NL\text{-}G\text{-}F}$ mice. This subset is known as interferon response microglia (IRM), due to its elevated levels of type I-interferon response genes (144). Moreover, the upregulation of the type-I interferon in response to Trx80 seems to be mediated by Trem2, since its silencing decreased the expression of genes involved in this response in Trx80-treated microglia compared to control. Trem2 is a receptor expressed by microglia and involved in various responses such as inflammation, phagocytosis and proliferation and some genetic variants of this protein have been shown to increase the risk of developing AD (192). Previous studies suggest that IRMs are associated with basal surveillance of neuronal stress and damage during aging (193). Our results showing that Trx80 increases in the brain with age, suggest that Trx80 could be a mediator of the appearance of IRMs in aged brains. Interestingly, a microglia subset that is enriched in interferon response genes has recently been found in MCI and AD brains (145, 194, 195). Moreover, one of the aforementioned studies identified a microglia cluster particularly abundant in AD that is not only enriched in type-I interferon genes but also in genes related to lysosomal and vesicular function (195). This cluster presents upregulation of pathways related to unfolded protein response and autophagy. Thus, this microglia gene cluster may be displaying a gene expression profile that explains the roles of Trx80 in both autophagic processes and type-I interferon response reported in **Papers III** and **IV**. We report an early increase of Trx80 in a mouse model of amyloid pathology, supporting an association of this axis with AD pathology, however, whether the presence of type-I interferon response microglia promotes or hinders the development of AD remains unknown.

4.3 REGULATION OF TRX80 AND ITS ROLE IN INFLAMMATION

In **Paper IV** we compared Trx80 levels between different brain cells; neurons, astrocytes and microglia and identified neurons as the main source of Trx80 in the brain. Although Trx1, precursor of Trx80, is expressed in all cell-types, mostly

neurons seem to have the capacity to cleave Trx1 into Trx80 under basal conditions. A previous study identified the metalloproteases ADAM10/17 as responsible for the processing of Trx1 into Trx80 (131). Our results suggest that the levels or the activity of these enzymes might be higher in neurons than in other brain cell types.

As previously mentioned, Trx80 levels in the brain are affected by aging, alterations in cholesterol metabolism and amyloid pathology. These factors are known inducers of oxidative stress (86, 104, 196, 197) and thus, the increase of Trx80 in response to these factors may be caused by oxidative stress itself. Since Trx80 is the product of an antioxidant protein, we hypothesized that an oxidative stimulus in neurons would induce the expression of Trx1 and hence a higher production of Trx80. Indeed, *in vitro* treatments with either rotenone or 27-OHC-elicited a Trx80 increase in neurons. Interestingly, the cleavage of Trx1 into Trx80 renders the peptide devoid of its redox capacity (135). This, together with our results, suggests that neurons increase Trx80 production under stress conditions with other purposes than mere antioxidant defense.

In the brain, Trx80 not only activated a type-I interferon response in microglia but also induced the expression of cytosolic pattern recognition receptors and interleukin-6 signaling pathway, among others, supporting the notion that Trx80 triggers a pro-inflammatory response on microglia.

These results mirror the reported effects of Trx80 in the periphery, where Trx80 triggers innate immunity by inducing the activation and differentiation of human monocytes and the upregulation of cell surface pathogen recognition receptors (128, 130, 135-138). In addition to this, we report that in serum, Trx80 levels are associated with higher levels of pro-inflammatory cytokines such as IL-8, IL-13, IL-5, IL-6, interferon-α2 and interferon-γ in old participants (**Paper II**). Also, ApoE4-carriers have higher serum Trx80 levels than non-carriers. Given that ApoE4 can contribute to inflammation (198, 199), it may be speculated that higher Trx80 levels are being produced as consequence.

Importantly, the ApoE4 carriers show higher Trx80 levels in serum and lower in the brain as compared to non-ApoE4 carriers. This is in apparent disagreement with the increased Trx80 levels in serum from the AD patients reported in the study. One possible explanation for this divergency is that the brain and peripheral Trx80 represent two independent pools, produced from different sources of Trx80 in the

body. Trx80 in the brain, produced by neurons and astrocytes (131), becomes progressively depleted as neurodegeneration occurs in AD, likely due to a reduction in the production of Trx80 precursor (Trx1), a downregulation of the enzymes that cleave Trx1 to Trx80 or neuronal death. Conversely, peripheral Trx80 is produced by innate immune cells and probably by other cell types in the body.

5 CONCLUSIONS AND FUTURE CONSIDERATIONS

The recurrent failures of disease-modifying drugs for AD might reflect, in part, that participants enrolled in clinical trials present a pathology that is too advanced to achieve a clinical benefit. Therefore, effective and reliable biomarkers for early detection and acute diagnosis of the preclinical stages of AD are fundamental for therapeutic progress. Clinical studies have shown that AD patients have significantly higher levels of 27-OHC in the brain and CSF. Additionally, associations between high serum 27-OHC levels and reduced cognitive performance have been described in MCI patients. In **Paper I** we report that 27-OHC correlates with lower hippocampal volume and cognitive test scores in a healthy but at-risk older population, suggesting that alterations in cholesterol metabolism might be affecting the brain years before the disease onset. Most importantly, the multidomain lifestyle intervention reduced 27-OHC serum levels primarily in younger in individuals with the highest serum 27-OHC levels, and this reduction correlated with improved cognition. This indicates that 27-OHC should be further studied not only as a potential AD and dementia biomarker, but also for monitoring the effects of preventive interventions. Importantly, a specific inhibitor of Cyp27A1, Anastrozole has been shown to decrease 27-OHC levels without altering cholesterol levels in mice (200). However, to our knowledge, no clinical trial is testing the effect of this compound in AD. Pharmacological treatments, in combination with lifestyle interventions can be a promising strategy to regulate 27-OHC levels and to prevent or delay the onset of dementia caused by alterations in cholesterol metabolism.

In this thesis we also propose serum Trx80 as an early biomarker for AD. In **Paper II** we report that serum levels of Trx80 are increased in AD patients and are associated with age and ApoE4 genotype in early AD stages. In healthy individuals at risk of dementia, serum Trx80 levels correlate with higher pro-inflammatory cytokine levels and lower hippocampal volume. Importantly, ApoE4 carriers show higher Trx80 levels in serum and lower in the brain as compared to non-ApoE4 carriers. Understanding how age and ApoE4 genotype, two main risk factors for AD, affect Trx80 levels in serum and the brain in contradictory manners might allow for a better understanding of early events in AD neurodegeneration. Moreover, further studies with a larger number of serum and CSF samples might be helpful to assess whether Trx80 can be used as a biomarker for early detection of AD-related changes.

In **Papers III** and **IV** we describe Trx80 roles in the brain: an anti-amyloidogenic peptide that enhances autophagic activity both *in vitro* and *in vivo* and as a trigger of inflammatory interferon response in microglia. Although apparently different, both roles could be related. A previous study speculated that Trx80 could participate in the control of intracellular infections by inducing autophagosome formation to trap and degrade pathogens (136). Virus and bacteria share mechanisms for their elimination by the immune system with misfolded and aggregated proteins present in neurodegenerative disease like AD. Thus, Trx80 may activate microglia to respond against Aβ oligomers or aggregates in the same way as Trx80-activated peripheral monocytes respond against pathogens: inducing an inflammatory response that activates neighboring microglia to provide a coordinated immune response (**Figure 4**). Once phagocytosed, Aβ is transported through a vesicular system to the lysosomes where it is finally degraded. Some of the proteins taking part in this dynamic vesicular process are involved in the autophagosome formation that is upregulated in Trx80-overexpressing cells, as we report in **Paper III**.

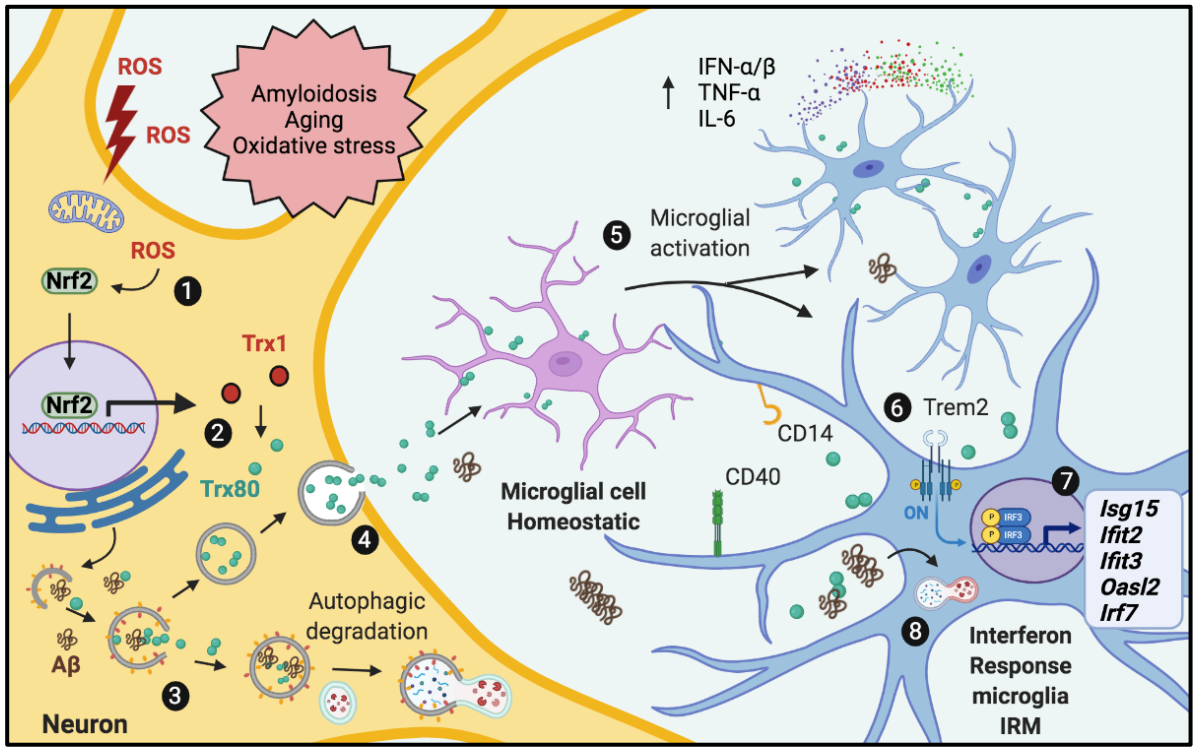


***Figure 4**. Mechanisms of Trx80 production, secretion and biological roles in amyloid pathology. **1)** ROS-mediated activation and nuclear translocation of Nrf2 in neurons. **2)** Trx80 production from the cleavage of Trx1 by the activity of ADAM10/17. **3)** Trx80 facilitates autophagosome formation and enhances Aβ degradation. **4)** Neuron derived Trx80 is released to the extracellular space. **5)** Microglia responds to extracellular Trx80. **6)** Trx80-activated microglia is regulated by Trem2. **7)** Trx80-*

activated microglia induces the expression of type-I interferon response genes. ***8)*** *Trx80-activated microglia activates cellular pathways related to engulfment of misfolded proteins and pathogens.*

From the APP^{NL-G-F} mouse model of amyloid pathology (**Paper IV**), we know that neurons increase Trx80 levels in response to Aβ at early stages. However, this increase in Trx80 is not enough to eliminate the high Aβ load in this mouse model and thus, Aβ accumulates in their brain. As the mice age they show widespread Aβ depositions, gliosis and synaptic loss accompanied by advanced cognitive decline, which could be translated into altered neuronal function. This can possibly trigger a decline at the levels of Trx1 and the levels and activity of the metalloproteases responsible for the cleavage of Trx80 precursor, exhausting the Trx80 production system. Deficient Trx80 production, together with increasing Aβ levels might, among other factors, shift the proportion of other microglia phenotypes towards disease-associated microglia (DAMs) that express many AD-risk genes contributing to disease progression (**Figure 5**). Additionally, widespread Aβ accumulation can promote other detrimental processes such as abundant oxidative stress and uncontrolled inflammation that accelerate the AD development.

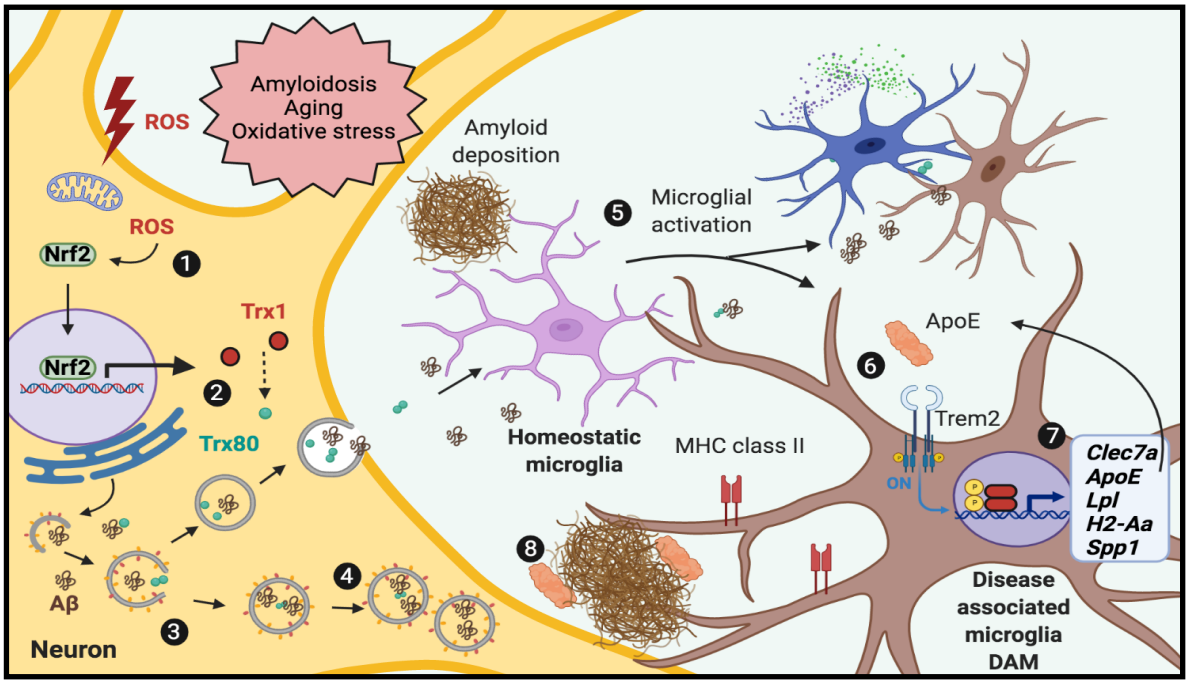


Figure 5*. Advanced amyloid pathology in the brain and low Trx80 levels.* ***1)*** *ROS-mediated activation and nuclear translocation of Nrf2.* ***2)*** *Low Trx80 production from the cleavage of Trx1.* ***3)*** *Reduced autophagosome formation.* ***4)*** *Impaired resolution of autophagy and autophagosome accumulation caused by Aβ.* ***5)*** *Microglia activation in response to increase levels of Aβ depositions in the brain.* ***6)*** *ApoE-Trem2 axis mediated activation and inflammatory response.* ***7)*** *Disease associated*

microglia (DAM) gene expression profile in response to Aβ. ***8)*** *Increased amyloid deposits and ApoE. Plaque associated microglia.*

From the evidence presented in **Papers III** and **IV** it would be tempting to suggest strategies to boost Trx80 levels in the brain as a way of dealing with Aβ pathology. One possibility would be to increase the activity of α-secretases that cleave Trx1 into Trx80 by using activators of these enzymes. However, this would not be ideal since α-secretases not only cleave Trx1 but have many other substrates, what could lead to undesired off-target effects. Another possibility would be to increase the levels of Trx80 precursor, Trx1. However, this approach could as well be challenging, since Trx1 is an antioxidant and under healthy redox homeostasis the cells use ROS as signaling molecules, thus it is not desirable to deplete them. Moreover, Trx1 is overexpressed in several types of tumors, therefore increasing Trx1 levels in proliferating cells could pose an additional risk. Since neurons are generally post-mitotic, a selective overexpression of Trx1 in neurons might be considered a better approach. However, even if Trx80 levels were to be successfully increased in the brain, this could lead to microglial inflammatory responses contributing to neuroinflammation. In other words, Trx80 function may be a double-edge sword as the same ability that can potentially lead to an efficient Aβ degradation may also cause undesirable inflammatory responses in the brain. Therefore, further work needs to be done to fully understand the molecular mechanisms that triggers the observed Trx80 effects. In this regard, attempts to dissociate the Aβ degradation mechanism from the inherent inflammatory response Trx80 triggers would be ideal. Alternatively, a combination of Trx80 overexpression in neurons together with an anti-inflammatory treatment would be an interesting strategy.

Single cell transcriptomic studies have provided the means to uncover and decipher microglial heterogenicity. Thanks to this rapidly evolving field, our view on the biology and function of microglia has evolved from a simplistic view of homeostatic versus activated microglia to a complex variety of microglial phenotypes that respond differently to stressors. These tools have identified interferon response microglia as an important phenotype in neurodegeneration. Although IRMs have been found in the proximity of neurons harboring DNA damage as well as amyloid plaques containing nucleic acids and present in the brains of MCI and AD patients, their potential role in the brain is still unknown. In this thesis we have shown that Trx80-treated microglia displays a significant gene expression profile overlap with IRMs.

Not only in commonly upregulated type-I interferon response genes but also in commonly downregulated genes. Thus, we provide an *in vitro* method that allows for a deeper characterization of this microglia subset as well as their involvement in AD.

In this thesis we describe and link the effects of two molecules involved in inflammation, cholesterol metabolism and oxidative stress: Trx80 and 27-OHC. In the periphery, both molecules are found in lesions associated with chronic inflammation and oxidative stress, such as atherosclerotic lesions. 27-OHC, a hallmark of altered cholesterol metabolism is the most abundant oxysterol in atherosclerosis (80) and Trx80 is produced by the macrophages present in such lesions, contributing to the inflammatory environment (134, 141). In AD, both 27-OHC and Trx80 are increased in serum of patients (134, 201) and seem to be affected by the presence of ApoE4 (202).

In the brain, similarly to the increased production of the inflammatory protein S100A8 and its receptor RAGE (84), high levels of 27-OHC increase neuronal Trx80 production *in vitro* and *in vivo*. 27-OHC, previously reported to cause oxidative stress and to induce the activation of Nrf2 (86), activates the Nrf2-Trx1-Trx80 axis in neurons. These findings link excessive 27-OHC to neuroinflammation by triggering both an alarming response and Trx80 production that may serve as a warning sign of dysregulation of brain homeostasis. In fact, excessive 27-OHC has been found to promote Aβ accumulation (72, 203), whereas Trx80 seems to protect against Aβ toxic effects and induce its degradation. However, in brains of advanced stages of AD, the levels of 27-OHC and Trx80 go in opposite directions. 27-OHC accumulates (65) in AD brains whereas Trx80 is completely depleted (131). Our data suggests that the ongoing processes are very dependent on the disease progression and thus, further research is required to clarify the timeframe of these events.

Altogether the results from this thesis suggest that, in the future, it may be fundamental to conceive a multi-target and more personalized and timed treatment to cure this multifactorial and heterogenous disease. Our results, describing the interplay between new factors that might indeed have a causative role in the disease years before its onset, could prove to be of great importance for the development of new, effective therapeutic targets and biomarkers against dementia.

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