# Preclinical and Clinical Early Alzheimer’s Disease (AD) Biomarker Discovery Using Proteins and MicroRNAs Carried by Small Extracellular Vesicles (sEVs)

## Thesis

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Preclinical and clinical early Alzheimer’s disease (AD) biomarker discovery using proteins and microRNAs carried by small extracellular vesicles (sEVs)

Soraya Moradi Bachiller

Istituto di Ricerche Farmacologiche Mario Negri – IRCCS

A thesis submitted to The Open University (UK) for the academic degree
Doctor of Philosophy

Discipline of Life and Biomolecular Science
October 2020
ABSTRACT

Alzheimer’s disease (AD) begins several years before the onset of symptoms. Unfortunately, current therapies treat too late and have limited efficacy.

To improve this, clinical trials need to test a potential drug earlier in the disease progression by increasing the diagnostic accuracy of an early or even asymptomatic stage for which reliable and minimally invasive biomarkers are mandatory.

In the context of blood-based biomarker discovery for early dementia, we focused on small extracellular vesicles (sEVs). SEVs contain specific cargoes that may reflect brain disease. They can cross the blood-brain barrier and can be isolated from biofluids, which may improve the detection of AD outside the brain.

Therefore, we investigated NFL, TREM2, and a possible novel AD candidate called ECSIT in mouse and human plasma-sEVs.

We found that TREM2, ECSIT and NFL are carried in mouse and human sEVs. Surprisingly, ECSIT showed lower levels in subjects with cognitive deficits, suggesting the possible role of this protein as sEVs-candidate biomarker for the early stage of AD, and prompting us to study its role in a cellular model for AD, where we found a possible link between ECSIT and APP.

We also focused on a different approach to reveal the presence of dysregulated microRNAs in human plasma-sEVs across AD stages.

To pursue this aim, we compared sEV-microRNA content from cognitively normal controls (Ctrl), mild cognitive impaired (MCI) and demented (AD) subjects. We showed a great number
of dysregulated miRNAs in the AD stage. However, we were unable to detect them for the MCI stage.

We conclude that our research contributed to assess the importance of plasma-sEVs as biomarker source and provided insights in the field of blood-based biomarker discovery for AD, that, we hope, may help to increase the diagnostic accuracy of the early stage of dementia, thus, enabling drug discovery progress.
PREFACE

The work described here was done at the Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy, from October 2017 to October 2020. This PhD was part of and was funded by a 3-years Marie Sklodowska-Curie Innovative Training Networks (ITN-ETN) - Blood Biomarker-based Diagnostic Tools for Early Stage Alzheimer’s Disease [BBDIAG - 721281] project. The PhD research project was conducted under the supervision of Dr. Diego Albani and Dr. Gianluigi Forloni as directors of studies, and Dr. J. Mark Cooper as external supervisor.
DECLARATION

I declare that this PhD research project has not been submitted in whole or in part for a degree or diploma to any other university. I submit to The Open University for examination in consideration of the award of a degree PhD. Where any of the content presented is the result of input from a collaboration with another group this is acknowledged here and, also, in chapter V. In this way, it is possible to know how much of the work is my own. The work is original and, to my knowledge, does not breach copyright law. When a work was taken from other sources, such work has been cited within the text.

The experimental work here described was performed by me, Soraya Moradi Bachiller, and it also includes work done in collaboration with:

- Annalisa Grimaldi and Nicolò Baranzini, Department of Biotechnology and Life Sciences, Università degli Studi dell’Insubria (Varese, Italy), who performed the transmission electron microscopy of sEVs-enriched samples.

- Luisa Benussi, Miriam Ciani and Roberta Zanardini, Molecular Markers Laboratory, IRCCS Istituto Centro San Giovanni di Dio Fatebenefratelli (Brescia, Italy), who performed nanoparticle tracking analysis of sEVs-enriched samples (from cells, mice and humans).

- Stefano Fumagalli, Istituto di Ricerche Farmacologiche Mario Negri IRCCS (Milan, Italy), who performed structured illumination microscopy of our sEVs-enriched samples.

- Veronica Caddeo, Istituto di Ricerche Farmacologiche Mario Negri IRCCS (Milan, Italy), who performed immunofluorescence studies.
- Sergio Marchini and Ilaria Craparotta, Istituto di Ricerche Farmacologiche Mario Negri IRCCS (Milan, Italy), who prepared miRNA libraries for next-generation RNA-sequencing.

- Javier De Las Rivas, Óscar González Velasco and Alberto Berral González, Bioinformatics and Functional Genomics Research Group, Cancer Research Center (CiC-IBMCC) from CSIC and University of Salamanca (CSIC/USAL) (Salamanca, Spain), who performed RNA-sequencing data analyses of sEV-associated miRNAs.

- Pierluigi Quadri, Geriatric Division, Mendrisio and Lugano Regional Hospital (Switzerland), who provided human plasma samples and Mauro Tettamanti, Istituto di Ricerche Farmacologiche Mario Negri IRCCS (Milan, Italy), who provided clinical data.

All the illustrative figures were designed by me, Soraya Moradi Bachiller.
“High voltage rock 'n' roll”
Cómo es la vida, ¿verdad?


¡Ay, Abuela! Sé que lo primero que te escucharía decir sería “¡¡Sorayita!!” y tu risa de fondo. Siempre lo hacías cuando regresaba a casa. Es como si aún pudiera darle vida a ese recuerdo.

Este camino empezó cuando tú te fuiste de mi lado. Ahora que ha terminado sé que te hubiera hecho muy feliz verlo. Espero que te guste.

Boyko, hoy te tengo que dar las gracias desde lo más profundo y sincero de mi corazón. Has sido una de las personas más bonitas que este camino me ha regalado. Gracias por enseñarme que la ciencia se hace con amor y pasión. Gracias por acompañarme siempre. Aunque hubiera querido, no habría podido tener mejor supervisión que la tuya durante estos años. Gracias.

Papá y mamá, os quiero infinito.

Por último, a ti, Lionna.

¡Este trabajo te lo dedico a ti! Es gracias a ti.
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Bartoli F, **Moradi Bachiller S**, Antigny F, Bedouet K, Gerbaud P, Sabourin J, Benitah JP.


PMID: 31878108

“Asymptomatic Carriers of Presenilin-1 E218G Variant Show No Cerebrospinal Fluid Biochemical Signs Suggestive of Alzheimer’s disease in a Family with Late-onset Dementia”

Artuso V, Benussi L, Ghidoni R, **Moradi-Bachiller S**, Fusco F, Curtolo S, Roiter I, Forloni G, Albani D.


PMID: 30381075
PUBLICATIONS BY THE CANDIDATE EMANATING FROM THE WORK DESCRIBED IN THIS THESIS

“Extracellular-vesicle ECSIT as possible biomarker for detection of early cognitive decline: in vivo evidence using Alzheimer’s disease transgenic mice and human plasma”


Neuroscience. 2020

Accepted for publication but minor revisions still need to be done.
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<tbody>
<tr>
<td>Aβ</td>
<td>β-amyloid</td>
</tr>
<tr>
<td>ABCA7</td>
<td>ATP binding cassette subfamily A member 7</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADE</td>
<td>Astrocyte-derived exosome</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Ago2</td>
<td>Argonaute 2</td>
</tr>
<tr>
<td>AICD</td>
<td>APP intracellular domain</td>
</tr>
<tr>
<td>Alix</td>
<td>ALG-2 interacting protein X</td>
</tr>
<tr>
<td>APOE</td>
<td>Apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-secretase 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BIPED</td>
<td>Burden of disease, investigative, prognostic, efficacy of intervention and diagnostic</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BP</td>
<td>Biological process</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C99 / βAPP-CTF</td>
<td>99-residue C-terminal fragment</td>
</tr>
<tr>
<td>C83</td>
<td>83-residue C-terminal fragment</td>
</tr>
<tr>
<td>CC</td>
<td>Cellular component</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDR</td>
<td>Clinical dementia rating</td>
</tr>
<tr>
<td>CD33</td>
<td>Siglec-3</td>
</tr>
<tr>
<td>CHMP</td>
<td>Charged MVB protein</td>
</tr>
<tr>
<td>CLU</td>
<td>Clusterin</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned medium</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CR1</td>
<td>Complement receptor 1</td>
</tr>
<tr>
<td>Ctrl</td>
<td>Cognitively normal</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damaged-associated molecular pattern</td>
</tr>
<tr>
<td>DE</td>
<td>Differentially expressed</td>
</tr>
<tr>
<td>D.I.</td>
<td>Discrimination index</td>
</tr>
<tr>
<td>DLB</td>
<td>Dementia with Lewy bodies</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DMT</td>
<td>Disease-modifying therapy</td>
</tr>
<tr>
<td>dSTORM</td>
<td>Direct stochastic optical reconstruction microscopy</td>
</tr>
<tr>
<td>ECSIT</td>
<td>Evolutionarily conserved signaling intermediate in Toll pathway</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>EOAD</td>
<td>Early-onset Alzheimer’s disease</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>EV</td>
<td>Extracellular vesicle</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FFT</td>
<td>Fast Fourier transform</td>
</tr>
<tr>
<td>FTD</td>
<td>Frontotemporal dementia/degeneration</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide association studies</td>
</tr>
<tr>
<td>HIS</td>
<td>Hachinski ischemic score</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulfate</td>
</tr>
<tr>
<td>ILV</td>
<td>Intraluminal vesicle</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin 1 receptor-associated protein kinase</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>5LO</td>
<td>5-lipoxygenase</td>
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<tr>
<td>LOAD</td>
<td>Late-onset Alzheimer’s disease</td>
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<tr>
<td>LRP1</td>
<td>Low density lipoprotein receptor-related protein 1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<tr>
<td>MAPT</td>
<td>Microtubule-associated protein Tau</td>
</tr>
<tr>
<td>MAP</td>
<td>Microtubule-associated protein</td>
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<tr>
<td>MCI</td>
<td>Mild cognitive impairment</td>
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<td>MCI-AD</td>
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<td>Mitogen-activated protein kinase kinase kinase 1</td>
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<td>MF</td>
<td>Molecular function</td>
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<td>Minimal information for studies of extracellular vesicles</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<td>miRISC</td>
<td>miRNA-RISC</td>
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<tr>
<td>MMSE</td>
<td>Mini-mental state examination</td>
</tr>
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<td>coding RNA</td>
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<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MVB</td>
<td>Multivesicular body</td>
</tr>
<tr>
<td>MVP</td>
<td>Major vault protein</td>
</tr>
<tr>
<td>NanoFACS</td>
<td>Nanoscale cytometry</td>
</tr>
<tr>
<td>ND</td>
<td>Neurodegenerative disease</td>
</tr>
<tr>
<td>NDE</td>
<td>Neuron-derived exosome</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NFL</td>
<td>Neurofilament light</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangle</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>Ng</td>
<td>Neurogranin</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>NIK</td>
<td>NF-κB inducing kinase</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>ncRNA</td>
<td>Non-coding RNA</td>
</tr>
<tr>
<td>NORT</td>
<td>Novel object recognition test</td>
</tr>
<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
</tr>
<tr>
<td>nSMase2</td>
<td>Neutral sphingomyelinase 2</td>
</tr>
<tr>
<td>NTA</td>
<td>Nanoparticle tracking analysis</td>
</tr>
<tr>
<td>NTg</td>
<td>Non-transgenic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NURR</td>
<td>N-terminal unit for RNA recognition</td>
</tr>
<tr>
<td>O/N</td>
<td>Overnight</td>
</tr>
<tr>
<td>padj</td>
<td>Adjusted p-value</td>
</tr>
<tr>
<td>PALM</td>
<td>Photoactivation localization microscopy</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDCD4</td>
<td>Programmed cell death 4</td>
</tr>
<tr>
<td>PDZ</td>
<td>Post-synaptic density protein 95/disks large homolog/zonula occcludens 1</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PF</td>
<td>Particles per frame</td>
</tr>
<tr>
<td>PLD2</td>
<td>Phospholipase D2</td>
</tr>
<tr>
<td>PI(3)P</td>
<td>Phosphatidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PMT</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>PPI</td>
<td>Protein-protein interaction</td>
</tr>
<tr>
<td>P-tau</td>
<td>Phosphorylated tau</td>
</tr>
<tr>
<td>PSEN1</td>
<td>Presenilin 1 gene</td>
</tr>
<tr>
<td>PSEN2</td>
<td>Presenilin 2 gene</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenilin 1 protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PS2</td>
<td>Presenilin 2 protein</td>
</tr>
<tr>
<td>RBP</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>3R/4R</td>
<td>Three/four semi-conserved tandem repeats</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>R-Smad</td>
<td>Receptor-regulated Smads</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>sAPP</td>
<td>Soluble and extracellular N-terminal ectodomain of the APP</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sEV</td>
<td>Small extracellular vesicle</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SIM</td>
<td>Structured illumination microscopy</td>
</tr>
<tr>
<td>Smad</td>
<td>Small mother against decapentaplegic</td>
</tr>
<tr>
<td>SMT</td>
<td>Symptoms-modifying treatment</td>
</tr>
<tr>
<td>SNARE</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SNAP</td>
<td>Soluble N-ethylmaleimide-sensitive factor attachment protein</td>
</tr>
<tr>
<td>SNAP25/23</td>
<td>Synaptosome associated protein 25/23</td>
</tr>
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</table>
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>sTREM2</td>
<td>Soluble TREM2</td>
</tr>
<tr>
<td>SYNCRIP</td>
<td>Synaptotagmin binding cytoplasmic RNA interacting protein</td>
</tr>
<tr>
<td>T-EM</td>
<td>Tetraspanin-enriched microdomain</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>3xTg-AD</td>
<td>Triple transgenic mouse model for AD</td>
</tr>
<tr>
<td>TYROBP</td>
<td>Transmembrane immune signaling adaptor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumor necrosis factor receptor-associated factor 6</td>
</tr>
<tr>
<td>TREM2</td>
<td>Triggering receptor expressed on myeloid cells 2</td>
</tr>
<tr>
<td>Tsg101</td>
<td>Tumor susceptibility gene protein 101</td>
</tr>
<tr>
<td>T-tau</td>
<td>Total tau</td>
</tr>
<tr>
<td>TXN</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin binding domain</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>VaD</td>
<td>Vascular dementia</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
</tr>
<tr>
<td>VPS</td>
<td>Vacuolar protein sorting</td>
</tr>
<tr>
<td>YBX1</td>
<td>Y-box binding protein 1</td>
</tr>
</tbody>
</table>
NON-BOOK COMPONENT

The non-book component consists in excel files complementary to chapter IX:

- “Alignment_data_RNAseq”. This file contains the alignment data between miRNA reads and miRBase 22 database.
- “diff-exprs-DESeq2-ADvsCtrl_Plasma_sEVs”. This file contains differentially expressed sEVs-miRNAs for the contrast Ctrl vs AD.
- “diff-exprs-DESeq2-MCIvsCtrl_Plasma_sEVs”. This file contains differentially expressed sEVs-miRNAs for the contrast MCI vs Ctrl.
- “GSE120584_gammaCtrl-MCI-AD_10bootstraps_miRNA_in serum”. This file contains gamma values for miRNAs in serum across AD stages.
- “TABLE_11miRNAs-search-x-GeneTargets_440interactions”. This file shows the mRNA-miRNA pairs for network visualization.
CHAPTER I

THE PROBLEM. ALZHEIMER’S DISEASE

1.1. Neurodegenerative diseases, Alzheimer’s and dementia

The molecular consequences of some protein abnormalities trigger progressive loss of neurons and this is one of the most frequent, although non-exclusive, characteristic of the so-called neurodegenerative diseases (NDs). The intra- or extracellular accumulation of proteins with an abnormal conformation produces, firstly, neuronal anomalies and, later, the primary clinical features and symptoms (e.g. difficulties with language, memory, thinking skills or changes in personality). This pattern of decline, that affects at least two cognitive domains, is referred to as dementia (McKhann et al., 2011). Amyloidosis, tauopathies and α-synucleopathies are common NDs characterized by the abnormal deposition of β-amyloid (Aβ), tau and α-synuclein, respectively, in the human brain. Moreover, in NDs such as the Alzheimer’s disease (AD), amyloidosis and tauopathy coexist in the brain (Jack et al., 2019).

The amyloidosis is the accumulation of extracellular deposits of amyloid fibrils, which are insoluble and aggregate into β-sheet conformations (Long and Holtzman, 2019) and has a central role in AD. This complex and multifactorial neurodegenerative and brain disease is the most common cause of dementia in elderly people (Gale et al., 2018). It may begin even 20 years before the onset of symptoms (Dubois et al., 2016). It consists in several characteristic symptoms regarding memory, learning and other thinking skills. And it represents one of the biggest challenges for the whole society (Bondi et al., 2017).
Chapter I. The problem. Alzheimer’s disease | Soraya Moradi Bachiller

The extracellular deposits of amyloid fibrils are formed by Aβ peptides. They represent, together with hyperphosphorylated tau protein accumulation inside neurons, the two most common changes in AD brains which interfere in axonal transport and cellular survival (Hughes et al., 2020; Zhang et al., 2004).

At the beginning of the disease, the abnormal deposits above described are unnoticeable because clinical symptoms are not visible yet. With the progression of AD pathogenesis, they become more noticeable. This shift consists in three phases known as preclinical AD, mild cognitive impairment due to AD (MCI-AD or prodromal AD) and dementia due to AD (AD dementia). Besides the three phases, symptoms are only evident in the clinical stages (MCI-AD and AD dementia) (Aisen et al., 2017).

These three phases in the AD progression are referred to as the AD continuum. In the preclinical phase of AD, brain changes can already be measured, such as abnormal Aβ(1-42) levels in cerebrospinal fluid (CSF) (Moonis et al., 2005). However, symptoms are not yet present because individuals are clinically normal (Aisen et al., 2017). The following stage (first described in 1999 (Petersen et al., 1999)) is called mild cognitive impairment (MCI). It represents an intermediate step between cognition of healthy and aged subjects and those with AD (Petersen et al., 2001). That is, individuals are not normal for age but neither demented. Within this group, two classifications are made to distinguish MCI from MCI-AD. Subjects suffering from MCI are divided in amnestic or non-amnestic (McKhann et al., 2011). In AD dementia, the most common is the amnestic presentation which is referred to as memory impairments, while the non-amnestic presentation concerns language, visuospatial and/or executive deficits/dysfunctions (McKhann et al., 2011). Amnestic MCI subjects are also divided in single domain or multiple domain depending on the number of cognitive domains affected. Single
domain amnestic MCI subjects suffer from episodic memory impairment only, while those multiple domains have deficits in at least one other cognitive domain (Winblad et al., 2004). However, not all the MCI subjects will develop AD, in some cases, they remain stable in their MCI condition (Castellani et al., 2010).

Huge efforts have been done to find an effective treatment to AD since it was discovered in 1907 by Alois Alzheimer (Hippius and Neundörfer, 2003). However, the number of people affected with this progressive and devastating disease keeps on rising, becoming a challenge for patients, families, caregivers and for everyone because of its huge socioeconomic impact (Bondi et al., 2017).

AD is complex and this should be considered when making efforts to discover a successful treatment. However, Aβ deposits and tau inclusions are still the centre of AD research, giving rise to the most known, and believed, hallmarks and hypotheses to explain AD pathogenesis (Bondi et al., 2017) or, at least, part of it.

1.2. The amyloid hypothesis

In 1906, Alois Alzheimer, a clinical psychiatrist and neuroanatomist, was the first one who reported AD in a 51-year-old woman (Auguste D.). This woman was affected by a “peculiar severe disease process of the cerebral cortex” (Hippius and Neundörfer, 2003). However, the first description of amyloid plaques, as circular accumulations in the brain of elderly patients, was made in 1892 by Paul Blocq and George Mannesco. A few years later, in 1984, George G. Glenner and Caine W. Wong succeeded in purifying the Aβ peptide from these circular accumulations in meningeal vessels of AD brains (Glenner and Wong, 1984). Some years later, in 1991, John Hardy and David Allsop proposed a central role for the amyloid deposition in the
AD pathogenesis (Hardy and Allsop, 1991). And finally, in 1992, the well-known “amyloid hypothesis” was presented by J. Hardy and G. A. Higgins (Hardy and Higgins, 1992). This Aβ-centred view of AD places amyloid isoforms as the most causative factors explaining the mechanisms of AD pathogenesis. The metabolism and processing of amyloid precursor protein (APP) and the Aβ deposition are the primary events guiding downstream tau phosphorylation, tangle formation and neurodegeneration (Fan et al., 2020; Hardy and Allsop, 1991; Selkoe and Hardy, 2016).

The amyloid hypothesis starts with the Aβ peptides, Aβ(1-40) and Aβ(1-42), derived from the proteolytic cleavage of the single transmembrane APP domain. APP has a full-length of 770 residues, a large ectodomain, a single transmembrane region and an intracellular domain. Apart from the important contribution of APP to AD pathogenesis, essential roles for this protein have also been described, as for the embryonic development (Joshi et al., 2009; Musa et al., 2001), neurogenesis (Caillé et al., 2004) and synaptogenesis (Castro et al., 2019; Kirazov et al., 2001; Priller et al., 2006), among others.

APP contribution to AD pathogenesis and to physiological processes are determined by the two proteolytic cleaving pathways that this protein can suffer; the amyloidogenic and the non-amyloidogenic pathways (Figure 1). The amyloidogenic processing of APP involves, firstly, the cleavage by the β-secretase (BACE1). This cleavage releases the soluble N-terminal ectodomain (sAPP) β into the extracellular space and leaves the 99-residue C-terminal transmembrane fragment of the APP (called C99 or βAPP-CTF) in the membrane. The C99 fragment is then cleaved by the γ-secretase complex. This enzymatic complex cuts C99 at the ε positions (ε-cleavage), releasing the APP intracellular domain (AICD). The rest of C99 is subjected to further single-residue cleavages (such as the presenilin-dependent cleavage or ζ-
cleavage (Zhao et al., 2004, 2005)) by the γ-secretase complex in which longer forms (Aβ(1-42) and Aβ(1-43)) and shorter forms (Aβ(1-37), Aβ(1-38) and Aβ(1-40)) of Aβ peptides are released (Castro et al., 2019; Chasseigneaux and Allinquant, 2012).

**Figure 1. Amyloidogenic processing of the APP.** Full-length APP is shown in black color. The amyloidogenic proteolytic pathway starts with the β-secretase which cleaves APP and releases the sAPPβ. The release of AICD and shorter or longer Aβ peptides is carried out by the γ-secretase complex. APP, amyloid precursor protein; sAPPβ, soluble and extracellular N-terminal ectodomain β of the APP; Aβ, β-amyloid; C99, 99-residue C-terminal fragment; AICD, APP intracellular domain. *Figure designed by Soraya Moradi Bachiller and adapted from Castro et al., 2019.*

APP is also physiologically processed in the non-amyloidogenic pathway (*Figure 2*). The cleaving enzymes involved in this pathway are the α-secretase and the γ-secretase complex. The
α-secretase is also called disintegrin and metalloproteinase domain-containing protein 10. Under physiological conditions, it cleaves human APP at an extracellular position, releasing the sAPPα ectodomain and the C83 (83-residue C-terminal fragment) (which is still membrane-anchored) (Gallardo and Holtzman, 2019). A further processing of this C83 fragment by the γ-secretase complex releases the p3 fragment and the AICD. The α-secretase cleavage occurs within the Aβ sequence and produces Aα peptides. Therefore, the production of Aβ peptides is avoided in the non-amyloidogenic pathway (Chasseigneaux and Allinquant, 2012).

The γ-secretase complex is the common protease in the amyloidogenic and the non-amyloidogenic pathway, being APP one of its most studied substrates. This aspartyl protease is a tetrameric complex that consists in nicastrin, the anterior pharynx defective 1 and both presenilin 1 and 2 proteins (PS1 and PS2). The presence of all the subunits and a stoichiometric assembly are essential for the correct function of the complex. Both presenilins are the catalytic subunits responsible for the substrate cleavage. Anterior pharynx defective 1 and nicastrin are responsible for the complex assembly and the correct substrate interaction (Escamilla-Ayala et al., 2020). In the amyloidogenic pathway, the amyloid formation departs from the C99 fragment by the PS1. The PS1 cleavage usually takes place at the plasma membrane (PM), releasing Aβ into the extracellular space. Additionally, PS2 cleavage happens in endosomes, releasing Aβ into the endosomal lumen. Indeed, there are some C99 cleavages that occur in the lumen of other organelles, delivering Aβ in these compartments (Castro et al., 2019).
Figure 2. Non-amyloidogenic processing of the APP. Full-length APP is shown in black color. The non-amyloidogenic proteolytic pathway starts with the α-secretase which cleaves APP and releases the sAPPα fragment. AICD and p3 are then released thanks to the further processing of the γ-secretase complex. APP, amyloid precursor protein; sAPPα, soluble and extracellular N-terminal ectodomain α of the APP; C83, 83-residue C-terminal fragment; AICD, APP intracellular domain. Figure designed by Soraya Moradi Bachiller and adapted from Castro et al., 2019.

Independently of the compartment in which Aβ peptides are released, all of them belong to the class of intrinsically disordered proteins and can be post-translationally modified by isomerization (Kuo et al., 1998), oxidation (Dong et al., 2003), phosphorylation (Kumar et al., 2011), pyroglutamate-modifications (Mori et al., 1992; Nussbaum et al., 2012) and racemization (Mori et al., 1994).
Aβ(1-42) peptide is also involved in physiological processes. For example, at low concentration, it has been shown to be neuroprotective against excitotoxic cell death and to promote the survival of neurons undergoing death in trophic deprivation conditions (Giuffrida et al., 2009, 2010).

In pathological conditions, Aβ(1-38), Aβ(1-42) and Aβ(1-40) are the predominant Aβ peptides. Although Aβ(1-38) is soluble and does not have the tendency to aggregate, Aβ(1-40) and Aβ(1-42) can associate to form higher order structures, such as oligomers, protofibrils or β-sheet-enriched fibrils. The most aggregation-prone Aβ peptide is Aβ(1-42) and, because of two additional hydrophobic amino acids, it also shows more neurotoxicity than Aβ(1-40). Its increased production over Aβ(1-40) is a common feature in AD (Chévez-Gutiérrez et al., 2012; Hellström-Lindahl et al., 2009).

In the aggregation process, Aβ monomers are the starting point of the nucleation-dependent polymerization phase. In this process, Aβ(1-42) and Aβ(1-40) adopt a random-coil formation in their soluble state and assemble into a nucleus. This nucleus has numerous sites for monomer addition, allowing the formation of oligomers (higher order structures composed of monomer units). At this stage, the addition of more monomers results in β-sheet structures, called protofibrils which are considered an important intermediate in the amyloid fibrillogenesis (DaSilva et al., 2010; Walsh et al., 1997).

Oligomeric species and protofibrils are the most neurotoxic forms in AD (Klein et al., 2001; Ono and Tsuji, 2020; Viola and Klein, 2015). They interfere with cell signaling pathways, affect membranes and cause neuronal death (Lee et al., 2017). Also, oligomeric species can be internalized in the mitochondria, the endoplasmic reticulum, the Golgi apparatus, endosomes
and lysosomes causing damages in the mitochondrial respiratory chain, endoplasmic reticulum stress and altered apoptosis (Hillen, 2019; Lee et al., 2017).

As the end step in the Aβ aggregation process, the insoluble amyloid plaques whose main component are Aβ peptides. They can appear as diffuse (which may be present in the brains of cognitively normal adults) and mature senile type plaques (also called dense-core plaques and commonly present in the AD brain) (Gallardo and Holtzman, 2019). These clumps of β-sheets are, according to the amyloid hypothesis, a hallmark of brain changes and clinical symptoms in subjects suffering from AD.

1.3. The tau hypothesis

The other pathological hallmark of AD is the intracellular accumulation of neurofibrillary tangles (NFTs), which are formed of misfolded tau protein. Tau protein is encoded by the microtubule-associated protein tau gene (MAPT) and binds to the polymers of tubulin of the cytoskeleton or microtubules (MTs) via the MT-binding domains. Together with the microtubule-associated proteins (MAPs) (Dehmelt and Halpain, 2005), tau protein gives MTs the stability they need to carry out their functions (Drechsel et al., 1992; Weingarten et al., 1975). In the brain and, specifically in neurons, MTs have a crucial role since they are essential for the axonal transport, dendritic simplification and, thus, for the neuronal integrity (Brandt and Bakota, 2017).

Six tau isoforms are expressed in human adult brain, derived from alternative splicing. All the isoforms contain a carboxy-terminal region with three (3R) or four (4R) semi-conserved tandem repeats of 31 or 32 amino acids and an amino-terminal projection region. The carboxy-terminal region represents the MT-binding domain (Dehmelt and Halpain, 2005; Duan et al., 2012;
Goedert et al., 1989; Kametani and Hasegawa, 2018). In physiological conditions, tau protein localizes in the neuronal axon, interacts with the MAPs family members, MAP2 and MAP4, and maintains MT stability and assembly (Duan et al., 2012).

In tauopathies, some MAPs become pathogenic, generating an abnormal MT dynamic and a reduction in the MT abundance (Brandt and Bakota, 2017). Both are a consequence of the dissociation of tau protein from MTs because of the abnormal phosphorylation of this protein (Duan et al., 2012). In AD, the 3R and 4R tau isoforms are accumulated in hyperphosphorylated state (approximately three times more phosphorylated than normal tau (Blennow and Zetterberg, 2018)), that makes them fall off from the MTs, which, in turn, become destabilized and more fragile (Brandt and Bakota, 2017).

In addition, hyperphosphorylated tau proteins aggregate in the cytosol into insoluble β-sheet structures called paired helical filaments which form pre-tangles and NFTs (Figure 3) (Gallardo and Holtzman, 2019).

NFTs are present in the neuronal cell body (Goedert et al., 1989; Kametani and Hasegawa, 2018) in different and connected brain areas following the Braak stages (Duan et al., 2012). According to it, tau appears first in the transentorhinal region (stage I and II), moving then to the limbic areas (stages III and IV) and, lastly, to the neocortex (stages V and VI) (Braak and Braak, 1991).
Figure 3. Tau protein phosphorylation and aggregation. When tau protein is phosphorylated (P), it becomes detached from the microtubules, leading to their destabilization. Then, hyperphosphorylated tau monomers aggregate and form paired helical filaments and, consequently, neurofibrillary tangles. *Figure designed by Soraya Moradi Bachiller and adapted from Liu et al., 2019.*

Surprisingly, the hyperphosphorylation and spreading of tau pathology are linked and correlate with neurodegeneration in the AD human brain (Brandt and Bakota, 2017; Duan et al., 2012; Kametani and Hasegawa, 2018; Šimić et al., 2016) better than Aβ aggregates. Indeed, previous studies have shown that NFTs appear earlier than amyloid accumulation (Braak and Del Tredici, 2014; Weigand et al., 2020). And thus, the idea of tau as the main factor underlying the development and, also, the progression of the AD seem a plausible alternative to the amyloid hypothesis.
1.4. Amyloid, the controversial hallmark of the Alzheimer’s disease

The amyloid hypothesis is a single gene disease hypothesis that exists since 1992. Since then, huge attention and focus have been given to it. Definitely, APP, Aβ peptides and the secretases are great contributors of AD pathophysiology (O’Bien and Wong, 2011) which makes them reasonable druggable targets for the pharmaceutical industry. In fact, the pharmaceutical industry has invested and invests great amounts of money on the development of drugs targeting amyloid burden (Sabbagh et al., 2019).

However, this over-reliance on amyloid as the driven force in AD progression and as the core element from which AD arise, led to several failures over the years. Indeed, of “52 anti-Aβ targeted therapeutics, 31 have failed in the clinic to date with none reaching the point of being submitted for regulatory approval” (Mullane and Williams, 2020). It is difficult to understand why we are failing therapeutically, though we are targeting the prime factor from which plaques, tau tangles, neurodegeneration and dementia occur.

Probably, amyloid is not sufficient to explain AD since “there can be plaques without AD” and “there can also be AD without plaques” (Herrup, 2015). Amyloid-targeting therapeutic drugs, such as aducanumab, have failed to stop or improve AD progression though they were able to reduce amyloid burden (Liu et al., 2019). That is, when amyloid burden is reduced or cleared, the progression of the neurodegeneration process is not prevented (Holmes et al., 2008) and improvements in cognition do not happen (Doody et al., 2014). Moreover, even if amyloid is present in healthy human brains, these subjects do not necessarily develop AD (Aizenstein et al., 2008; Jansen et al., 2015; Petrone et al., 2019; Roberts et al., 2018; Villemagne et al., 2011). In other words, in aged and non-demented subjects, amyloid accumulation may exist without
cognitive impairment or decline, and removing amyloid from the brain does not cure AD or prevent its progression.

Apart from the discrepancies regarding the amyloid burden in AD subjects, there are other controversial facts, such as the brain atrophy which is also found in AD-prone areas of aged subjects at low risk of AD, the amyloid-independent tau deposition (Bouras et al., 1993), and the amyloid accumulation due more to a decrease clearance than to an increase production of this peptide (Fjell et al., 2014; Jeong, 2017).

When human AD-related transgenes are introduced into mouse genome, huge amounts of amyloid are produced in the mouse brain, allowing an altered behaviour and cognition (Samaey et al., 2019). However, the exposure to Aβ aggregates is not sufficient to cause memory dysfunction in some mouse models (Kim et al., 2013). Instead, when the opposite is done, and plaques are removed from mouse brain, their behaviour and the Aβ-induced deficits improve (Cramer et al., 2012; Schenk et al., 1999), confirming the neurotoxicity of Aβ.

However, when AD is modelled and anti-amyloid therapies are proved in animal models, it is important to note that rodents do not naturally develop AD. Genetic engineered mice are necessary to obtain models with an increased production of Aβ(1-42). All these engineered mice are widely used and accepted by the scientific community. However, the cognitive deficit and functional defects we see on them are not irreversible, at difference from what happens in human brain where we see neuronal loss. Animals do not suffer from the same brain damages and neurodegeneration as well as humans, suggesting the need for a carefully interpretation of the results that show deficit reversion when amyloid is cleared (Herrup, 2015). Therefore, a better understanding of the AD pathology in each specific mouse model is necessary when correlating it with findings in human AD (Neff, 2019).
In parallel, the amyloid hypothesis has disregarded the beneficial roles that amyloid has in several tissues, brain included (Kent et al., 2020). For example, Aβ acts as a vascular sealant in the blood-brain barrier (BBB) (Atwood et al., 2003), and as an antimicrobial and virucidal agent (Lukiw et al., 2010; Robinson and Bishop, 2002; Soscia et al., 2010). It can also participate in the brain recovery after traumatic injuries (Brody et al., 2008) and it is also important in synaptic plasticity/transmission and memory (Abramov et al., 2009; Puzzo et al., 2011). It seems that Aβ is not only a deleterious peptide. Aβ also exists in the healthy brain. And this is important to consider, especially, when using amyloid deposits as a biomarker for the diagnosis of preclinical AD in subjects that maybe are not in the preclinical stage but are healthy. In other words, “plaques define risk, not disease” (Herrup, 2015).

All this suggests a debate about whether the abnormal protein accumulation, within high plasticity brain areas, is the pathological substrate for cognitive decline in AD and, also, in healthy cognitive elderly subjects. It is clear that age affects the brain and, sometimes, independently of NDs. Understanding naturally age-related changes could help us to better understand AD, the pathological AD-related changes and the feasible proteins to be therapeutically targeted (González-Velasco et al., 2020).

Definitely, Aβ has a central role in AD pathogenesis, but Aβ pathology does not occur alone, it also happens in benign cognitive aging decline and, it is only partially responsible of the disease. Even if the amyloid hypothesis is well-represented in mouse models, the complexity of the disorder is not (Drummond and Wisniewski, 2017; Esquerda-Canals et al., 2017; Morris et al., 2014, 2018; Selkoe, 2011). AD is no longer considered a liner disease, where we progress from Aβ to AD. It is likely heterogeneous and complex, where autophagy (Di Meco et al., 2020), endolysosomal function (Van Acker et al., 2019), Ca\(^{2+}\) homeostasis (Galla et al., 2020),
inflammation (Kinney et al., 2018), DNA damage (Lin et al., 2020), mitochondrial function (Wang et al., 2020) and glucose metabolism (Cisternas and Inestrosa, 2017), among others, are implicated as relevant pathogenic players.

1.5. Clinical features and symptoms

According to the amyloid hypothesis, AD is sometimes diagnosed on the basis of clinical observations (cognitive deficits) that follows the first pathological changes (amyloid and tau deposition). Indeed, this disease can be described as a continuum. That is, as a sequence of elements going from the clinically asymptomatic subjects with AD pathology until the symptomatic stages. AD continuum can discriminate the preclinical phase from the clinical one. Before arriving to the AD continuum, a transition from normal aging to preclinical AD is needed. A mix of genetic and environmental factors are involved, although how this transition happens is not well understood (Aisen et al., 2017).

Once arrived in the continuum, the first stage is the preclinical AD. This early phase is represented by cognitively normal subjects with the abnormal levels of Aβ(1-42) and neuronal injury markers (such as phosphorylated tau) detected in CSF or by positron emission tomography (Vos and Visser, 2018).

The following phase, MCI-AD (or prodromal AD) is characterized by the abnormal presence of some biomarkers (Aβ(1-42) and tau), which reflect brain changes and minor difficulties with memory and thinking that can only be detected by the closest relatives. At this stage, subjects generally maintain their independence when carrying out daily living activities (whose assessment can be measured by the Bristol activities of daily living (Bucks et al., 1996) and the instrumental activities of daily living scales (Lawton and Brody, 1969)). MCI-AD subjects are
not cognitively normal when cognition is assessed using the mini-mental state examination (MMSE) (Folstein et al., 1975), the clinical dementia rating (CDR) (Hughes et al., 1982) and/or the Alzheimer’s disease assessment scale – cognitive subscale (Rosen et al., 1984). They do not meet the criteria for dementia due to AD but have a great probability to become AD (M. S. Albert et al., 2011).

Not all MCI subjects are MCI-AD. The MCI clinical condition can be divided in amnestic and non-amnestic MCI and single-domain and multiple-domain presentations. The most indicative of AD is the amnestic presentation while the non-amnestic suggests other NDs such as frontotemporal degeneration (FTD) or dementia with Lewy bodies (DLB). The first classification regarding the clinical presentation of amnestic or non-amnestic is done on the basis of the memory impairment. If subjects suffer from it, they are classified into amnestic. If they also present impairments in other cognitive areas apart from memory, they are multiple-domain amnestic MCI. When only memory deficit occurs, amnestic MCIs are single-domain. The same thing happens with non-amnestic subjects and other non-memory deficits (language, visuospatial and executive dysfunctions) (Petersen, 2004).

The dementia due to AD is the last stage of the disease where subjects suffer from moderate (mild and moderate dementia due to AD) to serious (severe dementia due to AD) memory, thinking and behavioural symptoms. When mild dementia due to AD starts, individuals are independent for most of the daily tasks, and they require assistance only for some of them. When subjects progress to moderate dementia due to AD, serious changes affect their personality in such a huge way that they also lose their personal interests, empathy, sociability and initiative. Mood also changes, anxiety, apathy and agitation increase and the sleep decreases. All these symptoms are grouped within the behavioural and psychological symptoms of dementia.
Chapter I. The problem. Alzheimer’s disease | Soraya Moradi Bachiller

(Cerejeira et al., 2012) which can be assessed and measured by the BEHAVE-AD assessment system (Reisberg et al., 1987) and the neuropsychiatric inventory (Cummings, 1997). They also have problems with communication and with the daily tasks, such as dressing. In the last stage of the disease (severe dementia due to AD) symptoms become more serious. The disorientation, confusion, hallucinations and impaired judgement, among others, become clearer (Atri, 2019; Bondi et al., 2017). Damages in several areas involved in movement make more apparent their physical state. They start suffering from blood clots (Cortes-Canteli et al., 2012), and pneumonia (Degerskär and Englund, 2020) is also common at this stage.

1.6. Risk factors

Multiple environmental- and co-morbidity-related factors contribute to AD pathogenesis and hereafter, we will comment some of those included in the AlzRisk database http://www.alzrisk.org/): alcohol, B vitamins, blood pressure, cognitive activity, diabetes mellitus, dietary pattern, homocysteine, nutritional antioxidants, obesity, physical activity and statin use.

Several authors have studied the role of blood pressure in AD and whether an antihypertensive therapy has beneficial properties to slow down the progression of AD. No consensus has been reached yet and numerous controversial results have been published about an intensive blood pressure control and its association with AD (Power et al., 2011). For example, it has been recently reported that lowering blood pressure reduces the vascular disease progression (Nasrallah et al., 2019) while it does not reduce the risk of having probable dementia (Williamson et al., 2019). Instead, antihypertensive drugs (such as angiotensin-1 receptor blockers and calcium channel blockers) are able to decrease AD risk by reducing blood pressure.
fluctuations (Lattanzi et al., 2018), and are also capable to decrease AD-associated neuropathological features (Hoffman et al., 2009).

On their behalf, Mediterranean dietary patterns may be neuroprotective and may reduce the risk of having dementia (Bartochowski et al., 2020; Morris et al., 2015). Alcohol consumption association with AD risk varies depending on the assumption (moderate drinking may reduce dementia risk, while excessive drinking is related to higher dementia risk with regard to non-drinkers) and on the type of drink (intake of beer is associated with higher risk while wine may reduce the risk of dementia) (Deng et al., 2006). Obesity and overweight increase the risk (Whitmer et al., 2005) while physical activity increases blood flow, reduces blood pressure and thus reduces AD risk (Buchman et al., 2012).

Insulin resistance and hyperglycaemia have an important role in AD brain (De Sousa et al., 2020). For example, reduced peripheral insulin sensitivity and hyperinsulinemia exist in subjects suffering from AD, affecting insulin receptors in the brain. Moreover, insulin participates in the Aβ clearance via the insulin-degrading enzyme and peripheral insulin resistance is correlated with Aβ deposition in the brain (Kellar and Craft, 2020). Therefore, insulin can be considered another probable risk factor in dementia and AD, which is consistent with several studies (Ahtiluoto et al., 2010; Cheng et al., 2011).

The cognitive reserve is another risk factor that needs to be considered. It is referred to the ability to use the brain network and make it flexible despite brain changes. Social interaction and engagement contribute to the maintenance of the cognitive reserve (Seeman et al., 2001), and may also protect against cognitive decline.

Little support has been provided for the association between vitamins and nutritional antioxidants supplements and AD risk in subjects without vitamins deficits. For example, Zandi
et al. studied vitamin C, B and E as supplements. They did not see any protective effect when using vitamin C and E but a trend towards reduced AD risk was observed (Zandi et al., 2004). Morris et al. instead, examined the associations between AD risk and the intake of folate, vitamin B12 and B6. They showed that, in aged subjects, the intake of these three supplements was not associated with the AD risk (Morris et al., 2006). The same conclusion was provided by another study, where the authors did not see any correlation between the incidence of AD and the intake of vitamins B6 and B12. Moreover, the authors suggest that further studies that correlate AD biomarkers fluctuations with the dietary intake of vitamin supplements are necessary to confirm their possible association with the risk of dementia or AD (Nelson et al., 2009). Instead, elevated levels of homocysteine in plasma (that reflects low levels of folate, vitamin B12 and B6), increase dementia and cognitive impairment risk. Of relevance, homocysteine-lowering treatment with B vitamins is capable to slow down cognitive decline (Smith et al., 2018).

Lastly, statin exposure has been also studied in AD and dementia. A recent study examined the association between statin and the risk of dementia in subjects with hypercholesterolemia. The authors proved that the risk of dementia and AD was reduced in subjects exposed to statin (Lee et al., 2020). The same effect was shown in the meta-analysis performed by Poly et al. (Poly et al., 2020). Others suggest insufficient the available data to consider statins as beneficial to delay the progression of AD (Davis et al., 2020).

1.7. Alzheimer’s disease. Early-onset vs. late-onset

Subjects suffering from AD can have the clinical onset before the age of 65 years. These subjects are diagnosed with early-onset AD (EOAD) and differs significantly form the late-
onset AD (LOAD) where pathogenesis appear later in life (over 65). EOAD has a significant genetic predisposition in its familial form. In ~10% of subjects suffering from familial EOAD, the disease is inherited in an autosomal dominant manner (Cacace et al., 2016) and is the consequence of high-penetrant mutations in \textit{PSEN1}, \textit{PSEN2} or \textit{APP} (Bird, 2008). However, these high-penetrant mutations only account for the ~10% of EOAD cases (Wisniewski and Drummond, 2020).

LOAD, instead, is a multifactorial syndrome not directly due to genetic mutations but to several comorbidities (Gerritsen et al., 2016; Kellar and Craft, 2020; Solis et al., 2020), dysfunctions in susceptibility genes, deficits in soluble Aβ clearance, and the gradual accumulation of malfunctions related with the aging process.

AD subjects belonging to the EOAD category commonly represent the non-amnestic component of AD (Koss et al., 1996). In EOAD, semantic memory is better preserved than in LOAD (Joubert et al., 2016). However, they also have more atrophy (located in different areas of the brain; neocortical areas for EOAD and medial temporal lobe areas for LOAD (Frisoni et al., 2007)) and tau burden (Migliaccio et al., 2012; Schöll et al., 2017; Whitwell et al., 2019) compared to LOAD subjects. These characteristics are related with the more aggressive clinical course of EOAD (Koedam et al., 2008). EOAD subjects also show deficits in attention and lower verbal learning performance (Palasí et al., 2015) and have a longer duration of the disease before it is diagnosed (Van Vliet et al., 2013).

Within the early-onset category of dementias, other neurodegenerative conditions rather than AD are included (such as vascular dementia (VaD), FTD, DLB, Huntington’s disease or prion disease), depending on different genetic or environmental triggers (Kuruppu and Matthews, 2013).
1.8. Alzheimer’s disease susceptibility genes

*PSEN1, PSEN2* or *APP* are the three causative genes (Giau et al., 2018; Park et al., 2017; Van Giau et al., 2018) for the familial form of EOAD whose genetic variants (http://www.alzforum.org/mutations) are related to amyloid processing (Janssen et al., 2003).

For the LOAD, instead, the greatest genetic risk factor identified is the inheritance of the ε4 allele of apolipoprotein E gene (*APOE*) (Strittmatter et al., 1993). ε2 and ε3 alleles also exist for *APOE*. However, only the inheritance of one or two *APOE* ε4 alleles increases the risk of having LOAD (Corder et al., 1993; Farrer et al., 1997). It seems that this effect is derived from the ability of *APOE* ε4 allele to promote and stabilize Aβ oligomerization and fibrillization, which, consequently, increases amyloid toxic species (Wisniewski and Drummond, 2020).

However, *APOE* genotype alone is not able to explain all LOAD cases (Lambert et al., 2013), which suggests that LOAD has a strong and multifaceted genetic component (Rocchi et al., 2003). Indeed, several genetic variations, or single-nucleotide polymorphisms (SNPs), have been identified next to, within or near *ABCA7, CLU, CR1, CD33, CD2AP, EPHA1, BIN1, PICALM, MS4A, CASS4, CELF1, DSG2, FERMT2, HLA-DRB5-BBR1, INPP5D, MEF2C, NME8, PTK2B, SLC24H4-RIN3, SORL1* and *ZCWPW1* (Karch and Goate, 2015) in genome-wide association studies (GWAS) and/or whole genome/exome sequencing. And some of them are within the top 10 list (http://www.alzgene.org/TopResults.asp) of AlzGene database (Bertram et al., 2007).

Among the above cited genes, clusterin (*CLU*), adenosine triphosphate (ATP) binding cassette subfamily A member 7 (*ABCA7*) and sortilin related receptor L 1 are implicated in cholesterol metabolism (Karch and Goate, 2015).
CLU encodes for CLU protein or apolipoprotein J. It participates in lipid transport and membrane protection, apoptosis and cellular interactions (Karch and Goate, 2015). GWAS revealed CLU SNPs that reduce AD risk, such as rs11136000 in Caucasian populations (Han et al., 2018). Moreover, CLU was showed to prevent Aβ aggregation (Matsubara et al., 1996), bind Aβ oligomers (Narayan et al., 2011) and reduce Aβ toxicity only when it is present in an excess with regard to Aβ (Yerbury et al., 2007). In addition, elevated plasma levels of this apolipoprotein are associated with more severe AD (Schrijvers et al., 2011).

On his behalf, ABCA7 protein belongs to the ABC transporter family and have an important role in the maintenance of the lipid metabolism, the lipid cellular efflux, and the phagocytosis of Aβ (Surguchev and Surguchov, 2020). Several ABCA7 SNPs (such as rs78117248 and rs4147929) have been identified in GWAS (Cuyvers et al., 2015; Del-Aguila et al., 2015) that contribute to AD risk. Moreover, ABCA7 levels are associated with plaque burden (Shulman et al., 2013) and cognitive decline (Karch et al., 2012) in AD brain.

Instead, sortilin related receptor L 1 gene encodes SorLA protein which belongs to the Vsp10p domain receptor family. It is involved in vesicle trafficking and SNPs near this gene have been previously associated with AD (Lambert et al., 2013). The SNP rs11218343 and rs689021 are associated with a protective effect for LOAD in Caucasian and Chinese populations and lower AD risk in Caucasians, respectively (Cong et al., 2018; C.-C. Zhang et al., 2017), while rs1010159 is associated with higher AD susceptibility in Asian population (Cong et al., 2018). Moreover, SorLA binds and regulates the differential trafficking and metabolism of APP, and connects these processes to LOAD. When SorLA is present, APP is directed to the recycling pathway while being released into late endosomal pathways (where it is cleaved and Aβ is generated) when SorLA is absent (Rogaeva et al., 2007).
Other susceptibility genes have been associated with the immune response and neuroinflammation, such as the complement receptor 1 (CR1) and Siglec-3 (CD33). CR1 encodes CR1 type-I membrane glycoprotein, which is a component of the complement response system, plays a role in host defense (Morgan and Gasque, 1996). It was first identified to contribute to AD by GWAS thanks to SNPs such as rs3818361 and rs6656401 (Lambert et al., 2009). Moreover, its genetic variants have been shown to influence the amyloid deposition (Biffi et al., 2012) and its expression is associated with severe cognitive decline (Karch et al., 2012).

CD33 is a lipid and acid-modified protein receptor highly expressed in immune cells and implicated in immune and inflammatory responses (Crocker et al., 2012) and endocytosis mechanisms (Tateno et al., 2007). Genetic polymorphisms have been also identified for CD33 by GWAS. For example, some studies have detected rs3865444 as a CD33 genetic polymorphism associated with AD (Hollingworth et al., 2011; Naj et al., 2011), while others showed the opposite (Lambert et al., 2013; Siokas et al., 2020).

There are also other genetic variants, such as those in the phosphatidylinositol binding clathrin assembly protein gene. Its genetic polymorphisms rs3851179 A allele and rs541458 protect against cognitive decline (Masri et al., 2019; Ponomareva et al., 2020) and associate with reduced AD risk (Zeng et al., 2019), respectively. The protein encoded by this gene also participates in Aβ clearance via the autophagic degradation pathway (Tian et al., 2013). Others like the triggering receptor expressed on myeloid cells 2 (TREM2), and its R47H and R62H alleles (Jin et al., 2014), UNC-5 netrin receptor C or phospholipase D3 also contribute to the huge genetic component of LOAD (Carmona et al., 2018; Rocchi et al., 2003).
Chapter II
CHAPTER II

ABOUT THERAPIES, BIOMARKERS AND NETWORKS

2.1. Symptoms-modifying available therapies

Unfortunately, there are no drugs to cure AD. However, some pharmacological treatments that can help with symptoms, are available. All these symptoms-modifying treatments (SMTs) are based on the same principle; in the AD brain, synapses are lost and communication among nerve cells is destroyed, thus favouring neurodegeneration, cell death and imbalance among neurotransmitters, such as glutamate or acetylcholine (ACh). Thus, the available treatments try to counterbalance this major disruption in the brain by targeting the cholinergic and the glutamatergic systems (Yiannopoulou and Papageorgiou, 2020).

Two types of medicines are approved for specific AD stages only; that is, to treat mild, moderate and severe dementia due to AD. These drugs are cholinesterase inhibitors (donepezil, rivastigmine and galantamine) and the antagonist of the N-methyl-D-aspartate-(NMDA) receptor (memantine) (Conway, 2020; Sharma, 2019).

The development of donepezil, rivastigmine and galantamine is based in the cholinergic system and its deterioration as one of the major cause of cognitive decline. In AD brain, there is a decrease of ACh availability. Therefore, these three drugs are designed to increase available ACh by inhibiting the acetylcholinesterase (the enzyme that catalyse the hydrolysis of ACh) (Sharma, 2019). As the cholinergic system is essential for memory, learning, thinking and reasoning problems, these medicines help to manage memory loss and all the related symptoms (Yiannopoulou and Papageorgiou, 2020).
Donepezil, approved in 1997, is used to treat all stages of dementia due to AD. It does not act only at neurotransmission level, but also by protecting against the glutamate toxicity (Akasofu et al., 2006), modulating inflammatory pathways (Conti et al., 2016; Maroli et al., 2019) and protecting against lipid peroxidation (Meunier et al., 2006) or oxidative stress (Klann et al., 2020; Saxena et al., 2008).

On his behalf, rivastigmine was approved in 2000 for mild to moderate dementia due to AD. It is a reversible inhibitor of both acetylcholinesterase and butyrylcholinesterase. The last cholinesterase inhibitor, galantamine, was also approved for the treatment of mild and moderate stages of the disease. This drug was firstly designed for its use in the treatment of myopathy, sensory and motor deficits and, for the treatment, in general terms, of NDs. And, in 2001 was finally adopted for AD (Sharma, 2019).

Instead, memantine was approved in 2003 to treat moderate to severe AD. This drug works by regulating the activity of glutamate as neurotransmitter. In AD brains, Aβ blocks glutamate uptake by astrocytes (Matos et al., 2012; Vincent et al., 2010). This increased concentration of glutamate at the synapses enhances the entry of calcium in neurons via NMDA receptors, leading to the generation of reactive oxygen species (ROS) and nitrogen ones. Cytoskeleton in neuronal cells becomes disrupted and membranes degenerate and, finally, neuronal cell death occurs. Memantine is an antagonist of NMDA receptors. It blocks them and reduces the influx of Ca^{2+}, improving memory, attention, language skills and the ability to perform simple daily tasks (Conway, 2020; Yiannopoulou and Papageorgiou, 2020).
2.2. Failed and ongoing clinical trials for disease-modifying therapies

Donepezil, rivastigmine, galantamine and memantine are SMTs. Treatments that help to prevent and produce a change in the course of AD, by interfering in the underlying pathophysiological mechanisms, are called disease-modifying therapies (DMTs). DMTs for the treatment of AD are mainly based on the tau and Aβ hypotheses; that is, they are anti-tau or anti-Aβ agents. Aβ and tau proteins are appealing targets in dementia due to AD and, from this point of view, one could expect a change in AD trajectory by decreasing levels of these proteins, by preventing their aggregation and/or by removing the toxic aggregates of them in abnormal conformations (Yiannopoulou et al., 2019).

DMTs, unfortunately, do not yet exist. Indeed, since 2003, no drug (neither SMTs nor DMTs) has been approved by the Food and Drug Administration (FDA) for the AD treatment (Cummings et al.2018; Yiannopoulou et al., 2019), though there are several drugs (DMTs and cognitive enhancers) that are being tested in the early-stages in clinical trials against Aβ, inflammation and oxidative stress, among others (Table 1).

Here, we will discuss some DMTs which have so far been developed and have failed for the treatment of AD.

In the Aβ targeting agents’ category, three major groups depending on the main therapy aim can be distinguished. The first one aims to reduce Aβ(1-42) production and are the so-called γ- and β-secretase inhibitors or α-secretase modulators. The second group consists in the aggregation inhibitors, which are dedicated to reduce Aβ plaque burden. And, the last one is based in passive immunotherapy via the Aβ clearance through intravenous immunoglobulins (IVIGs) or monoclonal antibodies (mAbs) (Yiannopoulou et al., 2019).
β- and γ-inhibitors and α-secretases modulators are directed to modify the amyloidogenic and non-amyloidogenic pathways. Aggregation inhibitors interact with Aβ(1-42) to inhibit fibrils and oligomers formation. In their behalf, passive immunotherapy is based on the use of exogenous anti-Aβ mAbs, with great affinity for single Aβ molecules, or the use of immunoglobulins. In contrast with mAbs, IVIGs are purified plasma polyclonal immunoglobulins (mainly of the IgG-type) from more than 1000 healthy donors which have lower affinity for Aβ monomers but higher for several epitopes present in oligomers and fibrils (Mehta et al., 2017; Yiannopoulou et al., 2019; Yiannopoulou and Papageorgiou, 2020).

<table>
<thead>
<tr>
<th>Agent (mechanism of action)</th>
<th>Population</th>
<th>Completion date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posiphen (APP processing inhibitor)</td>
<td>Amnestic MCI or mild AD</td>
<td>2021</td>
</tr>
<tr>
<td>CAD106 (Anti-Aβ immunotherapy)</td>
<td>Cognitively healthy subjects with two copies of the APOE ε4 gene</td>
<td>2025</td>
</tr>
<tr>
<td>CNP520 (β-secretase inhibitor)</td>
<td></td>
<td></td>
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<tr>
<td>CT1812 (σ2R antagonist)</td>
<td>Mild to moderate AD</td>
<td>2022</td>
</tr>
<tr>
<td>PTI-125 (Filamin A inhibitor)</td>
<td>Mild to moderate AD</td>
<td>2020</td>
</tr>
<tr>
<td>PQ 912 (Glutaminyl cyclase inhibitor)</td>
<td>MCI or mild probable AD</td>
<td>2023</td>
</tr>
<tr>
<td>Docosahexaenoic (Lipid supplementation/neuroprotective)</td>
<td>Healthy adult carriers and non-carriers of APOE ε4</td>
<td>2022</td>
</tr>
<tr>
<td>Nicotine (Nicotinic receptor agonist/neurotransmitter-based)</td>
<td>MCI</td>
<td>2021</td>
</tr>
<tr>
<td>(-)-Phenserine (Neural programmed cell death inhibitor)</td>
<td>Probable AD</td>
<td>2025</td>
</tr>
<tr>
<td>Agent (mechanism of action)</td>
<td>Population</td>
<td>Completion date</td>
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<tr>
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<tr>
<td>Levetiracetam (Synaptic vesicle protein modulator/neuroprotective)</td>
<td>Healthy adults, carriers of APOE ε4</td>
<td>2021</td>
</tr>
<tr>
<td>Levetiracetam (Synaptic vesicle protein modulator/neuroprotective)</td>
<td>Probable AD</td>
<td>2024</td>
</tr>
<tr>
<td>Allopregnanolone (Anti-inflammatory and mitochondrial function stimulator/neuroprotective)</td>
<td>APOE ε4 carriers and diagnosed with mild AD</td>
<td>2025</td>
</tr>
<tr>
<td>Bryostatin-1 (Hippocampal PKCε stimulator)</td>
<td>Advanced AD</td>
<td>2022</td>
</tr>
<tr>
<td>Benfotiamine (Synthetic derivative of thiamine)</td>
<td>MCI or AD</td>
<td>2020</td>
</tr>
<tr>
<td>T3D-959 (PPARγ and PPARδ agonist)</td>
<td>Mild to severe AD</td>
<td>2023</td>
</tr>
<tr>
<td>Candesartan (Angiotensin II receptor blocker)</td>
<td>Hypertensive adults with MCI</td>
<td>2020</td>
</tr>
<tr>
<td>Candesartan (Angiotensin II receptor blocker/neuroprotective)</td>
<td>Non-hypertensive adults with MCI</td>
<td>2022</td>
</tr>
<tr>
<td>LM11A-31 (It increases survival signaling/neuroprotective)</td>
<td>Mild to moderate AD</td>
<td>2020</td>
</tr>
<tr>
<td>Lupron (Gonadotropin-releasing hormone receptor agonist)</td>
<td>Women with mild to moderate AD and who are also taking AChE inhibitors</td>
<td>2024</td>
</tr>
<tr>
<td>Lenalidomide (Anti-inflammatory modulator)</td>
<td>Amnestic MCI</td>
<td>2024</td>
</tr>
<tr>
<td>MW151 (Proinflammatory cytokines suppressor)</td>
<td>Healthy volunteers</td>
<td>2021</td>
</tr>
<tr>
<td>2-hydroxybenzylamine (γ-ketoaldehyde scavenger)</td>
<td>Healthy volunteers</td>
<td>2020</td>
</tr>
<tr>
<td>Glutathione (Antioxidant)</td>
<td>MCI</td>
<td>2022</td>
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**Table 1. Early-stage clinical DMT or cognitive enhancer trials.** For full details on each clinical trial, please refer to [https://www.nia.nih.gov/research/ongoing-AD-trials#section1](https://www.nia.nih.gov/research/ongoing-AD-trials#section1).
From all these categories, several drugs have already been tested in clinical trials with patients from mild to severe dementia due to AD and with MCI-AD. Some of them are γ-secretase inhibitors, such as semagacestat and tarenflurbil, or β-secretase inhibitors, like lanabecestat, verubecestat and atabcestat. Others such as bapineuzumab, crenezumab, aducanumab and solanezumab are anti-Aβ mAbs, or IVIGs in the case of gammagard liquid.

From the γ-secretase inhibitors cited above, semagacestat led to a decline in cognition and to several side effects, such as weight loss, skin cancers and infections (Doody et al., 2013), and tarenflurbil did not improve cognition while producing dizziness and anemia (Green et al., 2009).

The β-secretase inhibitor verubecestat was able to reduce Aβ levels in CSF of AD subjects (Kennedy et al., 2016) but it did not improve cognitive decline (Egan et al., 2018). Lanabecestat and atabcestat also reduced Aβ levels. However, their clinical trials were discontinued due to the lack of efficacy regarding cognition and the side effects (hepatotoxicity for atabcestat; weight loss and psychiatric adverse events for lanabecestat) (Henley et al., 2019; Novak et al., 2020; Wessels et al., 2020).

Within the anti-Aβ mAbs drugs, solanezumab was proven to reduce free Aβ concentration in plasma (Honig et al., 2018) and, although a trend to improve cognition was identified in mild AD subjects, it did not reach statistical significance (Doody et al., 2014). In addition, solanezumab did not either slow down brain atrophy in mild AD individuals (Schwarz et al., 2019). On his behalf, bapineuzumab targets aggregated fibrillary Aβ but it did not show any relevant benefit in mild-to-moderate AD subjects (Salloway et al., 2014), while causing cerebral edema (E. Liu et al., 2018) when delivered at high doses and among APOE ε4 carriers. The termination of the phase 3 trial was also announced for crenezumab at the beginning of 2019,
though it was capable to bind Aβ oligomers, fibrils and plaques with high affinity and avoid vasogenic edema or microhemorrhage among treated subjects (Adolfsson et al., 2012). Aducanumab targets Aβ aggregates. This drug was able to clear soluble and insoluble Aβ and stabilize cognitive decline, while causing cerebral edema in APOE ε4 carriers and in a dose-dependent manner (Sevigny et al., 2016b). However, in March 2019 and due to the previous data collected, its sponsor decided to discontinue aducanumab clinical trial. Then, in October 2019, and following the re-analysis of additional data, aducanumab seemed to be effective in reducing the cognitive decline at the highest doses tested. And consequently, the sponsor sought FDA approval for the treatment (Howard and Liu, 2020). Several opinions surround the possible efficacy and the resurrection of aducanumab, since this is another amyloid-targeting drug similar to others that have already failed (Mullane and Williams, 2020).

Anti-Aβ or anti-tau are not the only drugs developed for DMTs. When Aβ and tau proteins start accumulating and aggregating, the pro-inflammatory response increases and so does the neuronal damage. Therefore, in ongoing clinical trials, there are drugs which might prevent neuroinflammation and neuronal death that have attracted great interest. These DMTs include anti-inflammatory (MW151 in healthy adults), neuroprotection (levetiracetam in MCI subjects) and growth factor support agents (LM11A31 in mild-to-moderate AD subjects) (Huang et al., 2020; Yiannopoulou and Papageorgiou, 2020).

Azeliragon is one of them. This small molecule is an antagonist of the receptor for advanced glycation end products. This receptor binds Aβ and mediates its toxic effects, enhancing glia inflammatory responses, increasing oxidative stress and the transport of Aβ in the brain (Huang et al., 2020; Lue et al., 2005). Azeliragon seems to decrease Aβ load in the brain of transgenic mice improving their performance in different behavioral tests. Indeed, to confirm its efficacy
in humans, subjects with mild dementia due to AD and impaired glucose tolerance have started (in 2019) phase 3 trial that will finish at the end of 2023. TRx0237 (LMTX) is a tau-aggregation inhibitor. In the first trial, LMTX revealed not to be an effectively treatment for AD. Right now, the phase 3 trial is being carried out with the aim to compare the efficacy of LMTX when different doses are administered to subjects with early dementia due to AD. This trial is supposed to be finished by the end of 2020 (Huang et al., 2020). The *Ginkgo biloba* extract has been proposed as a neuroprotector agent. It may suppress ROS, block platelet activating factors, decrease neuroinflammation and cell damage, and improve brain circulation (Tian et al., 2017). Phase 2 and 3 trials have started in 2016 with mild-to-moderate dementia due to AD subjects and it is expected to continue until mid-2020 (Huang et al., 2020).

DMTs and failed clinical trials have belonged together in the history of AD. The multiple failures of clinical trials for DMTs in AD might indicate the drug effectiveness or the failed clinical trial by itself. That is, there are clinical trials- or drug-related reasons of failure. In the first category, a given clinical trial could fail because of the delayed beginning of the treatment and the inappropriate subjects’ enrolment (misclassification). A drug fails when it is not effective, when the wrong pathological substrates (that is, the wrong main target of the disease) are targeted, when the drug dose is inappropriate and/or because of its excessive toxicity. And, in general, failures are also due to the incomplete understanding of the complex and heterogeneous pathophysiology of AD (Blennow and Zetterberg, 2018; Cummings et al., 2018; Mehta et al., 2017; Sevigny et al., 2016a; Yiannopoulou et al., 2019).
2.3. Blood-based biomarkers are needed. Biomarkers for Alzheimer’s disease diagnosis or therapy

As stated above, despite the huge investment in the development of AD therapies, there have been more failures than successes. To avoid the delayed beginning of the disease treatment, clinical trials are trying to enrol subjects in the early stages of AD, that is, before the clinical presentation (signs and symptoms).

From this perspective, clinical trials are trying to shift their focus towards preclinical stages of NDs through the discovery of accessible, sensitive and specific biomarkers (Obrocki et al., 2020).

On the road of reaching this milestone, two things have to be considered, what biomarkers are and what the biomarker classification system is.

In 1998, the Biomarkers Definitions Working Group of the National Institute of Health (NIH) defined a biomarker as a “characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Biomarkers Definitions Working Group, 2001). In AD, biomarkers must reflect the core hallmarks in the brain (Aβ plaques and NFTs tangles) and the pathophysiological mechanisms associated to them (i.e. inflammation, regional brain volume loss, synapse damage or destruction of brain circuit levels).

The National Institute on Aging and Alzheimer's Association stresses the importance of a biological definition of the AD based on the A/T/N system (Jack et al., 2016). This system stands for Aβ (A), tau (T) and neurodegeneration (N) biomarkers (Jack et al., 2016). Biomarkers can be also sorted following the Burden of disease, Investigative, Prognostic, Efficacy of intervention and Diagnostic (BIPED) classification system. Five categories exist in BIPED.
Burden of disease groups biomarkers that can assess the severity of the disease (at a single time point, that is, for cross-sectional studies). Investigative category contains biomarkers with pathological relevance that can be used as an explorative setting. Prognostic contains biomarkers that predict the future onset of the disease (for longitudinal studies). Efficacy of intervention group of biomarkers provide information about the efficacy of a treatment (also for longitudinal studies). And, diagnostic biomarkers are those who classified individuals as either having or not having the disease (useful in cross-sectional studies). BIPED classification allows the biomarker discovery process, from the target identification to the clinical trial (Henriksen et al., 2014).

2.3.1. Cerebrospinal fluid and blood diagnostic biomarkers for Alzheimer’s disease

Already in the target identification stage, the most studied and core biomarkers for AD are Aβ, total tau (t-tau) and phosphorylated tau (p-tau) proteins in CSF (Olsson et al., 2016). Moreover, several studies have measured the levels of these biomarkers in CSF and tested their accuracy in AD diagnosis; in discriminating AD from Ctrl subjects, in discriminating AD from other type of dementia and in predicting the conversion from MCI to AD (Engelborghs and Le Bastard, 2012; Hansson et al., 2006; Le Bastard et al., 2010b; Mattsson et al., 2009; Ritchie et al., 2014).

Regarding amyloid, the Aβ(1-42) peptide and the Aβ(1-42)/Aβ(1-40) ratio have gained huge attention in this field. The reason why this ratio is measured is because Aβ(1-40) represents the most abundant Aβ peptide produced or secreted by neurons, helping to normalize the amount of Aβ(1-42) and compensate for individual variations of amyloid production. In addition, CSF Aβ(1-40) is also most abundant than Aβ(1-42) and, combined with it, it shows higher accuracy to identify AD than CSF Aβ(1-42) as a single biomarker (Hansson et al., 2019; Janelidze et al.,
Both of them, Aβ(1-42) and Aβ(1-42)/Aβ(1-40) ratio show reduced CSF levels in subjects with AD or prodromal AD (Olsson et al., 2016; Parnetti et al., 2012). Moreover, CSF Aβ(1-42) levels are inversely correlated with the amyloid plaque load (Seppälä et al., 2012; Strozyk et al., 2003).

However, when using CSF Aβ(1-42) as a biomarker for AD, there are some limitations that need to be considered. For example, CSF Aβ(1-42) levels drop in different NDs, such as in VaD (Bjerke et al., 2009), DLB (Slaets et al., 2013b) or FTD (Koopman et al., 2009), and this may affect the discrimination capacity of this peptide.

On his behalf, plasma Aβ(1-42) does not show any correlation with CSF Aβ(1-42) levels (Olsson et al., 2016), since no differences (or minor changes (Hanon et al., 2018; Janelidze et al., 2016a; Rembach et al., 2014)) in the former have been reported when comparing AD and Ctrl subjects (Le Bastard et al., 2009; Le Bastard et al., 2010a; Lövheim et al., 2017). One of the reasons could be the contribution from peripheral tissues to plasma Aβ (Blennow and Zetterberg, 2018; Henriksen et al., 2014).

Plasma Aβ(1-42)/Aβ(1-40) ratio, instead, shows decreased levels in AD with regard to Ctrl subjects, correlates with amyloid burden (Rembach et al., 2014) and is associated with an increased risk of having dementia (Verberk et al., 2018).

Regarding tau-related pathology, increments in CSF t-tau and p-tau isoforms have proven to be good biomarkers for AD (Olsson et al., 2016). Increments in CSF t-tau levels are correlated with disease progression (Roe et al., 2013). And, the higher this increment is, the more aggressive and severe is the neural damage (Degerman Gunnarsson et al., 2016; Lashley et al., 2018).

Plasma t-tau and p-tau levels are higher in AD and MCI with regard to Ctrl subjects (Shekhar et al., 2016; Tatebe et al., 2017; C.-C. Yang et al., 2018), regardless of CSF t-tau or p-tau (Fossati
et al., 2019; Zetterberg et al., 2013), and are associated with worse memory and lower cortical thickness (Dage et al., 2016), lower grey matter density (Deters et al., 2017) and cognitive decline (Mielke et al., 2017).

Since high t-tau or p-tau levels reflect damaged neurons and the disruption of tau protein functionality, abnormal levels of both proteins are also present in other tauopathies. That is, p-tau and t-tau only reflect the intensity and severity of neurodegeneration and do not seem to be specific for AD pathology (Blennow and Zetterberg, 2018; Obrocki et al., 2020).

Apart from Aβ(1-42), Aβ(1-42)/Aβ(1-40) ratio, t-tau and p-tau (the core AD/neurodegeneration markers), numerous biomarkers are being investigated for their added value in the differential and early diagnosis of AD. All of them reflect processes that are also altered in AD and sometimes correlate with Aβ and tau pathology. Below the most studied are described:

- TREM2 is a type-1 transmembrane glycoprotein, with an immunoglobulin-like extracellular domain and a cytosolic tail. It is expressed in microglial cells and helps them to modify their number, size and distribution when the brain is injured (Jay et al., 2019). It also undergoes a proteolytic processing and releases its ectodomain into the extracellular space as a soluble variant called sTREM2. In CSF, higher sTREM2 levels have been detected specifically for MCI-AD with regard to healthy controls or dementia due to AD subjects (Lashley et al., 2018) and seem to correlate with CSF markers for neuronal injury (Heslegrave et al., 2016; Rauchmann et al., 2019; Suárez-Calvet et al., 2016, 2019). However, no changes in plasma sTREM2 levels have been identified in AD subjects (Ashton et al., 2019b).

- In combination with sTREM2, progranulin, which is a protein expressed by microglia in the brain, might reflect microglia activation during AD. Both proteins levels proved to be higher in CSF when pathology was present (Suárez-Calvet et al., 2018).
- Other synapse- and inflammation related proteins, such as synaptotagmin-1, synaptosome-associated protein 25 (SNAP25), chitinase-3-like protein or neurogranin (Ng) have been detected in CSF and seem to be promising synaptic degeneration-related biomarkers in AD. For example, chitinase-3-like protein is an inflammatory glycoprotein with a role in tissue remodelling that has already been shown to be increased in CSF in preclinical stages of AD (Janelidze et al., 2018). However, it is not a specific biomarker for any ND (Baldacci et al., 2019). SNAP25 is an essential component of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex that mediates synaptic communication. CSF levels of SNAP25 were higher in dementia due to AD and in MCI-AD than in non-demented controls (Brinkmalm et al., 2014). The same happens with synaptotagmin-1, a protein found in the presynaptic PM which is implicated in neurotransmitter release (Brinkmalm et al., 2014). On his behalf, Ng, a calmodulin-binding postsynaptic dendritic protein expressed by excitatory neurons, is highly expressed in the amygdala and the hippocampus (Lashley et al., 2018). Ng increased levels in CSF of dementia due to AD and MCI-AD subjects are a consequence of synaptic disruption/loss (Hellwig et al., 2015; Kester et al., 2015). In addition, these changes correlate with cognitive decline, impairments in cortical glucose metabolism and brain atrophy (Kvartsberg et al., 2015). However, plasma Ng concentration remains unchanged in AD subjects compared with healthy controls (Obrocki et al., 2020).

- Apart from synaptic degeneration biomarkers, there are biomarkers that reflect the intensity and severity of the disease. One of these is the neurofilament light (NFL). NFL is a type of intermediate filament seen in the cytoplasm of axons that plays an important role in axonal homeostasis and synaptic transmission. CSF and plasma NFL concentrations are increased in dementia due to AD and are highly correlated (Lewczuk et al., 2018; Mattsson et al., 2017).
However, higher levels of this protein are also a characteristic of other NDs, such as FTD or VaD, pointing out that NFL is a neurodegeneration biomarker rather than a specific biomarker of AD (Blennow and Zetterberg, 2018).

- The neutrophil gelatinase-associated lipocalin or lipocalin-2 is a secreted glycoprotein implicated in neuroinflammation, cognition and memory, among others. This protein has appeared as a possible early-blood biomarker negatively correlated with CSF Aβ(1-42) in preclinical AD (Eruysal et al., 2019) and also in MCI (Choi et al., 2011).

- Lastly, microRNAs (miRNAs) have also been proposed as biomarkers for AD since their control over gene expression can be directly related to the progression of the pathology. However, miRNA studies in the AD biomarker field have reported controversial results. In fact, only 17 out of 120 miRNAs were found to be dysregulated in AD in more than one publication (Cătană et al., 2020).

As imagined, CSF sampling has several limitations even if lumbar puncture is safe and well-tolerated procedure. That is why some diagnostic biomarkers (such as tau, Aβ, Ng, NFL or sTREM2) have started to be studied in blood, which is more accessible and could be repeatedly obtained in longitudinal studies to track disease progression. However, disease biomarkers are lower outside the CSF (Obrocki et al., 2020), as only a little fraction of these molecules enters the blood-stream. Also, there are several factors that affect the amount of these molecules in blood, such as the smoking status, gestation, diet, medication, physical activity level or fasting length, among others (O’Bryant et al., 2015). And, lastly, blood is a more complex matrix than CSF. Brain proteins have to be measured in a matrix containing very high levels of blood proteins such as albumin, which means a high interference in analytical methods, they could be degraded by proteases, metabolized in the liver or cleared in the kidneys. All this introduces a
2.4. Serendipity, drug repurposing and human complexity

The discovery of new biomarkers is a highly complex task. And, in the case of AD is even more tricky because of the complexity of this disease. Therefore, choosing only a causative protein, gene, lipid, miRNA or metabolite, makes us lose all the information that surrounds the core of the disorder.

This loss of information happens and hardens the development of new drug treatments, either a SMT or a DMT, and can affect the so-called drug repurposing (the reuse of drugs for something different for which they were initially created). Repositioned drugs offer many advantages, because they are developed faster and cheaper than new drugs, and medicine is plenty of them. The most well-known example is aspirin (Jourdan et al., 2020).

The discovery of these new-old drugs arose, as Jourdan et al. said, by “serendipity” but not because of a completely rational thinking. They happen when you were not expecting them to happen (Jourdan et al., 2020). Without going very far, the novel coronavirus is the clearest example of what serendipity is all about (Cherian et al., 2020). In absence of an approved drug against a disease, drug repurposing is the most effective treatment. If we go further, there are also several examples for AD. One of them is zileuton. This drug was initially developed to treat chronic asthma. Asthma is an inflammatory disease in which airflow obstruction plays a crucial role. Bronchoconstriction, microvascular permeability and mucus secretion are all dysregulated in asthma. Leukotrienes are eicosanoid inflammatory mediators produced by leukocytes. In their synthesis, the activation of 5-lipoxygenase (5LO) (enzyme implicated in the arachidonic acid
metabolism) is involved. Leukotrienes induces inflammation, affects microcirculation, vessel permeability and, lastly, bronchoconstriction (Sirois, 2019). Therefore, the inhibition of the leukotriene pathway seems a feasible target for the treatment of asthma. There are two ways to inhibit the leukotriene pathway. The first one consists in blocking the receptors for leukotrienes on bronchial cells. The other mechanism is with 5LO inhibitors, such as zileuton. This second approach reduces inflammation, mucus secretion and bronchoconstriction, among others (Bouchette and Preuss, 2020).

However, this is not the only use for zileuton. Even if no clinical trial has been developed for this drug yet, it seems to be a possible good one for the treatment of AD. In fact, Chu et al. first proposed zileuton as a possible treatment for memory deficits and Aβ deposition in the triple transgenic mouse model for AD (3xTg-AD) (Chu et al., 2013). Actually, chronic asthma and AD have something in common. The 5LO is the essential source of inflammatory leukotrienes. In the brain, instead, 5LO is widely expressed. And in AD subjects, this enzyme is upregulated in hippocampus compared to healthy controls (F. Chen et al., 2020; Chu et al., 2013). After these authors, several groups have demonstrated the beneficial effects of targeting the 5LO pathway in several diseases, such as ischemia (Silva et al., 2015) or tauopathy (Giannopoulos et al., 2015).

The reason why these disorders can be treated with the same drug, in this case an inhibitor of 5LO pathway, is because they are inflammatory diseases and zileuton has an inhibitory effect on inflammation processes (O’Byrne, 1997; Webers et al., 2020).

That is, repurposing or serendipity sometimes allow researchers to attack diseases and to discover drugs (new-old ones). When diseases share the same dysfunction is reasonable to think that they can also share drug. However, serendipity does not allow us to understand what is
really happening in the complexity underlying the dysfunction. As long as we all are complex systems, most of human diseases are too. Bearing in mind this complexity, an alternative approach taking into account and trying to model the complexity of our body and diseases will lead to better pharmacological approaches and, also, to better drug repurposing. This is only possible when passing from the serendipity to the study of networks behind complex diseases.

This complexity is easy to understand when looking at the human interactome (the ensemble of the interactions among proteins that happen in an organism and under a specific biological context). 25000 protein-coding genes, large amount of non-coding RNAs (ncRNAs) and diseases that are not usually due to a single mutation in a single gene (Barabási et al., 2011; Beermann et al., 2016) underscore and confirm the complexity. Therefore, non-monogenic diseases depend on multiple gene products that do not act alone but interact in a complex network. That is, interactions between components of the complex system create magnificent networks that are present in the real world, from new technologies (such as Instagram or Facebook) to complex human diseases. And it seems clear, as A.L. Barabási said “we will never understand complex systems unless we develop a deep understanding of the networks behind them”.

2.4.1. Networks do matter. The importance of the Seven Bridges of Königsberg problem

*Before we start talking about networks, let’s define the following terms (Barabási and Oltvai, 2004):*

**Graph theory.** Mathematical field in charge of studying graphs.
**Graph.** Mathematical representation that models relations between two objects. Objects are represented by vertices and the connections among them are the edges. Graphs do not care about the data they represent.

**Network.** It is a graph that refers to real systems. Objects are represented by nodes and links are the connections. For example, society is a network of subjects linked by friendship or family.

**Degree.** The degree of a node is defined by $k$ and tells us how many links it has to other nodes.

**Degree distribution.** The degree distribution is $P(k)$ and gives the probability that a node has exactly $k$ links.

**Short average path length.** Number of links along the shortest path between two connected nodes.

**Clustering coefficient.** It is the degree to which nodes tend to cluster in the network. It measures the tendency of the nodes in a network to form clusters or groups.

Complex systems are those composed of a huge number of components that interact among them. Single components interact forming networks. However, to better understand how interactions in a network take place, we need to move to the 18th century and the city of Königsberg.

This city consists of two main lands and two islands in the middle. Connecting all of them, seven bridges (Figure 4 A). If you were in Königsberg and you would like to have a walk through the city, could you find a continuous path to do that, crossing each bridge only once and returning to the starting point? To give it a solution, the mathematician Leonhard Euler represented each part of the city with a node, and each bridge between two lands with a link (Figure 4 B). He
built a graph (Figure 4 C) and observed that nodes with odd number of links must exist only at the starting or the end point of the continuous path. Otherwise if you arrive to a node with odd number of links, you would not find an unused link to leave the land. This problem does not have a mathematical solution and therefore, the continuous path does not exist.

This was the first time that a math problem was solved using graph theory and, also, the first time that someone showed that problems become simpler when you represent them using graphs (Petrella and Doraiswamy, 2013).

**Figure 4. The seven bridges of Königsberg problem.** A schematic map of Königsberg (Kaliningrad, Russia) during Euler’s time with the four land pieces (named 1, 2, 3 and 4) and the seven bridges (represented in brown) connecting them across the river Pregel is represented (A). Euler solved the seven bridges of Königsberg problem building a graph that has four nodes (the four land pieces) and seven links (each one corresponding to each bridge) (B). With the graph he built (C), he demonstrated that there is not a continuous path to cross every bridge only once. *Figure designed by Soraya Moradi Bachiller.*

Graphs are the roots of networks and networks happen randomly. That was what Paul Erdős and Alfréd Rényi proposed in their random graph model in the 20th century. The Erdős-Rényi model arose with the aim to represent graphs with unknown organizing principles in which edges were distributed randomly. In random graphs, the vast majority of vertices have the same number of
edges and, thus, the same degree. Therefore, they follow a Poisson distribution (Figure 5). This type of distribution is represented with a peak, which refers to the same degree of most of the vertices in the graph (Barabási and Oltvai, 2004).

**Figure 5. Random networks are Poisson-distributed.** The degrees (k) in a random network follow a Poisson distribution, that is, a bell curve where every node has the same degree (nodes are represented in violet color while links are green lines). Most of them have the same number of links in the network and, consequently, highly-connected nodes do not exist. *Figure designed by Soraya Moradi Bachiller and adapted from Barabási, A. L. 2016, Network science, section 4.3.*

Random graphs also have small-world properties. The small-world character was first described in the Watts-Strogatz model (Watts and Strogatz, 1998) to represent random graphs with short average path lengths between two connected vertices and higher tendency to cluster than random graphs. The most well-known representation of the small-world property is the “six degree of separation” concept. This rule establishes that people are six or fewer connections away from
each other, that is, there is a short path length between pairs of people. The small-world property can be applied to cells, where each two chemicals are separated by three biochemical reactions or to actors in Hollywood that are, on average, three actors distant from each other (Albert and Barabási, 2002). And this characteristic is therefore present in real networks.

However, random graphs were not ever tested for the large amount of data that real and complex systems usually have. Albert-László Barabási and Réka Albert were the first to realize that, in complex networks, the data is highly self-organized and connections do not happen randomly. In 1999, they discovered the need of scaling in real networks. They identified a common property of large networks in which node degree follow a power-law distribution rather than a Poisson one (Figure 6). A power-law distribution does not have a peak, as the Poisson distribution has in random graphs, but it is described by a decreasing function. This is what they called the scaling property of real networks. Scaling property is a consequence of two mechanisms that allow networks to expand; growth and preferential attachment. Scale-free networks suffer from continuous addition of new nodes. For example, the publication of new manuscripts allows research literature to grow. The addition of new nodes is not a random process because new nodes attach to well-connected nodes. That is, new nodes are more likely to attach to existing nodes with a large number of connections. This characteristic is also known as the “rich get richer” concept; new manuscripts will cite well-known and much-cited papers than less-known and less-cited ones. These two natural events allow the existence of two properties of nodes in scale-free networks; higher tendency to cluster and inhomogeneous degree distribution. Clustering is something really common in social networks where there are different groups of friends. On his behalf, the degree distribution is the direct consequence of the preferential attachment. Some nodes are more likely to become highly-connected nodes.
These nodes are the so-called “hubs”. The vast majority of nodes, instead, are less likely to connect and, thus, they are the less-connected nodes with only few connections in the network. The power-law degree distribution also reflects this phenomenon. There is not a peak as in the Poisson distribution, but a decreasing function where two groups of nodes are identified; few hubs with so many connections and lots of nodes with few connections (Figure 6) (Albert and Barabási, 2002; Barabási and Albert, 1999).

**Figure 6. Scale-free networks are characterized by the power-law degree distribution.** The degrees (k) in a scale-free network follow a power-law distribution where most nodes have few links while only some of them have many links and held the less-connected nodes together in the network (nodes are represented in violet color while links are green lines). The degree of the nodes correlates to its size. *Figure designed by Soraya Moradi Bachiller and adapted from Barabási, A. L. 2016, Network science, section 4.3.*
2.4.2. **Error tolerance of scale-free networks. Which node should be attacked?**

The Barabási-Albert model gave rise to the power-law degree distribution that characterizes real networks, such as the world wide web, internet, actor collaboration, science collaboration, human sexual contact, cellular, ecological, phone call and citation networks (Barabási and Albert, 1999).

The power-law distribution property is easy to understand in every cellular network. One common example are the metabolic networks, where each node represents a biomolecule and the biochemical reactions are represented by links. Water plays a role in most of these reactions (so, it is a hub), while the rest of the biomolecules participate in fewer reactions. As for water, the same happens, for example, with ATP in metabolic networks (Barabási and Bonabeau, 2003).

Robustness and vulnerability are the two most common properties of cellular networks with scale-free properties. Robustness is the resistance to “random damage”, and vulnerability is the susceptibility to “malicious attacks”. Scale-free networks are characterized by the presence of few hubs and many less-connected nodes. And this leads them to behave in a very predictable way, being robust to accidental failures but vulnerable to direct attacks. The accidental failures or random damages will take out less-connected nodes, since they are more abundant than hubs in the network. This kind of damage does not alter the remaining nodes (at least, not many of them) since less-connected nodes only have few links. Consequently, it does not have a great impact in the network function since they do not lead to the loss of connectivity (Figure 7) (Albert, 2005; Albert et al., 2000). That is, the lethality of a node removal correlates with the number of interactions that given node holds (Jeong et al., 2001).
Instead, the direct or malicious attacks lead to the loss of hubs (highly-connected nodes) which also lead to a major loss of connectivity and to the network disruption since the removal of few hubs breaks up a complex network into many disconnected components (Barabási and Bonabeau, 2003; Loscalzo et al., 2007) (Figure 7). This suggests that cellular networks are robust to avoid their disruption when random attacks happen. In other words, the cell is vulnerable only when it losses hubs.

Figure 7. Scale-free networks are robust under “random damage” while vulnerable under “malicious attack”. In this schematic network, the size of the node correlates with the degree. In scale-free networks, when hubs are removed, most of the connections are lost. In fact, removing only 2 hubs (violet-colored) of this imaginary network, produces the loss of 26 connections. If we then removed 1 hub and 3 less-connected nodes (violet-colored), the sum of 11 links disappears. 6 out of the 11 links which get lost are due to one hub removal while only 5 are due to the loss of 3 less-connected nodes. This confirms that the removal of less-connected nodes is not sufficient to break apart the network. Instead, if we remove the last 2 remaining hubs, we lose the connectivity and, consequently, destroy the network. This also confirms that
removing hubs leads to a great loss of connections and to the network to break into disconnected components. *Figure designed by Soraya Moradi Bachiller.*

Consequently, to change the behaviour of real networks without destroying them, the removal of hubs should be avoided (Kovács and Barabási, 2015) That is, every complex system requires networks to provide every single component the resources it needs, allowing the flux of information. For the information to flow, the connectivity has to be maintained and this is only possible because of the presence of hubs (Barabási, 2017).

The error tolerance of scale-free networks and, also, the existence of hubs and less-connected nodes, have beneficial properties for real world. They allow natural selection and evolution of complex systems. It is known that cells have an impressive capacity of adjusting themselves in response to changes in their developmental state and, also, in the environment because changes happen in less-connected nodes. However, changes in hubs (such as mutations or deletions) cause the elimination of every link these highly-connected nodes have, destroying network connectivity and leading to embryonic lethality. In this way, scale-free networks minimize the consequences of genetic errors unless hubs are the targets of these perturbations. In addition, as deletion of hubs leads to larger phenotypic outcomes, hubs tend to be encoded by essential genes which are necessary for early development and whose dysfunctions lead to spontaneous abortion. Less-connected nodes, instead, tend to be encoded by disease genes since people can tolerate disease-causing mutations quite well (Barabási and Bonabeau, 2003; Barabási et al., 2011; Goh et al., 2007; Loscalzo et al., 2007).

The deletion of hubs leads to serious dysfunctions because they prevent the flux of information. For the correct function of a system, this flux of information and the connectivity cannot be disrupted. Therefore, hubs should not be the target of drug treatments. Firstly, because in a
disease we do not want to destroy the network, we only want to change how the information flows among their components. And, secondly, because hubs do not accumulate non-lethal disease genetic errors or dysfunctionalities, less-connected nodes, instead, do it.

Some researchers have already used network-based approaches to study how cellular connections affect human disease progression, with the aim of identifying disease genes and better targets for drug treatments. Systems biology is the interdisciplinary field of science that study networks underlying complex systems (Barabási et al., 2011). This field has already shown how complex systems can be controlled and driven from the healthy to the disease stage (Vinayagam et al., 2016). It has also been demonstrated that networks allow the discovery of novel drug targets, disease genes and biomarkers in breast cancer (Khan et al., 2020), AD (Yan et al., 2019), lung cancer (Kaushik et al., 2020) or cellular senescence (Avelar et al., 2020). In addition, the network for a given disorder is specific but more than one disorder share disease-associated biomolecules (Gamba et al., 2020). Subsequently, shared symptoms are also a common feature for different diseases. This allows networks to provide rational strategies for drug repurposing (F. Cheng et al., 2018; Menche et al., 2015; Pournoor et al., 2019). One example is the re-use of anti-diabetic agents to prevent insulin resistance in AD (Esmaeili et al., 2020). Also, new-node disease associations can be also detected based on the guilt-by-association strategy. This approach consists in the finding of new disease candidates that are already associated with participants of the pathological process studied (Singh-Blom et al., 2013).
Chapter III
CHAPTER III

EXTRACELLULAR VESICLES

3.1. Extracellular vesicles and the transferrin receptor in sheep red cells

The secreted membrane-enclosed by a lipid bilayer receive the name of extracellular vesicles (EVs). They are secreted by all cells and released into the extracellular space. They are naturally delivered in body fluids, such as urine (Street et al., 2017), CSF (Stuendl et al., 2016), blood (Hornung et al., 2020), saliva (Katsiougiannis et al., 2017), tears (Pieragostino et al., 2019) and breast milk (Mirza et al., 2019), among others.

They were first discovered in the late 1960s under the name of “platelet dust” (Wolf, 1967). That is, EVs were not first identified as what they actually are, but as biological material originated from platelets, which were able to sediment at high speed centrifugation.

Some years later, two independent groups (Harding et al., 1983; Pan and Johnstone, 1983) discovered that cells were able to secrete membrane vesicles. In brief, in the red cell maturation process, from reticulocytes to erythrocytes, the transferrin receptor seemed to disappear. It only seemed. The transferrin receptor was being released into the extracellular space within vesicles.

This loss of PM proteins, as it happens with the transferrin receptor in reticulocytes was only the reflection of the removal of unwanted and unnecessary material out of the cell. And this common process for all the cells mostly happen through the inward and outward budding of the PM (Johnstone, 2006).

Membrane curvature or budding is the common mechanism implicated in EV biogenesis. Specifically, endosomal membrane invagination, cell surface PM budding and cell
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fragmentation are the three different ways of biogenesis for each type of EV; exosomes (~30-150 nm), ectosomes (also called microvesicles or shedding vesicles; ~100-1000 nm) and apoptotic bodies (~800-5000 nm), respectively. Each one of them represent a heterogeneous population of EVs with specific physical properties and functions, that sometimes overlap (Iraci et al., 2016; Kalra et al., 2016).

However, from the late 1960s, something has changed. We now know that the utility of EVs is something more than the mere fact of releasing unwanted material. They are enriched in proteins (Raposo et al., 1996), nucleic acids (Valadi et al., 2007) and lipids (Record, et al., 2018), among others. They are also capable of transferring this functional material to the target recipient cell, in health and disease. And these two conditions, biological cargo selectively chosen and packaged, and specifically delivered to the target cell, define EVs as a communication tool among cells.

3.2. Small-extracellular vesicles. Exosome and ectosome biogenesis

Exosomes, ectosomes and apoptotic bodies are all EVs. However, the most known and studied vesicle in EV field has been the “exosome”, leading, sometimes, to inaccurate and wrong definitions. In an effort to clarify EV terminology and support EV-related work, the International Society for Extracellular Vesicles renewed the Minimal Information for Studies of Extracellular Vesicles (MISEV) in 2018 (Théry et al., 2018). As suggesting in MISEV2018 guidelines, one way for referring to EV subtypes are their physical characteristics (such as their diameter). However, the overlapping diameters between exosomes and ectosomes make a very difficult task to isolate them separately. Therefore, from now, the term small-extracellular
vesicles (sEVs) is used to refer to EVs with less than 200 nm in diameter, that is, exosomes and ectosomes.

Figure 8. Exosome and ectosome biogenesis. MVB are formed during the process of endosomal maturation. In this process, the limiting membrane of the early endosomes invaginates sequestrating the material present in the cytosol and forming ILVs. MVBs can fuse either with the lysosome for degradation or with the PM to release ILVs, that once outside the cell receive the name of exosomes. Instead, microvesicles or ectosomes bud directly from the PM. MVBs, multivesicular bodies; ILVs, intraluminal vesicles. Figure designed by Soraya Moradi Bachiller.

The different, although overlapping, size of exosomes and ectosomes reflect their diverse biogenesis and sorting machineries involved. Exosomes originate from the endosomal system by inward budding, while ectosomes designate sEVs shedding from the PM by outward budding (Figure 8). For exosomes, as they shed from the multivesicular bodies (MVBs), they have a similar diameter to the intraluminal vesicles (ILVs). However, ectosomes do not have that
limitation in size (Juan and Fürthauer, 2018; Meldolesi, 2018; Tkach et al., 2018; Van Niel et al., 2018). This inward and outward invagination allow exosomes and ectosomes to have the same topology than the parental cell; that is, the cellular lumen is inside sEVs.

Curiously, exosome and ectosome biogenesis, although at different sites of the cell, start in the same region, the cellular lipid bilayer, and in a similar manner. The exosome biogenesis starts with the invagination of the PM, to form an early endosome and, subsequently, the inward budding of the late endosome PM. Ectosome formation also happens by PM deformation, but by outward curvature. In both cases, lipids (such as cholesterol and glycosphingolipids) that cluster in discrete PM microdomains (also called lipid-rafts regions) and transmembrane proteins (tetraspanins, integrins and metalloproteinases) that group into the tetraspanin-enriched microdomains (T-EMs), have a role in membrane curvature. Sometimes, these two microdomains (lipid-rafts and T-EMs) coalescence since both are essential for the adhesion of proteins, nucleic acids, lipids or metabolite recruited and sorted into sEVs (Meldolesi, 2018; Van Niel et al., 2018; Villarroya-Beltri et al., 2014; Yáñez-Mó et al., 2009).

### 3.2.1. Exosomal ESCRT-dependent biogenesis

After the PM curvature, the early endosome is formed. This early endosome matures into a late endosome. The PM of this late endosome (also called MVB or multivesicular endosome) suffers inward budding generating ILVs in its lumen (Van Niel et al., 2018).

The most accepted and known machinery involved in the formation and sorting of ubiquitinated cargo into ILVs is the endosomal sorting complex required for transport (ESCRT).
ESCRT components were first characterized in yeast and are encoded by class E vacuolar protein sorting (VPS) genes (Ahmed et al., 2019). Here, we will use yeast nomenclature when referring to ESCRT components (the human nomenclature of some of these proteins, instead, is included in parentheses). Note that some of these subunits consist of several isoforms.

Four complexes (ESCRT-0, -I, -II and -III) and accessory and essential proteins, such as ALG-2 interacting protein X (Alix) and Vps4 have been described in the ESCRT-dependent pathway (Egea-Jimenez and Zimmermann, 2019).

ESCRT components act sequentially (Figure 9). The ESCRT-0 recognizes and sequesters ubiquitinylated proteins, ESCRT-I, -II and –III direct the cargo sorting and facilitates membrane curvature, and Vps4 allows membrane scission and ILV detachment from the MVB membrane (Ahmed et al., 2019; Hurley, 2010; Juan and Fürthauer, 2018; Kalra et al., 2016; D. Li et al., 2018; Mosesso et al., 2019).

ESCRT-0 recognizes monoubiquitinated proteins via its ubiquitin binding domains (UBDs) (Frankel and Audhya, 2018; Takahashi et al., 2015). For this recognition to start, the FYVE domain of the Vps27 binds phosphatidylinositol 3-phosphate (PI(3)P) with high affinity. This allows the recruitment and approach of ESCRT-0 heterodimer onto late endosomes (Hurley, 2010). Once there, both subunits of ESCRT-0, Vps27 and Hrs-STAM, via their 4 UBDs (each subunit has two UBDs), recruit ubiquitinated cargo. Subsequently, and because of the interaction between Vps27 and Vps23, ESCRT-0 recruits ESCRT-I. ESCRT-I consists of Vps23 (tumor susceptibility gene protein 101, Tsg101), Vps28 (VPS28), Vps37 (VPS37A-D) and Mvb12 (UBAP1 and MVB12A-B). At this stage the ESCRT-0/ESCRT-I complex is already formed in the late endosomal membrane and is capable to act as a bridge to recruit the ESCRT-II through the interaction between Vps28 and Vps36 (thanks to the lipid-binding domain of
Vps36, also called GLUE). The ESCRT-II is composed of two Vps25 (EAP20), one Vps22 (EAP30) and one Vps36 (EAP45) and is able to start the membrane budding and contribute to the uptake of ubiquitinated cytosolic cargoes (only at one UBD). Vps25 contains winged helix motifs that allow the recruitment of the ESCRT-III via the interaction with Vps20. The recruitment of Vps20 is followed by Snf7, Vps2p and Vps24p (in this order). ESCRT-III is the only one having no UBD but implicated in membrane scission (Wollert et al., 2009). It consists of Vps20 (charged MVB protein 6, CHMP6), Snf7 (CHMP4A-C), Vps24 (CHMP3) and Vps2 (CHMP2A-B) as its core subunit proteins. They are all in the cytosol as inactive monomers while associating as an active polymer only on the endosomal membrane and assembling into the ESCRT-III complex. Vps20, Vps24 and Snf7 are enough to promote membrane scission (that occurs without ESCRT components internalization (Wollert and Hurley, 2010)), while Vps2 is required for coupling the last ESCRT component (Vps4) to the ESCRT-III. Vps4 (VPS4A-B) is a type I AAA; that is, an ATPase that hydrolyses ATP to disassemble ESCRT-III subunits, releasing them back to the cytosol and finishing the ESCRT cycle (Jackson et al., 2017).
Figure 9. The exosome biogenesis mediated via the ESCRT-dependent mechanism. Several proteins form the ESCRT machinery. All of them act sequentially and are recruited to the endosomal membrane (which is also the membrane of the future MVB). The process starts with the recognition of ubiquitinated proteins by the Hrs and its lipid – PI(3)P – binding domain (FYVE). ESCRT-I, II and III direct the cargo sorting and start the endosomal membrane curvature. ESCRT-III interacts with its accessory protein Alix, completing the endosomal membrane budding and recruiting DUBs (that deubiquitinate cargoes) and the Vps4 (that drives ILV scission within the MVB and recycles ESCRT components). ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body; ILV, intraluminal vesicle; DUB, deubiquitinated enzyme. Figure designed by Soraya Moradi Bachiller.

Together with these subunits, there are several accessory proteins which mediate ESCRT functions, such as Vps60 (CHMP5), Ist1 (IST1), Doa4 (CHMP1A-B), Vps20 (LIP5) and Bro1/Alix (Ahmed et al., 2019; Frankel and Audhya, 2018; Hurley, 2010; Hurley and Hanson, 2011; Jackson et al., 2017; Villarroya-Beltri et al., 2014).
Another important feature when ubiquitinated cargoes are sorted in ILVs, is the recycle of ubiquitin when MVB fuse with the PM and not with the lysosomes to be degraded. This is a process that happens before the disassembly of the ESCRT-III. The interaction between the ESCRT-III subunit Snf7 and the ESCRT accessory protein Alix, recruits Doa4. Doa4 is a deubiquitinated enzyme (DUB) in yeast capable of preventing the degradation (in vacuoles/lysosomes) of sorted proteins by catalysing deubiquitination before they are sorted into ILVs (Ahmed et al., 2019; Nikko and André, 2007).

3.2.2. Exosome biogenesis. Besides ESCRTs

ESCRT-dependent pathway is the most common and well-described for exosome biogenesis. However, MVB and ILV formation also occur in an ESCRT- (totally or partially) independent manner. In fact, some previous studies have demonstrated that not all ESCRT subunits are essential for exosome biogenesis, since MVB formation is conserved after their depletion (Stuffers et al., 2009; Trajkovic et al., 2008).

Syndecans and syntenins (Baietti et al., 2012), heparanase (Roucourt et al., 2015), small GTPase ADP ribosylation factor 6 (ARF6) (Ghossoub et al., 2014) and lipid-modifying enzymes (such as sphingomyelinase or phospholipase D2 (PLD2)) (Trajkovic et al., 2008) also participate in ILVs generation.

Baietti et al. first described the syndecan-syntenin pathway. This mechanism of exosome biogenesis is not totally ESCRT-independent, since it needs Alix (Baietti et al., 2012). Therefore, it usually receives the name of syndecan-syntenin-Alix pathway. Syntenin is a cytosolic protein that contains two post-synaptic density protein 95/disks large homolog/zonula occludens 1 (PDZ) domains. On his behalf, and as shown in Figure 10, syndecan is a cell surface
Figure 10. Syndecan-syntenin-Alix pathway for exosome biogenesis. The syndecan-syntenin-Alix pathway starts with the non-trimmed HS syndecans and their associated cargoes (represented in black circles). Then, in the early and late endosomes (MVB), the heparanase (represented with the Pac-Man emoji) trims the HS chains on syndecans and, then, syntenin molecules binds to syndecans (represented with green squares). At this stage and already in the MVB membrane, syndecan undergoes proteolytic cleavage promoting its clustering with other syndecan molecules. The intraluminal budding of syndecan-syntenin and the corresponding cargo depends on the interaction of syntenin with Alix (represented with orange circles) and on ARF6/PLD2 activation. MVB, multivesicular body; HS, heparan sulfate; ARF6, ADP ribosylation factor 6; PLD2, phospholipase D2. Figure designed by Soraya Moradi Bachiller and adapted from Friand et al., 2015.

transmembrane co-receptor that contains heparan sulfate (HS) and chondroitin sulfate chains for the binding of adhesion molecules and growth factors. At the cell surface, syndecans are continuously internalized by the cell where it forms assemblies with ligands attached to its HS chains, allowing the recruitment of syntenin through PDZ domains and Alix, and supporting
membrane budding. HS and heparanase are essential in the syndecan-syntenin-Alix pathway. Heparanase cleaves HS chains on syndecan, forming trimmed HS chains that are necessary to generate exosomal syndecan secretions. It enhances syndecan clustering at the MVB membrane and increases syndecan and syntenin internalization in the lumen of exosomes (Roucourt et al., 2015). In addition, syntenin-enriched exosomes and ILVs formation in this pathway are also controlled by ARF6 and its effector, PLD2 (Ghossoub et al., 2014) (Figure 10). The latter metabolizes phosphatidylcholine into phosphatidic acid. Phosphatidic acid induces negative membrane curvature (Friand et al., 2015).

In this process of negative domain-induced budding, ceramide is also important since it produces spontaneous membrane invagination (Skryabin et al., 2020). Ceramide-dependent mechanism for exosome biogenesis consists in the hydrolysis of sphingomyelin by the neutral sphingomyelinase 2 (nSMase2). This enzyme catalyses the hydrolysis of sphingomyelin to generate ceramide and phosphorylcholine. This mechanism requires the presence of lipid-raft-based microdomains from which ceramides will be formed (Trajkovic et al., 2008).

In addition to lipids, syntenins and syndecans, tetraspanins and TEMs are also involved in ESCRT-independent mechanisms of exosome biogenesis (Buschow et al., 2009; Van Niel et al., 2011).

3.2.3. Ectosome biogenesis. ESCRT proteins in cell surface budding

Every process that induces the budding of the PM away from the cytosol needs the membrane remodelling process.
ILVs and MVB formation were the first processes described in which ESCRT machinery was necessary (Katzmann et al., 2001). However, ESCRT proteins have been also identified to allow viruses (one of them, the human immunodeficiency virus) to bud from the PM of infected cells (Garrus et al., 2001; Martin-Serrano et al., 2001), in cell division in mammals (Carlton and Martin-Serrano, 2007), in nuclear envelop reformation, in micro- and macro-autophagy and in repairing the PM. In the latter, Ca$^{2+}$, Alix, ESCRT-III and Vps4 patch the hole and release the damaged membrane in a mechanism similar to that used for viruses or ectosomes (Jimenez et al., 2014). All these processes need to complete membrane fission and, thus, involve the ESCRT machinery.

The first evidence showing that ESCRT proteins were also implicated in ectosome release arrived in 2011 (Gan and Gould, 2011). An inhibitory budding signal from the human immunodeficiency virus was shown to inhibit the interaction of the PM with Vps4 in the human cellular model HEK293T. This inhibition blocked the virus budding and, also, the release of non-viral proteins, demonstrating that PM vesicle shedding requires the localization of ESCRT proteins at the cell membrane.

Apart from the ESCRT machinery, lipid asymmetry and the cytoskeleton also play an essential role in cell PM membrane curvature for ectosome biogenesis.

Lipid composition changes because of lipid flippases, floppases (aminophospholipid translocases) and phospholipid scramblases. These enzymes perturb the bilayer asymmetry by mediating the bidirectional translocation of phosphatidylserine across the PM which usually resides at the inner leaflets. In the ectosome formation, the redistribution of the lipidic PM is necessary. To allow this redistribution, flippases protects phosphatidylserine to translocate to the inner leaflet, remaining exposed to the cell surface (in an ATP-dependent manner), and
scramblases are no longer able to translocate (in a Ca$_{2+}$-dependent manner) phospholipids to the inner leaflet (Nagata et al., 2020; Tricarico et al., 2017).

Apart from lipids, ectosome shedding may involve other proteins. That is the case of the arrestin domain-containing protein 1 which acts as an adaptor that binds the PM through its arrestin domain, and Tsg101 through a PSAP motif. The presence of the arresting domain-containing protein 1 makes Tsg101 goes to the PM and promotes the release of ectosomes (containing those proteins) in a ESCRT-III/Vps4-dependent manner (Alonso Y Adell et al., 2016; Juan and Fürthauer, 2018; Nabhan et al., 2012; Tricarico et al., 2017).

### 3.2.4. Ectosome biogenesis. ESCRT-independent mechanisms

Similarly, ectosome formation may occur in an ESCRT-independent way. That is the case of ectosomes released from ciliated sensory neurons in *C. elegans* (Wang et al., 2014) that is a constitutive and ESCRT-0/-I independent process.

*As previously stated, ectosomes and exosomes, because of their overlapping diameters and the current techniques, cannot be completely separated. Due to the aims of our work, from now, we will concentrate on mechanisms involved in the cargo sorting into exosomes, their secretion into the extracellular space and uptake by the recipient cells, as well as in their physiological and AD-related pathological functions.*
3.3. Exosome cargoes. Which mechanisms make proteins and microRNAs to be sorted into exosomes?

As recorded in ExoCarta database (exocarta.org), a large number of molecules are contained in exosomes. This suggests the heterogeneity of the sEVs. This specific enrichment of proteins, RNA species, lipids or metabolites within exosomes highlights, firstly, the existence of several sorting mechanisms that selectively incorporate these cargoes into exosomes. Secondly, exosomes do not exactly mirror their parental cells. Lastly, cells are capable of modulating their cargo depending on their state and perturbations in the extracellular milieu, such as stress, hypoxia or pathological processes happening in the context of a ND (Iraci et al., 2016).

Among all the cargoes that may target an exosome, we will focus on proteins and the small non-coding RNA species called miRNAs.

3.3.1. ESCRT machinery, tetraspanins, syndecan/syntenin and lipids in protein sorting into exosomes

Protein-cargo sorting mechanisms usually are reminiscent of those used in exosome biogenesis. That is, in the protein cargo sorting process, as for exosome biogenesis, ESCRT, tetraspanins, syndecan, syntenin and lipids are involved.

Monoubiquitinated proteins target the ESCRT-0 in order to be transported to the endosomal membranes. ESCRT accessory deubiquitinated enzymes remove ubiquitin before proteins are incorporated into ILVs to avoid protein degradation by the proteasome/lysosome (Henne et al.,
That is, in some cases, the monoubiquitination is the signal to direct MVBs to the lysosomal pathway.

For other proteins, instead, ubiquitination is necessary for their sorting into exosomes (Ageta and Tsuchida, 2019). This is the case of the ubiquitin-like protein 3. Ageta et al., identified ubiquitin-like protein 3 as a post-translational modification (PMT) that acts as a tag on which the incorporation of ubiquitin-like protein 3 and “half of all exosomal proteins” to MVBs depend (Ageta et al., 2018). Also, the recruitment of the viral protein Gag into late endosomes is a mechanism that depend on the ubiquitination by Nedd4 E3 ubiquitin protein ligase (Segura-Morales et al., 2005). Nedd4 E3 ubiquitin protein ligase is able to interact with and to ubiquitinate Alix, facilitating viral budding (Sette et al., 2010). Another example are proteins recruited and sorted into urinary exosomes (Huebner et al., 2016). In other cases, ubiquitination is not needed for the packaging within exosomes, as it happens with the major histocompatibility complex II (Gauvreau et al., 2009).

Ubiquitination is only one of the PMTs that allow proteins to regulate their localization and function. Phosphorylated proteins are also within exosomes and phosphorylation is needed for some proteins to be sorted into them. One example is Fas ligand. This protein needs two PMTs to be internalized into ILVs of MVBs, ubiquitination and, also, phosphorylation (Zuccato et al., 2007).

All this suggests that protein sorting without deubiquitination is also possible (Ageta and Tsuchida, 2019). Ubiquitination, in most cases, only marks proteins for degradation (Segura-Morales et al., 2005) and PMTs contribute to exosome biogenesis and sorting (Anand et al., 2019).
ESCRT-(partially) independent mechanisms also exist in sorting. Under depletion of some ESCRT subunits, such as Tsg101 or Vps4, MVB and ILV formation still happens. This ESCRT-independent sorting pathway includes tetraspanins, syndecan/syntenin and lipids. The family of transmembrane proteins, tetraspanins, which are able to interact with other transmembrane proteins, lipids and metalloproteases forming T-EMs, are enriched in exosome membranes. These regions serve as platforms for the compartmentalization of receptors and signaling proteins aimed to be sorted into exosomes (S.-P. Li et al., 2018; Perez-Hernandez et al., 2013; Skryabin et al., 2020).

In the tetraspanin family, CD63 participates in the premelanosome protein sorting during melanogenesis. In fact, melanosome (melanin-containing organelles) morphogenesis does not occur when the above mechanism is inactivated (Van Niel et al., 2011). B-catenin is also exported from the cell in association with exosomes and in a process regulated by tetraspanins CD9 and CD82 (Chairoungdua et al., 2010). On his behalf, the metalloproteinase CD10 is loaded into exosomes due to its interaction with other tetraspanin protein, CD9 (Mazurov et al., 2013). Other proteins, instead, are required to sort tetraspanins into exosomes. This is the case of Alix, ESCRT-III proteins and lysobisphosphatidic acid (Larios et al., 2020).

Lipids are also crucial in ESCRT-independent sorting pathways. NSMase2 and ceramide are necessary for the incorporation of ceramide and proteolipid protein into exosomes, sorting that was not affected after Tsg101 or Alix depletion (Trajkovic et al., 2008).

 Syndecans and syntenins are also involved in exosomal protein-cargo sorting, as syndecan is capable to bind to its HS chains chemokines, syntenin, growth factors and integrins, among other cargoes (Baietti et al., 2012; Skryabin et al., 2020). CD63 is sorted into exosomes because of the interaction between syntenin and the Alix-binding motif LYPX(n)L. When syntenin-
syndecan interaction does not happen, the exosomal accumulation of CD63 and Alix is reduced (Baietti et al., 2012).

3.3.2. **NSMase2, RNA-binding proteins, miRISC/Ago2, uridylation and endogenous mRNA in microRNA sorting into exosomes**

MiRNA species are the most studied ncRNAs. They are single-stranded with 21-23 nt in size and, together with the rest of ncRNAs, they correspond to the 98% of the entire genome. Their important role in intercellular communication was enigmatic until they were first shown to be carried by exosomes in 2007 (Valadi et al., 2007).

MiRNAs are not the only RNAs species enriched in exosomes. Coding RNAs (mRNAs) and ncRNAs (such as ribosomal RNA, long non-coding RNAs and circular RNAs) are also present (Iraci et al., 2016). All these species are functional and capable of modulating gene expression once they arrive in the target cell (S.-P. Li et al., 2018).

Exosomal miRNAs have gained both increasing attention and doubts of the scientific community (Sato-Kuwabara et al., 2015). Increasing attention because of their ability to modulate gene expression of the target cell. Doubts, because of the exosome size. According to Chevillet et al., for the medium size of an exosome (100 nm) and a cell (10 µm), an exosome may contain the 0.0001 % of the cellular volume. Moreover, only 1 miRNA is incorporated per 120 exosomes. Therefore, most of the exosomes are not functional in miRNA communication (Chevillet et al., 2014). In other words, miRNA sorting into exosomes is a specific type of communication that does not happen for all the miRNAs in the cells. And for this type of communication, specific mechanisms are necessary to enclose miRNAs (aimed to be sorted) within exosomes.
MiRNA sorting into exosomes starts with miRNA biogenesis. This is a complex process that involves several steps as shown in **Figure 11**. Firstly, long primary transcripts (pri-miRNA) are produced from miRNAs-coding genes in the nucleus by the RNA polymerase II. Pri-miRNAs, which have similar characteristics to the RNA polymerase II (such as the 5’ cap structure and the 3’ poly(A) tail), are then cleaved by the RNase III Drosha to produce the 70 nt-precurser miRNA with a stem-loop structure (pre-miRNA). Drosha does not have almost any enzymatic activity and is helped by DGCR8 microprocessor complex subunit to catalyse RNA substrates. Subsequently, pre-miRNAs are transported from the nucleus to the cytoplasm by the Ran-GTP-dependent nucleocytoplasmic transporter exportin-5, which recognizes and binds pre-miRNAs while protecting them during the transport from the nucleus to the cytoplasm. Once in the cytoplasm, the RNase III Dicer cleaves the stem loop generating two complementary miRNA molecules (of 21-23 nt in size). Only one of them binds to argonaute 2 (Ago2) and is incorporated to the RNA-induced silencing complex (RISC), forming the miRNA-RISC (miRISC). MiRISC recognizes and binds to the 3’ untranslated region (3’ UTR) of the target protein-coding mRNA target, causing the translational repression or the cleavage of the transcripts (D. Li et al., 2018; Sato-Kuwabara et al., 2015).
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Figure 11. MiRNA biogenesis. Figure designed by Soraya Moradi Bachiller.

Sorting mechanisms are specific for each miRNA and they are also influenced by cellular and environmental conditions. So much so that the miRNA repertoire contained in exosomes released by cells change depending on hypoxic conditions in breast cancer cell lines (King et al., 2012), on macrophages activation by interleukins (Squadrito et al., 2014), on chronic inflammation in immune cells derived from complex regional pain syndrome subjects (Ramanathan et al., 2019) or on mutations in the transcription factor KRAS in colon cancer cells (Cha et al., 2015).

Five mechanisms participate in miRNA sorting into exosomes; those dependent on nSMase2, RNA-binding proteins (hnRNPA2B1, SYNCRIP, YBX1, MVP and MEX3C), uridylation, Ago2 (also called miRISC-dependent mechanism) and endogenous mRNA (Table 2).
miRNA sorting mediated by | Mechanism
--- | ---
RNA-binding proteins (hnRNPA2B1, SYNCRIP, YBX1, MVP and MEX3) | K homology domain- or EXO-motif- dependent
nSMase2 | Ceramide-dependent
Uridylation | 3’ end-rich poly(U)-dependent
Ago2 | KRAS-MEK-ERK-dependent
Endogenous mRNA | Target transcripts-dependent

Table 2. List of miRNA sorting mechanisms.

The nSMase2 is the rate limiting enzyme in ceramide biogenesis. It is located at the inner leaflet of the PM and at the external and cytoplasmic side of the MVBs. Subsequently, its produces ceramide at the ILVs budding sites (T-EMs and lipid-rafts). The nSMase2/ceramide-dependent mechanism allows ceramide to trigger miRNA sorting into exosomes. Previous studies have demonstrated that miR-210 is incorporated into cancer cell-derived exosomes and can be transferred to endothelial cells thanks to nSMase2 (Kosaka et al., 2013). Consequently, when using the nSMase2 inhibitor, GW4869, exosome and exosomal miRNA release is reduced (Kosaka et al., 2010; Trajkovic et al., 2008; Yuyama et al., 2012).

MiRNAs have specific motifs in their sequences able to control their localization and, therefore, their sorting into exosomes. These short, seed and specific sequences, with a 4 nt core, are located in the 3’ region and are called EXO-motifs (for miRNAs aimed to be sorted into exosomes) and CL-motifs (for miRNAs staying within the cell). They facilitate the miRNA to bind a specific RNA-binding protein (RBP) (Villarroya-Beltri et al., 2013). This interaction allows the RBP to deliver the miRNA into T-EMs or lipid-rafts regions.

The most known RBPs are the heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1 and the synaptotagmin binding cytoplasmic RNA interacting protein (SYNCRIP). HnRNPA2B1
belongs to the hnRNP family which controls and regulates the nucleic acid metabolism, such as the formation of mature mRNAs and the regulation of gene expression (Geuens et al., 2016). It has affinity for the ceramide-rich regions (and, thus, they are able to bind to lipid-raft regions in the MVB limiting membranes). In the cytosol it is SUMOylated (D. Li et al., 2018; Villarroya-Beltri et al., 2013). SUMOylation allows hnRNPA2B1 to bind miRNAs through the recognition of a specific EXO-motif, which is different for each RBP (for hnRNPA2B1 the EXO-motif core is GGAG) (Villarroya-Beltri et al., 2013).

HnRNPA2B1 controls the loading of miR-198 and miR-939 into exosomes. Mutations in the EXO-motif GGAG blocked the sorting of miR-198 (S.-P. Li et al., 2018) while it does not happen the same for miR-939, suggesting other mechanisms contributing to its sorting (Ramanathan et al., 2019).

SYNCRIP is another RBP that also belongs to the hnRNP family, but unlike hnRNPA2B1, it pertains to the hnRNPQ subgroup (Geuens et al., 2016). Its EXO-motif (also called hEXO) sequence core is GGCU and it was identified as a component of the miRNA sorting machinery of hepatocyte-derived exosomes (Santangelo et al., 2016). For the specific recognition of the hEXO sequence, the SYNCRIP N-terminal unit for RNA recognition (NURR) is needed. NURR is a conserved sequence coupled with the RNA recognition motif to achieve high-affinity miRNA binding. Therefore, for SYNCRIP specific miRNA binding, both recognition of the hEXO sequence by the NURR and binding of the RNA recognition motif are needed (Hobor et al., 2018).

Curiously, hnRNPA2B1 and SYNCRIP bind to different EXO-motifs, suggesting that there might be a cooperation during miRNA sorting and different RBPs controlling the loading of
specific groups of miRNAs. And, in case of malfunction in one of them, the loading of miRNAs containing the EXO-motif for other RBPs will be not affected (D. Li et al., 2018).

Other components of the hnRNP family have been shown to participate in miRNA sorting into exosomes. This is the case of hnRNPA1 which promotes the sorting of miR-196a into cancer-associated fibroblast-derived exosomes by binding to the EXO-motif UAGGUA (Qin et al., 2019). Similarly, hnRNPC1 allows miR-30d incorporation into Ishikawa cell (cells derived from human adenocarcinoma)-derived exosomes. This sorting allows maternal-foetal communication in the endometrium at the first stages of the pregnancy. However, more research is needed to determine which is the EXO-motif implicated in the interaction between hnRNPC1 and miR-30d (Balaguer et al., 2018).

The Y-box binding protein 1 (YBX1) is another RBP that has been identified to have a role in miRNA sorting. YBX1 is a DNA- and RNA-binding protein which is involved in transcription regulation, DNA repair, stress response and mRNA splicing, among others (Lyabin et al., 2014). It was reported to bind and transport miR-223 into HEK293T cell-derived exosomes (D. Li et al., 2018; Shurtleff et al., 2016) and miR-133 into human endothelial progenitor cell-derived exosomes (Lin et al., 2019). Its EXO-motif is not yet known but it probably has high content of C/A. In addition, YBX1 might bind specific mRNA motifs, such as ACCAGCCU, CAGUGAGC and UAAUCCCA of exosomal mRNAs and long non-coding RNAs (Kossinova et al., 2017; Suresh et al., 2018).

Mex-3 RNA binding family member C (MEX3C) is another RBP with two K homology domains (a conserved sequence of 70 aminoacids) with single-strand RNA binding activity and ubiquitin E3 ligase activity. MEX3C interacts with the adaptor protein complex 2 (which participates in clathrin-mediated endocytosis). The association of this RBP with the adaptor
protein complex 2 facilitates the RNA-endosome association, by recruiting RNA target through the K homology domains or by interacting with other RBPs, such as Ago2. It allows the sorting of miR-451a into exosomes. Its inhibition, and that of the adaptor protein complex 2, decreases exosomal miR-451a, without modifying its cellular levels (Lu et al., 2017).

Ribonucleoprotein particles (which are complexes of RBPs and RNA) are also involved in miRNA sorting. This is the case of the major vault protein (MVP). MVP interacts with miR-193a and sorts it into exosomes from colon cancer cells. Silencing of MVP inhibits miR-193a and total RNA accumulation into exosomes (Statello et al., 2018; Teng et al., 2017).

Until now, five RBPs have been identified to allow the recognition and import of miRNAs into exosomes (hnRNPA2B1, SYNCRIP, MEX3C, YBX1 and MVP). However, RBPs may also inhibit these processes. This is the case of hnRNPA2B1, which inhibits the transport of miR-503 into endothelial cell-derived exosomes (Pérez-Boza et al., 2020). In this study, Pérez-Boza et al. showed that annexin A2 has affinity for miR-503 and could export it into exosomes. HhnRNPA2B1 does not bind directly to miR-503 because of the absence of the EXO-motif GGAG. However, hnRNPA2B1 showed great affinity for miR-503 and interacts indirectly with it thanks to annexin A2, inhibiting the miR-503 sorting into endothelial cell-derived exosomes. Reduce levels of hnRNPA2B1 increases the export of miR-503, confirming that RBPs are capable of negatively regulating miRNA sorting into exosomes (Pérez-Boza et al., 2020).

Ago2 is a component of RISC which accumulates into cytoplasmic processing bodies (P-bodies). It binds to miRNAs to form miRISC. Subsequently, miRISC associates with MVBs to promote miRNA sorting into exosomes along with Ago2 and its cofactor GW182 (Gibbings et al., 2009). That is why this mechanism is also called the miRISC-dependent pathway. Ago2 sorting into exosomes is regulated by MEK-ERK signaling. Inhibition of KRAS-MEK-ERK
signaling pathway increases the association of Ago2 with P-bodies while reducing it with MVBs and decreasing secretion into exosomes (McKenzie et al., 2016).

The 3’ end post-transcriptional modification of the miRNA sequence have been also described as a mechanism that favors miRNA sorting into exosomes (Koppers-Lalic et al., 2014). The 3’ end-rich poly(U) of miRNA has been shown to be more abundant in B cell exosomes while miRNAs with 3’ end-rich poly(A) are more abundant in B cells. That is, uridylation promotes miRNA sorting into exosomes (Koppers-Lalic et al., 2014) while adenylation does not. The miR-2909 is one example of miRNAs that use 3’ end PMTs to be recruited into cancer cells-derived exosomes (Wani and Kaul, 2018). The role of uridylation as a PMT to direct miRNAs into exosomes could be explained thinking about the miRNA sorting process as a mechanism to mark miRNAs for decay, considering that uridylation gives more instability to RNA transcripts (Reimão-Pinto et al., 2016; Scott and Norbury, 2013).

MiRNA activity is dependent on the cellular amounts of their target transcripts. Squadrito et al. demonstrated in bone marrow-derived macrophages that miRNA sorting into exosomes is also a process that allows the cell to dispose of miRNA in excess. That is, when miRNA targets (mRNA) are overexpressed in the cell, miRNAs are not released within exosomes. They need to stay in the cytoplasm in order to bind their targets. When endogenous mRNA levels are decreased in cells, miRNAs are sorted into exosomes, maybe through a relocation of RISC to endosomal membranes (Siomi and Siomi, 2009). This mechanism suggests that miRNA sorting into exosomes might serve the cells to arrange miRNAs in excess with regard to their targets in order to maintain the miRNA-mRNA homeostasis (Squadrito et al., 2014).
3.4. Exosome secretion. Transport, docking, fusion and fission

Once MVBs are completely formed, they have to avoid the lysosomal degradation pathway, reach the PM, attach to it, fuse with it and, finally, release the exosomes they contain into the extracellular space. That is, four processes must happen, the transport, the docking, the fusion and the fission.

Different mechanisms may direct the biogenesis of ILVs and MVBs. Those dependent on the ESCRT-machinery are able to select ubiquitinated cargoes. And, thus, as ubiquitin is a 8.6 kDa molecule that targets substrates to protein degradation pathways, cargoes selected through the ESCRT-dependent mechanism have more probabilities to target lysosomes. When targeting lysosomes, MVBs face an increase in the acidification level. A decrease in acidification, instead, is necessary for MVBs that are meant to fuse with the PM (H. Guo et al., 2017). Others, such as the covalent addition to proteins, also called ISIGylation, impairs exosome secretion by targeting MVBs to lysosomes for the degradation of their content (Villarroya-Beltri et al., 2016). Therefore, MVBs can fuse either with lysosomes or with the PM. And, curiously, the same cargo can target one pathway or the other (Buschow et al., 2009).

In this process of transport to the M, three main components are involved. The cytoskeleton, the motor proteins associated to them (actin, tubulin, dynein, kinesin and myosin), molecular switches (Rab GTPases), tethering proteins (soluble NSF attachment proteins, SNAPs) and their receptors (SNARE proteins).

Rab GTPase is a family of small GTPases composed of almost 70 members. They are essential regulators of intracellular vesicle transport and they participate in the vesicle mobility through the interaction with tethering proteins present in the membrane of the acceptor compartment. Through their localization in different membrane compartments and membrane regions of the
same organelle, they ensure the specificity of the membrane trafficking pathways and the correct
destination of the cargo they transport (Zhen and Stenmark, 2015).

Rab GTPases act as molecular switches. This means that they function alternating between the
“on” (GTP-bound) to the “off” (GDP-bound) form (Schwartz et al., 2008; Stenmark, 2009).

Three Rab GTPases have been identified to have a role in the exosome secretion pathway,
Rab11, Rab35 and Rab27a/b. However, their individual inactivation do not have a huge impact
on exosome secretion, suggesting that their role could be complementary (Raposo and
Stoorvogel, 2013).

The first Rab GTPase related with the exosome secretion pathway, Rab11, was discovered in
transferrin receptor-containing exosomes of K562 cells (Savina et al., 2002). Rab11 is located
in the Golgi membrane and early endosomes (Ren et al., 1998; Ullrich et al., 1996). It seems to
function in a coordinate manner with the luminal Ca\(^{2+}\) release of early endosomes (Savina et al.,
2005).

On their behalf, Rab35 and Rab27a/b were identified in screening studies using libraries of
GTPases activating proteins to identify molecular machineries involved in exosome secretion.
Rab35 accumulates on early endosomes (Sato et al., 2008) and it is essential for the secretion of
exosomes in oligodendroglia cells. The inhibition of Rab35 in these cells leads to the
intracellular accumulation of endosomal vesicles (Hsu et al., 2010). The other screening study
was performed by Ostrowski et al. in HeLa cells. The authors identified Rab27a and Rab27b as
crucial elements in the MVB docking at the PM and secretion of exosomes bearing CD63, CD81
and the major histocompatibility complex II. Silencing of each of them have different effects in
the size and localization of MVB while the exosomal protein composition remains unaffected
(Ostrowski et al., 2010).
With regard to Rab11 and Rab35, Rab27a and Rab27b are associated with late endosomes and secretory compartments (Stenmark, 2009). This suggests that in the maturation stages of MVBs different Rab proteins are involved (Kowal et al., 2014).

Once MVBs have approached the cell PM, the docking and fusion of both membranes are required. These processes are mediated by SNARE proteins and SNAPs. SNARE proteins are associated with the membrane through lipid modifications. On the vesicles, v-SNAREs and, on the target PM, t-SNAREs. V-SNARE and t-SNARE interact forming the trans-SNARE complex, bringing the two membranes closer and displacing water until they fuse. Once they have fused, the trans-SNARE becomes cis-SNARE, which is able to bind SNAPs and N-ethylmaleimide-sensitive factors (NSF). NSF hydrolyses ATP and disassembles the SNARE complex, recycling v- and t-SNAREs (T. Wang et al., 2017).

Several proteins are involved in vesicle tethering and fusion. For example, vesicle-associated membrane protein 7 is a v-SNARE that co-localizes with Rab11 and participates in the exocytosis of MVBs in K562 cell line (Fader et al., 2009). The synaptobrevin homolog YKT6 is involved in fusion process in the Golgi, endosomes and vacuoles (Meiringer et al., 2008). And it is required for the secretion of Wnt-bearing exosomes in Drosophila (Gross et al., 2012). The SNAP23 also promotes membrane fusion after histamine treatment (Verweij et al., 2018) or after its phosphorylation by pyruvate kinase type M2 in tumor cells (Wei et al., 2017). Syntaxin 5, instead, was reported to target MVBs to the PM through the Ral-1 small GTPase in C. elegans (Hyenne et al., 2015, 2016).

The last step, fission, requires actin polymerization and depolymerization under the PM. This mechanism is dependent on actin, myosin and ATP. GTPase RhoA, ARF6 and ARF1 seem to
have a role in the actomyosin contraction, which allow the vesicles to bud off. Tsg101 and Vps4 are also involved in this process of membrane scission (Van Niel et al., 2018).

Exosome secretion may be spontaneous (Di Vizio et al., 2009; Ponnambalam and Baldwin, 2003). However, several physical, biological and chemical stimuli are able to contribute and induce this process (e.g. KIBRA protein, cortactin and p53 as positive regulators; tetraspanin-6 and PI(5)P as negative regulators). This is the case of hypoxia, which promotes tumor progression, metastasis and angiogenesis through exosome release in breast cancer cells (King et al., 2012) and tumor glioblastoma multiforme cells (Kucharzewska et al., 2013). Also, in breast cancer cells, loss of nischarin (a tumor suppressor) increases exosome release and promotes tumor cell growth (McAndrews and Kalluri, 2019). When nischarin is present in cells, a triple co-localization among nischarin, CD63 and Rab14 happens, reducing the release of exosomes (Maziveyi et al., 2019). Other proteins, such as α-synuclein has also been shown to be secreted within exosomes by neuronal cells in a Ca^{2+}-dependent manner (Emmanouilidou et al., 2010). Under stress stimulus, p53 is also able to increase the exosome secretion by lung cancer cells (Yu et al., 2006). The human H-ras oncogene leads to an increase of exosome release by cancer cells (Lee et al., 2014), as well as the tumor suppressor-activated pathway 6 transmembrane protein by increasing the secretion of histamine-releasing factor bearing exosomes (Amzallag et al., 2004).

### 3.5. Exosome uptake. Recognizing the correct target

Exosomes, once in the extracellular space, need to target the recipient cell to carry out their function.
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Two mechanisms allow exosomes and other EVs to elicit functional effects in target cells. In some cases, the process is determined by the recognition and binding of ligands (proteins and adhesion molecules) in the vesicle and receptors in the target cell surface. In other cases, exosome content has to be transferred by fusion with the PM, where SNARE and Rab proteins are necessary to approach both membranes (Del Conde et al., 2005; Mulcahy et al., 2014; Parolini et al., 2009), or by endocytosis-based mechanisms (such as phagocytosis, clathrin-mediated endocytosis, caveolin-dependent endocytosis or lipid-raft-mediated endocytosis) (Morelli et al., 2004) (Figure 12). Therefore, not always the merging of cellular and exosomal content is necessary to induce a response in the target cell.

Some mediators of ligand-receptor interactions are transmembrane proteins enriched in exosome surface, that is, tetraspanins. Tetraspanin 8 (in association with the α4 integrin) has been found to promote exosomal uptake by endothelial cells (Nazarenko et al., 2010). Integrins, heparan sulfate proteoglycans, lipids, connexins (Cxs) or extracellular matrix components may also play a role in the interaction between exosomes and cells. Cellular but not exosomal heparan sulfate proteoglycans are used by cancer-derived exosomes for their uptake (Christianson et al., 2013). On his behalf, lipid-rafts domains are able to trigger the internalization of exosomes derived from cancer cells (Svensson et al., 2013). The exosomal Cx43 confers exosomes the ability to transfer information via Cx43-based hemichannels. These hexameric channels allow the docking between the exosome and its target cell. And not only with target cells expressing Cx43, but also with those that do not express it (Soares et al., 2015). Also, exosomal integrins and extracellular matrix components interact to allow exosome uptake by different tissues determining organ-specific metastasis (Hoshino et al., 2015). Integrins α6 and αX are also required for gastric-specific uptake of exosomes (Yoon et al., 2020). And integrin α4β7 is also
needed by gut trophic lymphocytes-derived exosomes to target the gut by binding to mucosal address in cell adhesion molecule 1 (Jeong Park et al., 2019). Therefore, some exosomal proteins, such as integrins support tissue distribution while others, such a Cxs, sustain the docking to target cells.

Within endocytosis several mechanisms are comprised. One of them is the clathrin-mediated endocytosis which involves cellular internalization of exosomes through the formation of clathrin-coated vesicles. Subsequently, these vesicles deform the membrane and generate a vesicular bud that pinch off the PM by dynamin, undergo clathrin un-coating and fuse with endosomes of the target cell (McMahon and Boucrot, 2011).

Phagocytosis is an actin-dependent mechanism which also mediate the internalization of exosomes. This process involves several receptors such as Toll-like (TLRs), scavenger, and complement receptors, which recognize the exosome, rearrange the cytoskeleton and form cup-shaped invaginations around them. Several proteins involve in phagocytosis are also necessary for exosome uptake. This is the case of the T cell immunoglobulin and mucin domain containing 4, which is used by macrophages to recognize and phagocytose exosomes (Feng et al., 2010).
Figure 12. Exosome uptake by a recipient cell. Exosomes are released from the parent cell’s MVBs in the extracellular space and interact with the recipient cell through endocytosis (clathrin-mediated, caveolin-mediated, phagocytosis, lipid raft-mediated, macropinocytosis) or fusion. Figure designed by Soraya Moradi Bachiller.

Macropinocytosis also contributes to exosome endocytosis. It happens when cytoskeleton rearranges and causes PM ruffling. This mechanism requires the participation of growth factors (Grimmer et al., 2002; Nakase et al., 2015).

Exosome endocytosis can also occur in lipid-raft regions at the target PM and independently of clathrin. They are independent of clathrin but might be caveolin-dependent since in the
colocalization of exosomes and lipid-rafts, caveolin proteins (such as caveolin-1) are sometimes needed to produce membrane invagination (Escrevente et al., 2011; Nanbo et al., 2013).

3.6. Exosome functions. Role in cell-to-cell communication

Exosomes were initially considered as cellular garbage bags able to discard unnecessary material out of the cell. However, it has become more obvious that exosome packaging is not a random process as it changes depending on the environmental and cellular conditions. Even if their content change depending on what the cell is undergoing, there are some proteins that are always enriched in exosomes. Probably, because they are implicated in their biogenesis and trafficking. In addition, their content is transferred to a specific cellular target/tissue to elicit a phenotypic response, which clearly reflects their role in intercellular communication (Saeedi et al., 2019).

Exosomes have been implicated in a broad range of functions in health and disease conditions where they make possible the communication among cells. However, most of their functions have been described in pathological conditions. For example, in cancer, exosomes promote metastasis (Peinado et al., 2012), tumorigenesis (Melo et al., 2014), tumor growth (Maziveyi et al., 2019), drug resistance (Hu et al., 2019) or tumor angiogenesis (Zeng et al., 2018). Exosomes also regulate and mediate autophagy after myocardial infarction (Santoso et al., 2020) or cardioprotection in sepsis (Wang et al., 2015). They can also trigger immune responses (Kitai et al., 2017) by disseminating viruses (Gu et al., 2020; Jiang et al., 2020) or transferring antigens (Vallhov et al., 2015).

Beyond their role in pathological conditions, exosomes are also involved in immunosuppressive responses by modulating the activity of natural killer and phagocytic cells. This is a well-known
described function for seminal exosomes that allows them to contribute to a successful fertilization (Vojtech et al., 2014). Not only exosomes but EVs in general seem to influence the embryo implantation, foetus-maternal immune tolerance and pregnancy success (Kurian and Modi, 2019). In several mammals, breast-milk exosomes also have potential beneficial effects as they are a source of immune-related properties that can be transferred to the new-born (Gu et al., 2012).

All these functions, either in physiological or pathological conditions, are due to the specific exosomal content and the ability these sEVs have to target specific cells and tissues in short and long distances.

### 3.7. Exosomes in Alzheimer’s disease pathophysiology

Several studies suggest that exosomes have a key role in the spreading of misfolded proteins; such as tau (Y. Wang et al., 2017), Aβ or α-synuclein (Ngolab et al., 2017), which are the core proteins of some NDs. In the context of AD pathogenesis, exosomes were first studied to understand the spreading of Aβ and tau proteins. Rajendran et al. were the first to demonstrate that β-secretase cleavage of APP occurs inside early endosomes in HeLa and the neuroblastoma cell line N2a, releasing Aβ peptides within exosomes. The authors also found that exosomal proteins accumulate in amyloid plaques. These data suggested, for the first time, a role for exosomes in AD (Rajendran et al., 2006). APP, APP-CTFs, β- and γ-secretases are also associated with exosomes, confirming that early and late endosomes are a site for APP cleavage (Sharples et al., 2008). After endolysosomal disruption, APP-CTFs are able to accumulate in exosomes (Miranda et al., 2018). These exosomes are able to bind to neurons or be engulfed by other specific recipient cell (Laulagnier et al., 2018). Moreover, in the acceptor cell, APP
products-bearing exosomes cause cytotoxicity and apoptosis and, consequently, spread the pathology (Sardar Sinha et al., 2018).

However, brain exosomes are not only able to spread disease-related proteins. They are also able to impair neuronal function, increasing mitochondrial damage (Elsherbini et al., 2020) or apoptosis. This last is the case of ceramide-containing exosomes whose secretion by astrocytes increases after exposure to Aβ(25-35). These exosomes are, then, captured by other astrocytes and cause apoptosis (G. Wang et al., 2012).

The evidence that tau is secreted in exosomes came in 2012. The authors of this study demonstrated that exosomes isolated from CSF samples of AD subjects contain p-tau, suggesting that this protein is secreted via exosome release (Saman et al., 2012).

In the spreading of tau, following the Braak stages along the brain, several mechanisms have been proposed. One of these secretory pathways of tau protein is the one involving exosomes. In fact, this has already been demonstrated in neurons, cultured cells (Y. Wang et al., 2017) and microglia (Asai et al., 2015).

Beyond the harmful effects, exosomes from healthy cells might have a protective function in AD pathogenesis. This is the case of neuronal exosomes enriched in glycosphingolipids, which induce clearance and uptake of Aβ by the microglia and, thus, decrease amyloid deposition (Yuyama et al., 2014, 2015). The same happens with human umbilical cord mesenchymal stem cells-derived exosomes, which may clear Aβ deposition by modulating the activation of microglia (Ding et al., 2018) or with exosomes derived from N2a cell line that are able to neutralize synaptic disruptions induced by Aβ (An et al., 2013).

Given these previous studies, it is clear that exosomes have a role in AD pathogenesis. A role that might be protective or harmful. Maybe this “double-edged sword” that define exosomes is
the reflect of the imbalance metabolism of some proteins, and the endosomal and autophagic
dysfunction in AD that lead to an unsuccessful degradation of these proteins (Arbo et al., 2020).
When the accumulation of toxic proteins goes beyond the clearance capacity of degradative
pathways of neuronal cells, they increase their secretion within exosomes and, at the same time,
spread the pathology.

3.7.1. Exosomes as biomarker carriers for the early detection of Alzheimer’s disease

Exosomes contain biological material which is selectively packaged. This cargo can change depending on the cellular and environmental features. This luminal cargo is protected from degradation (because of the membranous structure of an exosome) and is highly stable in storage conditions (Akuma et al., 2019). In AD, exosome-associated biomarkers may provide an early and accurate diagnosis before the symptoms onset (Kapogiannis et al., 2019). This allows them to reflect the disease progression from the early beginning. Moreover, since exosomes are able to cross the BBB (Alvarez-Erviti et al., 2011), they will be naturally present in biological fluids such as blood, eliciting a cellular response, also, at long distances. And biological fluids are abundant and easily accessible, allowing the repeatedly sampling collection needed in longitudinal studies. In addition, with regard to blood; that is, compared to the whole biofluid, exosomes are less complex. When we isolate sEVs, the complexity of the sample is reduced because we are depleting the sample of high abundant proteins in blood, such as albumin. And this helps in the identification and validation of biomarkers (Boukouris and Mathivanan, 2015).

In fact, blood/plasma and serum exosomes, derived from different cellular sources, have been shown to carry AD-related proteins and miRNAs. Most of these studies isolated neuron-derived
exosomes (NDE) or astrocyte-derived exosomes (ADEs) from plasma, and measured the concentration of proteins and miRNAs using ELISA, high-throughput next-generation sequencing (NGS) or quantitative reverse transcription polymerase chain reaction (qRT-PCR). This is the case of the most AD-related proteins P-S396-tau, P-T181-tau and Aβ(1-42). Fiandaca et al. isolated plasma NDEs where these three proteins showed higher concentration in AD than in controls, and in preclinical AD subjects up to 10 years before the clinical diagnosis (Fiandaca et al., 2015). These results were corroborated in AD and in MCI-AD in another cohort of subjects (Winston et al., 2016). The same year, Goetzl et al. isolated plasma NDEs and ADEs from AD, FTD and control subjects. They also measured the concentration of P-T181-tau, P-S396-tau and Aβ(1-42) and showed that levels of all of them were higher in ADEs than in NDEs. The same result was obtained in NDEs (higher in AD compared to control subjects). Instead, levels of Aβ(1-42) in ADEs were lower in AD subjects than in controls. The authors of this study also showed that levels of BACE1 and sAPPβ were higher within ADEs of AD subjects with regard to controls, while levels of glial-derived neurotrophic factor were lower. Also, ADE levels of septin-8, a protein implicated in synaptic vesicular trafficking and plasticity, were significantly lower in patients with AD compared to controls (Goetzl et al., 2016b). From these AD-related biomarkers, blood NDE-derived Aβ(1-42), t-tau and P-T181-tau concentrations correlate well with levels of these proteins found in CSF and are able to distinguish AD and amnestic MCI subjects from controls (Jia et al., 2019).

Concentration of synaptic proteins in NDEs have been also assessed in AD and MCI subjects. Winston et al. demonstrated decreased levels in MCI subjects-derived plasma NDEs of Ng, synaptophysin, synaptotagmin and synaptopodin (Winston et al., 2018). Also, in plasma NDEs, the presynaptic proteins neuronal pentraxin 2 and neurexin 2α, with their respective postsynaptic
partners’ glutamate ionotropic receptor AMPA type subunit 4 and neuroligin 1 have been studied. All of them showed a decreased concentration in dementia due to AD. In the preclinical period, that authors defined as the 6-11 years-period before the onset of dementia, all except neuronal pentraxin 2 showed significantly lower concentrations than in controls (Goetzl et al., 2018). In another study, plasma NDE levels of synaptophysin, synaptopodin, synaptotagmin-2, growth-associated protein 43, synapsin 1 and Ng had decreased concentrations in AD than in controls. In the preclinical period, synaptotagmin, synaptophysin, Ng, growth-associated protein 43 and synaptopodin were already decreased years before dementia (Goetzl et al., 2016a). SNAP25 is another synaptic protein whose concentration has been studied in serum NDEs of AD and healthy controls. This protein showed lower concentration in AD patients compared to controls, probably reflecting the altered synaptic integrity in AD brain (Agliardi et al., 2019).

Inflammation also plays an important role in AD and this could be reflected in levels of complement proteins. In another study, concentrations of C1q, C4b, factor D, fragment Bb, C5b, C3b and C5b-C9 were measured in plasma ADEs. All of them showed higher levels in MCI subjects converting to dementia than in those that do not progress to dementia. Instead, the decay-accelerating factor, CD46, CD59 and CR1 were lower in subjects with MCI converting to dementia (Winston et al., 2019).

As well as inflammation, degradative pathways are also key in AD, since they allow the correct clearance of toxic proteins. Lysosomal proteins involved in these pathways, cathepsin D, lysosomal-associated membrane protein 1 and ubiquitinated proteins, are also carried in plasma NDEs and are able to distinguish AD subjects from controls (Goetzl et al., 2015).

On their behalf, miRNAs have also been studied within exosomes in plasma. Plasma exosomal miRNAs show higher interest than non-exosomal miRNAs, since their signal in whole blood is
highly diluted. Moreover, exosomal miRNAs can be measured and might provide a disease-specific diagnostic profile for a given dementia-related disorder and its various stages (Barbagallo et al., 2020; Gámez-Valero et al., 2019; T. T. Yang et al., 2018). For example, Cheng et al. profiled serum exosome miRNAs and determined a set of them (miR-101-3p, miR-106a-5p, miR-106b-5p, miR-1306-5p, miR-143-3p, miR-15a-5p, miR-15b-3p, miR-18b-5p, miR-20a-5p, miR-30e-5p, miR-335-5p, miR-342-3p, miR-361-5p, miR-424-5p, miR-582-5p and miR-93-5p) differentially expressed (DE) between cognitively healthy and AD subjects. All these exosomal miRNAs were discovered using NGS and later validated with qRT-PCR (Cheng et al., 2015). Cha et al., instead, investigated plasma NDE miRNAs and showed a decrease in miR-212 and miR-132 concentrations in AD subjects with regard to controls (Cha et al., 2019). In another study, serum exosomal miR-135a and miR-384 were upregulated while miR-193b was downregulated in AD individuals compared to controls (T. T. Yang et al., 2018).

Unlike exosomal proteins, there is no consensus for any miRNA able to define the causality of AD or to follow its progression. Maybe, a panel of pathogenic-associated miRNA family combined with a disease-associated protein profile could improve the diagnostic value of both biomolecules in AD. Plasma and serum are better and more accessible sources of exosomal miRNA and protein biomarkers than CSF. However, it remains unclear which is the best source of exosomes for AD-related biomarkers; total plasma exosomes, NDEs or ADEs? And even most relevant, which neural proteins are contained in NDEs?
Chapter IV
CHAPTER IV

AIMS OF THE STUDY

AD is one of the most common NDs and the most frequent cause of dementia. Although new strategies for its early diagnosis and treatment are currently under investigation, AD is still characterized by its delayed diagnosis and high burden of mortality.

Nevertheless, the discovery of sEVs as mediators of intercellular communication and potential biomarker carriers open up a new field of research for an accurate and early diagnosis of AD and for monitoring its progression.

4.1. General hypothesis and aim

The general hypothesis of this PhD thesis is that sEVs are a potential source of candidate AD biomarkers (proteins and miRNAs) which vary their concentration within sEVs reflecting or anticipating AD progression and cognitive deficits in humans and 3xTg-AD mice.

Therefore, the aim of this study is to discover new candidate sEVs-associated biomarkers which can discriminate MCI from cognitively normal subjects, and 3xTg mice from their matched control strain. Moreover, with the aim of pursuing a low-invasive biomarker discovery strategy, the source of sEVs-biomarkers is plasma.

4.2. Specific aims and objectives

- To ascribe a specific biomarker to a subtype of EVs (in our case sEVs), it is necessary to separate them and be sure that what we have in our sEVs-enriched preparations are mostly sEVs.

Therefore, in chapter VI, we specifically aim to characterize our mouse and human plasma sEVs-enriched preparations with already validated techniques.
- Based on the previous hypothetically evidence that ECSIT protein is involved in AD, we address, in chapter VII, the possible role of this protein as specific sEVs-biomarker of cognitive impairment onset in 3xTg-AD mice and MCI subjects. To that end, we will study ECSIT protein in human (Ctrl, MCI and AD) and mouse (NTg and 3xTg) plasma sEVs-enriched preparations.

- Core (Aβ and tau) and candidate biomarkers have already been identified within sEVs in the field of dementia. TREM2 and NFL proteins have an important role in neurodegeneration and inflammatory responses and they have already been reported to change over AD progression in body fluids. However, to our knowledge, they have been never studied within sEVs in AD. Therefore, in chapter VIII, we aim to elucidate whether TREM2 and NFL proteins are released within sEVs in MCI subjects and 3xTg-AD mice, whether they are specifically associated with sEVs as biomarkers and whether their levels within sEVs reflect the cognitive deficit onset. To do so, we will measure NFL and TREM2 proteins in human (Ctrl, MCI and AD) and mouse (NTg and 3xTg) plasma sEVs-enriched preparations.

- sEV-miRNAs have also been proven to be a potential source of biomarkers for AD. However, a consensus about which of them are released and change specifically within sEVs along the AD progression has not yet been reached. In chapter IX, we aim to explore dysregulated sEV-miRNAs in MCI and demented subjects by measuring their levels in human plasma.

- In chapter X, we aim to confirm the link of ECSIT protein with neurodegeneration by studying its subcellular localization and how APP and Aβ peptides affect this localization. To do that, we will investigate ECSIT protein in the H4 cellular model for AD.
Chapter V
CHAPTER V

MATERIALS AND METHODS

5.1. The triple-transgenic mouse model for Alzheimer’s disease

The 3xTg mouse model for AD contains three overexpressed transgenes (APP Swedish, MAPT P301L and PSEN1 M146V) (Oddo et al., 2003), whose effects are restricted to the central nervous system. These three mutations give rise to plaque and tangle pathology. Strain-matched non-transgenic (NTg) mice were used as control line.

In every experiment carried out in the time line from 3 to 10 months of age, two groups of mice were included: 36 3xTg mice and 32 NTg mice. Experiments were concluded during the 10th month with the final number of 14 3xTg and 9 NTg females, and 19 3xTg and 20 NTg males. Animals were housed in groups of two up to five per cage and in a specific pathogen-free animal facility. They were fed with standard diet and tap water was available ad libitum.

5.2. Novel object recognition test for memory assessment

To test mice and their memory, an open-square grey arena (40 x 40 cm) was used. Firstly, in the habituation phase, mice explored the arena for 5 minutes (min). 24 hours (h) later, mice explored two identical objects for 10 min in the familiarization phase. And in the trial phase (when other 24 h passed), one of the familiar objects was replaced by the new object, and mice explored for 10 min. Memory was expressed as the discrimination index (D.I.) ((time spent with the novel object (seconds, s) - time spent with the familiar object (s)) / total time exploring (s)).

NTg and 3xTg mice were divided in sub-groups from 7 up to 9 mice. These sub-groups performed the novel object recognition test (NORT) once a month from 3 months to 10 months of age (time point 0 to time point 7, T0 - T7). The same sub-group of mice did not perform NORT for two consecutive months.

5.3. Collection and pre-processing of mouse plasma samples

The plasma collection from the vena facialis of 3xTg and NTg mice with a sterile lancet (Goldenrod Animal Lancet GR 5 mm and 5.5 mm, MEDIponit International, Inc) was performed once a month in the morning between 8:00 and 11:00 am, starting at 3 months of age till 10 months (T0 - T7).

We collected 250 µL of blood in K2/EDTA tubes (BD, #365975). Blood samples were not placed on ice at that moment (to avoid platelets activation and possible haemolysis) and were processed within the hour. Only after collection of blood droplets, samples were twisted up and down.
Samples were then centrifuged at 1500 g for 10 min at 15°C to separate the plasma fraction. The second one at 3500 g for 10 min at 4°C to separate additional cell debris from plasma. Plasma samples were then stored at -20°C during 1 h and then, at -80°C until analyzed.

5.4. Mouse plasma-sEVs separation

Ca²⁺- and Mg²⁺-free phosphate-buffered saline (particle-free PBS 1X, Thermo Fisher, #14190-094) was added to 30 µL of mouse plasma to 1 mL volume and mixed with 1% (v/v) protease and 1% (v/v) phosphatase inhibitor cocktails (Merck, #P8340 and #P5726, respectively). Plasma samples were mixed for 10 min at room temperature to allow the action of proteases and phosphatases.

Samples were then centrifuged twice at low speed, at 200 g for 10 min at 4°C and at 1200 g for 30 min at 4°C. Supernatants were centrifuged at 10000 g 1 h at 4°C twice and ultracentrifuged (Optima Max Beckman Coulter ultracentrifuge and TLA55 rotor) at 110000 g for 1 h 20 min at 4°C. This last step was repeated, washing the sEVs-containing pellet with particle-free PBS 1X.

sEVs-containing pellets obtained after the ultracentrifugation were resuspended in (a) 20 µL particle-free PBS 1X and lysed with 30 µL lysis buffer (50 mM Tris-HCl (Carlo Erba Reagents, #489983), 150 mM NaCl (Carlo Erba Reagents, #479687), 50 mM EDTA (Sigma, #ED4SS) and 1% Triton X-100 (Fisher Scientific, #BP151-100) at pH 7.4) for Western blotting; in (b) 20 µL particle-free PBS 1X for nanoparticle tracking analysis (NTA) (PBS 1X was added until the appropriate particles per frame (PF) count was reached); or in (c) 50 µL 2% glutaraldehyde or in 50 µL 2% paraformaldehyde and 2.5% glutaraldehyde (Sigma-Aldrich, #G5882) in particle-free PBS 1X for transmission electron microscopy (TEM).


5.5. Human subjects’ recruitment

This study included subjects consecutively recruited at Mendrisio and Lugano Regional Hospital (Switzerland). Subjects were classified into AD group (neurodegenerative dementia group) when the CDR score was ≥ 1, MCI group when CDR = 0.5, and Ctrl group when CDR = 0 (cognitively normal subjects). The design of the present study was approved by relevant local ethics committee of Mendrisio and Lugano Regional Hospital and, the written informed consents were obtained from all the patients. Specific demographic and clinic information about age, sex, education, CDR, Hachinski Ischemic Score (HIS) and MMSE test were also collected and provided in each chapter considering the human subjects included. Two exclusion criteria were followed. Participants should be older than 55 years (inclusive) and younger than 90 years (inclusive). Also, HIS should be ≤ 4.

5.6. Collection and pre-processing of human plasma samples

Ten ml of peripheral blood were collected in the morning and in fasting conditions from each subject. Blood volume was collected in EDTA tubes. Samples were centrifuged at 1500 g for 10 min at 4°C to separate plasma. Plasma samples (700 µL/aliquot) were stored at -80°C until required.

5.7. Human plasma-sEVs separation

We sampled 700 µL of human plasma and we completed them with particle-free PBS 1X up to 1 mL and supplied with 1% (v/v) protease and 1% (v/v) phosphatase inhibitor cocktails. Then, sEVs were separated as described for mouse plasma-sEVs. SEVs-containing pellets were
resuspended as for mouse for Western blotting, NTA and TEM. For structured illumination microscopy (SIM), pellets were resuspended in 500 µL of 4% paraformaldehyde (Carlo Erba, #XA11313) in particle-free PBS 1X and, for ELISA, sEVs pellets were resuspended in 100 µL lysis buffer.

5.8. Structured illumination microscopy

*The setting up of the protocol and the preparation of sEVs for structured illumination microscopy was performed by me. The visualization of sEVs and the creation of the ImageJ algorithm was done by Stefano Fumagalli.*

Plasma sEVs from cognitively normal subjects (Ctrl), MCI and demented (AD) subjects, were separated using the protocol described above. The sEVs-containing pellet was resuspended in 500 µL 4% paraformaldehyde solution for 20 min at room temperature. SEVs were washed with the blocking solution for 1 h at 4ºC and incubated overnight with the primary antibody (L1CAM). Negative controls were incubated with particle-free PBS 1X.

Between the blocking solution, the primary antibody and the secondary antibody steps, two ultracentrifugation steps at 110000 g lasting 1 h 20 min were done. The first one, to pellet the sEVs and the second one, to wash the sEVs-containing pellet with particle-free PBS 1X. We then resuspended the pellet in 20 µL particle-free PBS 1X.

Ten µL of the sEVs-enriched preparation were mounted with ProLong™ Gold antifade reagent (Invitrogen, #P36930). SEVs were then imaged at laser excitation of 488 nm with a 3D-SIM acquisition protocol. Two fields per condition were acquired with the Nikon SIM system with a 100x 1.49 NA oil immersion objective, managed by NIS elements software. Raw and
reconstructed images were validated with the SIM check plugin of ImageJ. SIM images were analyzed using an ImageJ algorithm originally designed to select L1CAM+ sEVs. For each identified sEV, a line passing at its centre was traced to calculate a pixel grey value histogram and measure particle size with the full width at half maximum method. All the primary and secondary antibodies are listed in **Table 3**.

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal anti-L1CAM</td>
<td>Abcam, #ab24345</td>
<td>1:100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibodies</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-Mouse IgG (H+L) Highly</td>
<td>Invitrogen, #A11001</td>
<td>1:500</td>
</tr>
<tr>
<td>Cross-Adsorbed Secondary</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody, Alexa Fluor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. SIM. Primary and secondary antibodies.**

### 5.9. Transmission electron microscopy

*The preparation of sEVs for transmission electron microscopy was done by me. The protocol was provided and performed by Nicolò Baranzini. Images were acquired by Annalisa Grimaldi.*

Human plasma sEVs were fixed with 2% glutaraldehyde in particle-free PBS 1X. A drop of the suspension (10 µL) was applied on 300 mesh formvar copper-coated grids (Pacific Grid Tech) and let them floated for 10 min. Samples were washed with milliQ water and stained with uranyl acetate (Fisher Scientific, #NC1375332) for eight min and then washed again in milliQ water. Finally, they were observed under a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan). Data were recorded with a MORADA digital camera system (Olympus, Tokyo, Japan).
5.10. Double immunogold labelling with transmission electron microscopy

*The preparation of sEVs for transmission electron microscopy was done by me. The protocol was provided and performed by Nicolò Baranzini. Images were acquired by Annalisa Grimaldi.*

Isolated sEVs were fixed in Karnovsky’s fixative (2% paraformaldehyde and 2.5% glutaraldehyde). Five µL of the eluted sEVs were transferred onto a 300 mesh formvar-carbon coated electron microscopy grids (Pacific Grid Tech). After 10 min, grids were placed on 50 µL particle-free PBS 1X drops with the sample membrane side facing down. Then, they were incubated for 30 min in the blocking solution containing particle-free PBS 1X, 1% bovine serum albumin (BSA) (Euroclone, #APA13910050), and 0.1% Tween-20 (Thermo Fisher, #BP337-100). Subsequently, samples were incubated with the polyclonal primary antibodies (rabbit anti-ECSIT and mouse anti-CD63) diluted in the blocking solution for 1 h. Negative controls were incubated with particle-free PBS 1X. Particle-free PBS 1X was used to wash the samples at least 10 times. Then, primary antibodies were visualized by immunostaining with goat anti-rabbit (particle size 10 nm) and the goat anti-mouse (particle size 6 nm) gold-conjugated secondary antibodies diluted in the blocking solution for 45 min. Samples were then counterstained with uranyl acetate (Fisher Scientific, #NC1375332) for 8 min and washed in milliQ water. Finally, they were observed under a Jeol 1010 EX electron microscope (Jeol, Tokyo, Japan). Data were recorded with a MORADA digital camera system (Olympus, Tokyo, Japan). All primary and secondary antibodies are listed in Table 4.
Table 4. Double immunogold labelling with TEM. Primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse monoclonal anti-CD63</td>
<td>Thermo Fisher, #10628D</td>
<td>1:40</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-ECSIT</td>
<td>Abcam, #ab21288</td>
<td>1:40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibodies</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat anti-rabbit IgG-gold conjugate antibody</td>
<td>Abcam, #ab27234</td>
<td>1:100</td>
</tr>
<tr>
<td>Goat anti-mouse IgG-gold conjugate antibody</td>
<td>Abcam, #ab39614</td>
<td>1:100</td>
</tr>
</tbody>
</table>

5.11. Nanoparticle tracking analysis

The preparation of sEVs for nanoparticle tracking analysis was performed by me. The protocol was performed by Miriam Ciani and Roberta Zanardini.

SEVs-enriched preparations were analyzed using NanoSight NS300 instrument (Malvern, Worcestershire, UK). Prior to analysis, sEVs-enriched pellets were diluted in particle-free PBS 1X to obtain a range of 20 to 200 PF in the field of view, and introduced into a chamber with a syringe. A laser beam of 488 nm was applied to the sEVs-enriched preparations and five 60 s-videos were recorded at camera level 15 and detection threshold 5. Data were processed using NanoSight NTA Software 3.2. Particle movement was analyzed by NTA software 3.2 version. Particle size distribution (D-values; D10, D50 and D90, a particle size value indicating that, respectively, 10%, 50% and 90% of the distribution is below this value) and the concentration (particles/mL) were the data obtained for each sample. Biological replicates for each condition with less than 20 or more than 200 PF were excluded. At least 3 biological replicates were...
analyzed for each condition (Ctrl, MCI and AD in human, and 3xTg and NTg from T0 - T7 in mouse).

5.12. SEVs-free fraction or sEVs-depleted pellet

In both mouse and human plasma, pellets obtained after the centrifugation step at 10000 g, were kept and washed with 500 µL particle-free PBS 1X. This suspension was centrifuged again at 10000 g for 1 h at 4ºC. The pellet obtained was called the sEVs-depleted pellet and, together with the sEVs-enriched preparations, was used to demonstrate the presence or the absence of typical (CD63, L1CAM, Tsg101) and non-typical (GM130, Albumin) sEVs markers.

5.13. Western blotting

Protein extracts, supplemented with 1 % (v/v) protease and 1 % (v/v) phosphatase inhibitor cocktails, were quantified using the bicinchoninic acid assay kit (Thermo Fisher #23227). Samples were stored at -80ºC until use. Equal amounts of proteins were loaded, separated on sodium dodecyl sulphate (SDS) polyacrylamide gels, and transferred onto a nitrocellulose membrane (BioRad, #162-0113). After blocking the membrane with 5% BSA, blots were incubated overnight at 4ºC with the appropriate primary antibody. Finally, HRP-conjugated secondary antibody for 1 h at room temperature was used.

Signal was developed by the Immobilon Western HRP (Millipore, #1909801) and detected with ChemiDoc Imaging System (BioRad). Densitometric analysis were performed using ImageJ. All the primary and secondary antibodies are listed in Table 5.
### Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody Description</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit monoclonal anti-CD63</td>
<td>Abcam, #ab134045</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit monoclonal anti-CD63</td>
<td>Abcam, #ab217345</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-L1CAM</td>
<td>Abcam, #ab24345</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-GM130</td>
<td>BD, #610822</td>
<td>1:250</td>
</tr>
<tr>
<td>Mouse monoclonal anti-albumin</td>
<td>Santa Cruz Biotechnology, #sc-271605</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-ECSIT</td>
<td>Abcam, #ab21288</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse monoclonal anti-Tsg101</td>
<td>Abcam, #ab83</td>
<td>1:1000</td>
</tr>
<tr>
<td>6E10</td>
<td>BioLegend, #803001</td>
<td>1:1000</td>
</tr>
<tr>
<td>Vinculin</td>
<td>Sigma-Aldrich, #V9264</td>
<td>1:5000</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-VDAC</td>
<td>Thermo Fisher, #PA1-954A</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit polyclonal anti-H3</td>
<td>Abcam, #ab1791</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit monoclonal anti-NFL</td>
<td>Cell Signaling, #C28E10</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit monoclonal anti-TREM2</td>
<td>Cell Signaling, #D8I4C</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

### Secondary Antibodies

<table>
<thead>
<tr>
<th>Antibody Description</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase-AffiniPure Goat Anti-Mouse IgG (H+L)</td>
<td>Jackson ImmunoResearch, #115-035-003</td>
<td>1:10000</td>
</tr>
<tr>
<td>Peroxidase-AffiniPure Goat Anti-Rabbit IgG (H+L)</td>
<td>Santa Cruz Biotechnology, #sc-2357</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 5. Western blot. Primary and secondary antibodies.
5.14. Enzyme-linked immunosorbent assay

SEV were assayed for ECSIT, L1CAM, TREM2 and NFL proteins. H4 cell culture medium was assessed for Aβ(1-42). ELISAs were performed following the manufacturer’s instructions. Information regarding each ELISA kit is provided in Table 6.

<table>
<thead>
<tr>
<th>Protein assayed</th>
<th>Supplier</th>
<th>Sensitivity limit</th>
<th>Detection range</th>
<th>Intra-assay CV</th>
<th>Inter-assay CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECSIT</td>
<td>MyBiosource, #MBS9321617</td>
<td>0.1 ng/mL</td>
<td>0.625 - 20 ng/mL</td>
<td>&lt; 15%</td>
<td>&lt; 15%</td>
</tr>
<tr>
<td>L1CAM</td>
<td>RayBiotech, #ELH-L1CAM</td>
<td>0.8 pg/mL</td>
<td>0.819 - 200 pg/mL</td>
<td>&lt; 10%</td>
<td>&lt; 12%</td>
</tr>
<tr>
<td>TREM2</td>
<td>MyBiosource, #MBS3801324</td>
<td>1.0 pg/mL</td>
<td>0 – 240 pg/mL</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NFL</td>
<td>MyBiosource, #MBS7200828</td>
<td>1.0 pg/mL</td>
<td>0 – 500 pg/mL</td>
<td>&lt; 10%</td>
<td>&lt; 10%</td>
</tr>
<tr>
<td>Aβ(1-42)</td>
<td>IBL International GMBH, #JP27711</td>
<td>4.03 pg/mL</td>
<td>12.5 – 800 pg/mL</td>
<td>&gt; 3%</td>
<td>&gt; 5%</td>
</tr>
</tbody>
</table>

Table 6. ELISA kits. The protein assayed, supplier, sensitivity limit, detection range, intra- and inter-assay CV are provided. CV: coefficient of variation.

5.15. MicroRNA isolation from human plasma-sEVs

Human plasma-sEVs were separated as already described and resuspended in 20 µl particle-free PBS 1X. The Maxwell® RSC miRNA Plasma and Serum Kit (Promega, #AS1680) was used to isolate microRNAs from the human plasma sEVs-enriched preparations according to the manufacturer’s instruction.
5.16. Library preparation and RNA-sequencing

The library preparation for RNA-sequencing was performed by Ilaria Craparotta.

For miRNA quantification the QuantiFluor ONE dsDNA System quantification (Promega, #E4871) was used according to manufacturer’s protocol. It was not possible to dose miRNA samples. Therefore, 5 µl of each sample were useful for library preparation.

The QIAseq miRNA library kit (Qiagen, #331502), the QIAseq miRNA NGS 12 Index IL (Qiagen, #331592), the PhiX Sequencing Control Kit v3 (Illumina, #FC-110-3001) and the NextSeq 500/550 High Output Kit v2.5 (75 cycles) (Illumina, #20024906) were used, following the manufacturer’s instructions, to prepare the library and to perform the single-end miRNA sequencing.

Elutes miRNAs were also studied for integrity and quality by running an screentape using the Agilent D500 TapeStation, according to the manufacturer’s instructions (Agilent, #5067-5593 and #5067-5592).

5.17. MicroRNA data processing

The processing of microRNA data was performed by Óscar González Velasco.

Data was processed by using the cutadapt tool (Martin, 2011) for the 3’ and the 5’ adapter removal. Low quality reads were filtered out using the same tool.

Bowtie 2 was then used (http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml#bowtie2-options-local) to align short reads to long reference sequences. MiRBase 22 (this miRNA database contains all known human and mature miRNA sequences, http://www.mirbase.org/) was used as the reference sequence.
Bowtie 2 tool looked for multiple alignment, but only the best one was reported. We used the end-to-end alignment with only one mismatch allowed. The former means that the alignment between the read and the reference sequence involves all the ending characters of the read sequence. This type of alignment is also called “untrimmed”. And the latter means that since the alignment was untrimmed, if a mismatch exists, it should be positioned in the middle of the reference sequence.

5.18. MicroRNA reads quantification

*The microRNA reads quantification was done by Óscar González Velasco.*

The quantification of miRNA reads was done using SAMTOOLS. This tool reports each mapped read to a miRNA as a valid count read while discarding reads that mapped in more than one region of the reference sequence. In this way, SAMTOOLS obtains the final raw reads (counts).

5.19. Differential expression analyses

*The differential expression analyses were done by Óscar González Velasco.*

The differential expression analysis for sEVs-miRNAs among the Ctrl, MCI and AD conditions were done using DESeq2 tool. DESeq2 works with raw reads and fits negative binomial linear models for each miRNA. It uses the Wald test for significance testing and it adjusts the p values for multiple testing using the procedure of Benjamini and Hochberg (Love et al., 2014).

DESeq2 provides the following output:
- BaseMean. It is the average of the normalized counts over all miRNA samples.
- Log2FoldChange. The log2FoldChange is provided between two conditions (in our case, Ctrl vs AD and Ctrl vs MCI). It is the effect size. It tells us how much the miRNA expression seems to change in MCI or AD compared to the Ctrl condition.
- LfcSE. It is the standard error of the log2FoldChange estimate between two conditions.
- Stat. It is the Wald statistic.
- P value. It is referred to the Wald test p value.
- NA. This reports that all counts for a miRNA were 0, and hence, no test was applied. P values can be also assigned NA if the miRNA contained an extreme count outlier and, therefore, it was excluded from the analysis.

The differential expression analysis for miRNAs in serum was obtained using the gamma correlation measure (Goodman and Kruskal, 1954). This measure compares miRNA expression throughout a set of ordered stages (in our case, Ctrl, MCI and AD subjects) and calculates, for each correlation, the corresponding p value based on cross-validation with 1000 iterations. We used the Goodman-Kruskal gamma correlation in which gamma values vary between -1 and +1. A positive gamma means a positive correlation between expression (up-regulation between stages) and the stages order (in our case, more expression is correlated with a more advanced disease stage). The opposite applies for negative gamma values. The gamma mean values were obtained by bootstrapping.
5.20. MRNA target selection and gene ontology analysis of sEV-microRNAs

MiRNAtap (Pajak and Simpson, 2020) and miRNAtap.db (Pajak and Simpson, 2016) were used to predict mRNA targets for the dysregulated sEVs-miRNAs.

TopGO was used to identify gene ontology (GO) (Alexa and Rahnenfuhrer, 2020) terms that are significantly enriched in the genes targeted by sEVs-miRNAs.

5.21. Literature search

Searches conducted for miRNA study excluded studies performed in cellular or animal models only, or in human brain tissue. Selected articles did not necessarily include a Ctrl group and used different technologies, such as NGS or RT-qPCR. The searches were initially run from 2004 and were updated including literature up to the beginning of 2020. References listed of the included studies were also evaluated. From the 58 accepted manuscripts, we recorded miRNAs that were identified as significantly dysregulated.

5.22. Network visualization

The mRNA target selection and the visualization of mRNAs-miRNAs interactions were done by Alberto Berral González.

MiRNAtap and miRNAtap.db were used to select the mRNA targets for each sEVs-miRNA. Cytoscape and the Cytoscape app aMatReader (Settle et al., 2018) were used to visualize miRNA-mRNA interactions.
5.23. Neuroglioma cell line (H4 cells)

The neuroglioma cell line (H4 cells) was used as the cellular model for AD. H4-SW cells overexpress the APP gene with the Swedish double mutation 670KM/671NL, leading to an increase cleavage of APP by β-secretase. This mutation allows this cellular model to secrete elevated amounts of Aβ(1-40) and Aβ(1-42) peptides. Non-transfected neuroglioma cells (H4-Native cells) were used as a control.

5.24. H4-conditioned medium. Culture and harvesting conditions

H4 cells were cultured in 8 ml of OptiMEM medium (Gibco, #51985026) supplied with 10% (v/v) fetal bovine serum (FBS) (Gibco, #10270098) and 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) mix (pen/strep) (Gibco, #15140148). Cell culture medium was sterilized by filtration and stored at 4°C. Cells were cultured in 75 cm² polystyrene flasks (Corning, #3135) at 37°C with a seeding density of 80 x 10⁴ cells/75 cm² flask, in a controlled atmosphere with 95% relative humidity and 5% CO₂. Medium was replaced every 2 days. Antibiotics, hygromycin B (Gibco, #10687010, 50 mg/mL) and blasticidin (Sigma-Aldrich, #15205, 5 mg/mL), were added in H4-SW cell culture. H4 cells were grown up to 90% confluence and then incubated in a serum-free culture medium for 48 h. The switch step from FBS-supplied OptiMEM and FBS-depleted OptiMEM occurred without any acclimatization step and two washing steps with 10 mL of particle free PBS 1X.

The conditioned medium (CM) obtained from five 75 cm² polystyrene flasks after 48 h was enriched in sEVs and then collected (1% (v/v) protease inhibitor and 1% (v/v) phosphatase inhibitor were added). Cells were washed with particle-free PBS 1X and then scrapped with 1
mL of lysis buffer supplemented with 1% (v/v) protease and 1% (v/v) phosphatase inhibitors and used as a negative control for the presence of sEVs markers.

5.25. Conditioned medium-sEVs separation

The separation of sEVs from the CM was done using a conventional ultracentrifugation protocol. The first step was performed at 200 g for 10 min at 4°C to discard dead cells. A second step at 1200 g was done for 30 min at 4°C to discard cell debris. Two steps at 10000 g for 1 h at 4°C were performed to discard larger EVs. Then, two final steps at 110000 g for 1 h 20 min at 4°C were done with the Optima L-90K ultracentrifuge (SW32 Ti rotor). The first one to concentrate sEVs. The second one to wash (with particle-free PBS 1X) sEVs and discard the possible contaminants in the sample.

Finally, sEVs were resuspended in 20 µL particle-free PBS 1X for NTA or in 20 µL particle-free PBS 1X and 30 µL lysis buffer for Western blotting.

5.26. Cell proliferation assay

Cell proliferation rate for H4-Native and H4-SW cells was measured in FBS-depleted OptiMEM with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay.

H4 cells were seeded into 96-well plates (seeding density = 4 x 10³ cells/well) containing 100 µL of FBS-supplied OptiMEM. When confluence was reached, H4-cells were washed twice with particle-free PBS 1X and FBS-depleted OptiMEM was added. The supernatant was
removed after 24, 48 and 72 h and changed with 100 µL of 10% MTS (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, #G3580) in cell culture medium. After 3 h incubation at 37°C, absorbance at a wavelength of 490 nm was measured in a microplate reader (Infinite® M200, Tecan) equipped with i-control Microplate Reader software (Tecan).

The average blank values were subtracted to the respective conditions.

5.27. Lactate dehydrogenase release assay

H4 cell death by short hairpin RNAs (shRNAs) was evaluated by measuring the release of lactate dehydrogenase (LDH), which is an indicator of cellular damage. Following experiments of lentiviral transduction, H4 cells were harvested and lysed for protein or RNA extraction. Supernatants (cell culture medium) were collected and LDH release in the supernatant was measured using the LDH kit (Promega, #G1780) following manufacturer’s instructions.

After 30 min incubation at room temperature, absorbance at a wavelength of 490 nm was measured in a microplate reader (Infinite® M200, Tecan) equipped with i-control Microplate Reader software (Tecan).

The average blank values were subtracted to the respective conditions.

5.28. Nuclear and cytoplasmic fractions extraction protocol

The nuclear fraction of H4 cells was prepared as follows. H4 cells were seeded in one 75 cm² polystyrene flask (seeding density = 80 x 10⁴ cells/75 cm² flask). When the 90% confluence was reached, they were washed twice with 10 mL of particle-free PBS 1X and
transferred into 1 ml of fractionation buffer (20 mM HEPES (Millipore, #391338), 10 mM KCl (Carlo Erba, #7447-40-7, 2 mM MgCl₂ (Sigma-Aldrich, #20833-7), 1 mM EDTA (Sigma-Aldrich, #ED4SS) and 1 mM EGTA (Sigma-Aldrich, #E3889) at pH 7.4) by scrapping and left 15 min on ice. The cell suspension was passed 10 times through a 27-gauge needle syringe. It was then left on ice for 20 min and centrifuged at 800 g for 5 min. The pellet contains nuclei and the supernatant contains the cytoplasmic fraction. The pellet was washed and dispersed with 500 µL fractionation buffer. A 25-gauge syringe was used to pass (10 times) the nuclear fraction. This fraction was then centrifuged at 800 g for 10 min. Only the pellet, the fraction that contains nuclei, was kept. This nuclear fraction was resuspended in 100 µL Tris-buffered saline 10X (242.29 g Tris(hydroxymethil)aminomethane (Carlo Erba, #489983), 180 g NaCl (Carlo Erba, #479687), 37% HCl (Carlo Erba, #403872) and distilled H₂O up to 2 L, pH 7.5) with 0.1% SDS 10% (50 g SDS (Sigma-Aldrich, #71729) + 500 mL distilled H₂O) and 1% (v/v) protease and 1% (v/v) phosphatase inhibitors were added. Nuclei were then sonicated. The supernatant instead was centrifuged at 10000 g for 5 min. The pellet was discarded and the supernatant, which is the cytoplasmic fraction, was kept on ice and 1% (v/v) protease and 1% (v/v) phosphatase inhibitors were added.

5.29. Mitochondrial fraction extraction protocol

Mitochondria were isolated from H4 cells using a mitochondria isolation kit for cultured cells (Abcam, #110170) as follows.

Cells were seeded (80 x 10⁴ cells/75 cm² flask) and 90% confluence was reached in four 75 cm² polystyrene flasks for each replicate. Cells were collected in 1 mL of trypsin 1X (Gibco, #15400-054) and centrifuged at 1000 g 5 min at 4°C. Supernatant was discarded and pellet was
resuspended in 400 µL reagent A for 10 min at 4°C. Pellet was homogenized with a potter homogenizer 30 times and centrifuged at 1000 g 10 min at 4°C. The supernatant (called “first supernatant”) was collected into a 2 mL-eppendorf and transferred into ice. Pellet was resuspended in 400 µL reagent B, homogenized 30 times and centrifuged at 1000 g 10 min at 4°C. This last supernatant was added to the first supernatant and was centrifuged at 12000 g 15 min at 4°C. The mitochondria-containing pellet was resuspended in 200 µL of reagent C, supplied with 1 % (v/v) protease and 1% (v/v) phosphatase inhibitor cocktail and stored at -80°C.

5.30. RNA extraction and quantitative reverse transcription polymerase chain reaction

H4-cells were seeded with a density of 80 x 10⁴ cells/75 cm² flask. For each replicate, total cellular RNA was extracted (Promega, #AS1270 and Maxwell® AS2000 instrument) from one 75 cm² flask when 90% confluence was reached.

For each 75 cm² flask, cells were trypsinized with 1 mL of trypsine. Cells were pelleted at 300 g for 5 min, the medium was then removed and 200 µL of chilled 1-thioglycerol/homogeneization solution mix (dilution 1:50) was added. Cells suspensions were vortexed and kept on ice.

To 200 µL of lysed cells, 200 µL of lysis buffer were added. Cell suspensions were vortexed for 15 s and the 400 µL of lysate were transferred to the Maxwell® 16 LEV Cartridges. The rest of solutions, plungers and elution tubes were added and positioned into the cartridges following manufacturer’s instructions. The RNA and its purity were quantified using the NanoDrop® ND-1000 spectrophotometer. Isolated RNA was kept at -80°C until complementary DNA (cDNA) synthesis.
Random primers initiated cDNA synthesis (Mastercycler® ep gradient S) using the high-capacity cDNA reverse transcription kit (Applied Biosystems, #4374966). RT-qPCR was performed (7300 Real Time PCR System, Thermo Fisher Scientific) with SensiFAST Probe Hi-ROX Kit (Meridian Bioscience, #BIO-82005) master mix. Specific primers were synthesized by Thermo Fisher. The real-time qPCR probes are shown in Table 7. The housekeeping gene β-actin was used as a reference. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta C_{T}}$ method. Each experiment was performed at least in triplicate except for the control shRNA lentiviral particles (scrambled shRNA sequence) that was done in duplicate.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Supplier</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECSIT</td>
<td>Thermo Fisher</td>
<td>Hs00933815_m1</td>
</tr>
<tr>
<td>B-actin</td>
<td>Thermo Fisher</td>
<td>Hs99999903_m1</td>
</tr>
</tbody>
</table>

Table 7. The primer sequences for RT-qPCR analysis.

5.31. Knockdown by lentiviral particles

ECSIT shRNA lentiviral particles (Santa Cruz Biotechnology, #sc-77224-V) were used to knockdown ECSIT expression in H4 cells. On day 1, H4 cells were plated with FBS-supplied OptiMEM and antibiotics (hygromycin (50 mg/mL) and blasticidin (5 mg/mL)) in a 6-well plate (seeding density = $10 \times 10^3$ cells/well) 24 h prior to viral infection. Cells were incubated O/N. On day 2, cell culture medium was removed from the plate wells and it was replaced with 2 mL of a mix of FBS-supplied OptiMEM and 5 µg/ml polybrene® (Santa Cruz Biotechnology, #sc-134220). Then, H4 cells were infected with the shRNA ECSIT lentiviral particles or the scrambled shRNA sequence (Santa Cruz Biotechnology, #sc-108080) (10³ infectious units per
virus to the culture medium). On day 3, cell culture medium was replaced with 2 ml of FBS-supplied OptiMEM without polybrene. H4 cells were then incubated for 48 h. On day 5, H4 cells were subcultivated 1:4 and O/N. On day 6, H4 cells containing the shRNA were selected by adding 5 µg/mL puromycin dihydrochloride (Santa Cruz Biotechnology, #sc-108071). On day 9, cell culture medium was replaced and changed with FBS-supplied OptiMEM and puromycin. On day 11, survived H4 cells were recovered for protein or RNA extraction. Cell culture media were also collected to perform Aβ(1-42) ELISA.

5.32. Immunofluorescence studies

*The immunofluorescence studies were performed by Veronica Caddeo.*

H4 cells were seeded (seeding density = 7 x10³ cells/well) onto glass coverslips in 24-well plates (Corning, #353047). They were incubated for 3 days until they reached 90% confluence.

To stain mitochondria, H4 cells were incubated with 100 nM MitoTracker™ Deep Red dye (Invitrogen, #M22426) in OptiMEM for 30 min at 37ºC and washed with particle-free PBS 1X for 5 min. Cells were fixed in 4% paraformaldehyde in particle-free PBS 1X at RT for 25 min, then washed with particle-free PBS 1X for 15 min.

Subsequently, H4 cells were blocked with 300 µL of 1% BSA and 0.25% Triton-X 100 in particle-free PBS 1X for 1 h. Then, they were incubated with the primary antibody (diluted in the blocking solution) O/N at 4ºC. Negative controls were incubated with particle-free PBS 1X.

All the samples were then incubated with the secondary antibody (diluted in the blocking solution). All the primary and secondary antibodies are listed in Table 8. Two washing steps
with particle-free PBS 1X (15 min each) were performed before and after the incubation with the secondary antibodies.

To stain nuclei, Hoechst dye (Invitrogen, #H3569) diluted 1:1000 in particle-free PBS 1X was used.

Finally, the coverslips were transferred onto slides and mounted with ProLong™ Gold antifade reagent. Cells were then observed using a confocal laser scanning microscope (Olympus, model IX81) and Fluoview imaging software (Olympus Life Science).

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit-polyclonal anti-ECSIT</td>
<td>Abcam, #ab21288</td>
<td>1:50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibodies</th>
<th>Supplier</th>
<th>Working Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal anti-mouse IgG-Alexa Fluor® 647</td>
<td>Invitrogen, #A32728</td>
<td>1:500</td>
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<tr>
<td>Polyclonal anti-rabbit IgG-Alexa Fluor® 488</td>
<td>Invitrogen, #A11034</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 8. Immunofluorescence study. Primary and secondary antibodies.

5.33. Statistical analyses

Statistical analyses were generally done using the software GraphPad PRISM (version 8.1.2) or online free resources (http://vassarstats.net/). The specific statistic test for the analyses of miRNA chapter are described in chapter V, 5.19. Clinical data. Included participant’s baseline demographic and clinic characteristics that were analyzed by Welch’s ANOVA or one-way ANOVA. Fisher’s exact test and χ² –test were used for categorical data. Experimental data. The D’Agostino-Pearson omnibus and the Shapiro-Wilks tests were used to assess the
normal distribution of each set of data. For data normally distributed, the significance of differences was established by one-way or two-way ANOVA followed by Tukey’s or Bonferroni’s multiple comparisons tests. For data sets that were not normally distributed, significance was determined with the Kruskal-Wallis test (followed by Dunn’s multiple comparisons test), Mann-Whitney U or Wilcoxon test. Equality of variances was also tested by F and Brown-Forsythe tests. For data sets which did not show equality of variances, significance was determined by Welch’s t test. For all analysis, a p value < 0.05 was considered as significant.
CHAPTER VI

MOUSE AND HUMAN PLASMA sEVs-ENRICHED SAMPLES ISOLATION AND CHARACTERIZATION

This chapter contains results included in the paper “Extracellular-vesicle ECSIT as possible biomarker for detection of early cognitive decline: in vivo evidence using Alzheimer’s disease transgenic mice and human plasma” (accepted for publication but under minor revisions).

6.1. Current isolation and characterization methods in EV studies

EVs have a specific cargo signature that does not necessarily depend on the parental cell (Iraci et al., 2016). Their content does rely on the cellular and environmental state and, therefore, on the healthy or disease processes the cell is going through. During these processes, the enrichment of some proteins and the depletion of others in a non-uniformly distributed manner for all EVs is very common (Iraci et al., 2016). Despite these changes, their content is well protected by a lipid bilayer and very stable under the appropriate storage conditions (Boukouris and Mathivanan, 2015). The analysis of EV content, in addition, has one main advantage with regard to the study of biological fluids. EVs reduce the complexity and background noise of the biological matrix from which they derive, leading to the detection of low abundance disease-related biomolecules (Boukouris and Mathivanan, 2015) and, thus, conferring them an immense potential for the biomarker detection in the clinical diagnosis with regard to the whole biofluid.

However, before ascribing a specific content or function to a subpopulation of EVs (apoptotic bodies, ectosomes or exosomes), it is necessary, firstly, to collect the matrix from which to
isolate them. Secondly, to isolate EVs. And thirdly, to characterize them according to their size and their membrane antigens. Concerning these three main steps, there are some points that need to be clarified, as established by the MISEV2018 guidelines (Théry et al., 2018).

Body fluids are different from culture medium and present distinct chemical and biophysical characteristics that need to be considered when isolating EVs. Plasma, serum and CSF are the most common biological fluids used in the EV field. However, which of the blood derivatives is the best for EV studies is not yet known. They both contain lipoproteins that can easily interfere with EVs. However, they differ in terms of yields of EVs isolated since the large majority of EVs released in the whole blood and serum are platelet-derived EVs which are not present in plasma (Tao et al., 2017; Weiss et al., 2018).

Also, when working with any of these blood products, information regarding donor age, sex, fasting or non-fasting state, exercise level, possible pregnancy, diet, medications and time of the day for the biofluid collection, among others, need to be provided. Volume, type of container, storage conditions, choice of anticoagulant are also some technical parameters that must be indicated. All these technical parameters vary depending on the biological fluid and the downstream analysis (Lucchetti et al., 2019; Théry et al., 2018).

Isolation and characterization of EVs are also necessary (Panagopoulou et al., 2020). However, isolation of pure EV samples, that is, complete purification of any EV subtype, is not possible. The reason is that available techniques allow the discrimination between larger EVs (e.g. apoptotic bodies) and sEVs (e.g. exosomes and ectosomes) while none of them distinguish exosomes from ectosomes, because they both overlap in size (Kalra et al., 2016). In addition, exosomes are a really heterogeneous population of sEVs, which makes even more difficult the isolation of their subpopulations with specific cellular origins and healthy/disease states.
Therefore, when talking about isolation techniques, the general term of EV or sEVs (if they have less than 200 nm in size) are preferred if the enrichment of a specific subtype of EVs (such as exosomes) with respect to another cannot be confirmed. In addition to the complexity and heterogeneity of EVs, biological fluids also represent by themselves a limitation for any isolation method, since they contribute to EV contamination with protein and lipid aggregates (Théry et al., 2018).

There are several methodologies developed to isolate EVs (Théry et al., 2018). The most common include differential centrifugation, density-gradient separation, size-exclusion chromatography (SEC), polymer-based precipitation, immunoaffinity capture, ultrafiltration and microfluidic methods (B-Y. Chen et al., 2019).

- **Differential centrifugation** is the gold standard method to isolate EVs (Gardiner et al., 2016). It utilizes centrifugal force to separate and remove non-EV components (dead cells and debris) and large EVs. The final ultracentrifugation step helps to pellet exosomes. The ability to work with either small or large quantities of solution and the absence of additional chemicals and polymers are the advantages of this method. However, its low cost is offset by the complex equipment required (ultracentrifuge), the possible vesicle rupture (Yu et al., 2018), the high risk of contamination with non-vesicle aggregates and lipoproteins that also precipitate at high-speed centrifugal force (B-Y. Chen et al., 2019) and its efficiency affected by the type of rotor and ultracentrifugation time (Cvjetkovic et al., 2014).

- **Density-gradient separation** is usually performed to better separate EVs that have already been isolated by differential centrifugation (Zhang et al., 2014). EV separation, based on size and density, happens into different solutions (sucrose, iohexol or iodixanol) with buoyant density layers. Even if this method allows purer preparations non-contaminated
with viral particles, density-gradient separation also requires an ultracentrifuge. If the centrifugation time is not the appropriate, some EVs and contaminating materials will not reach the equilibrium density, remaining in the wrong density layer (Taylor and Shah, 2015). In addition, because of the layering of gradients, high volume samples are required (B-Y. Chen et al., 2019).

- **SEC** allows EV separation based on their sizes using a column of porous beads with smaller diameter than EVs of interest through which the solution is filtered. This technique preserves better the vesicle integrity with regard to other EVs isolation methods (Gámez-Valero et al., 2016). However, it requires specialized equipment to be performed and, as well as the ultracentrifugation-based methods, SEC does not completely distinguish EVs from other soluble proteins present in the biofluid (Monguíó-Tortajada et al., 2019).

- **Commercial kits** are based on polymers which allow to decrease the solubility of EVs, making them to form aggregates and pellet at low-speed centrifugal force. This procedure is simple and does not require additional equipment (e.g. ultracentrifuge). However, the presence of impurities and contaminants in the sample is highly likely. Polymer-based precipitation or precipitation with chemicals methods are also popular. Within this group, we can distinguish precipitation with polymers, such as polyethylene glycol (PEG) (Konoshenko et al., 2018; Rider et al., 2016) in which commercial kits are also based on. However, the price, when using self-prepared PEG solution, is lower than current commercial kits. The positively charged molecule protamine is also able to isolate EVs (that are all negatively charged) in presence or not of PEG (Deregibus et al., 2016). Sodium acetate is another example. This compound may reduce the EV hydration, promoting hydrophobic interactions among them and, thus, increasing their aggregation and
precipitation at low-speed centrifugal force (Brownlee et al., 2014). The biggest advantage of precipitation methods with PEG, sodium acetate or protamine is the absence of ultracentrifuge. The biggest drawback, instead, is the contamination with residues of polymers and chemicals that affect downstream analyses of EV content.

- **Imunoaffinity capture** is based on the use of magnetic beads coated with antibodies against the non-tissue specific tetraspanins (CD63 or CD81) expressed in all EVs subtypes or another EV surface antigen (Théry et al., 2018). Every EV without the specific surface marker will elute within the solution in which EVs are resuspended. The high selectivity of this method is one of its biggest advantages, since selecting and isolating EVs based on their surface markers enhance the purity of the sample. However, this method is costly and only useful when a high proportion of EVs present the target antigen (B-Y. Chen et al., 2019).

- **Filtration** is another method to isolate EVs (Merchant et al., 2010). It uses porous membranes with different pore sizes (filters with pore diameters of 0.8, 0.45, 0.22 and 0.1 µm are commonly used (Konoshenko et al., 2018)) being able to concentrate an EV fraction of specific size. EVs smaller than the selected pore size pass through the membrane. It is a simple procedure and does not have limitations regarding the sample volume. However, as well as for SEC, it does not allow to distinguish between EVs and contaminants (e.g. protein aggregates) of similar size.

- **Microfluidic systems** are relatively new and consist of devices that separate EVs using immunoaffinity capture and filtration systems (Y.-S. Chen et al., 2019; Kanwar et al., 2014). Time processing in this new technology is faster than for the rest of the available methods. However, microfluidic devices are usually complex, costly and not designed for large amounts of sample volume.
Several methods have been also developed to test the presence, the integrity and the characteristics of EVs. Electron microscopy (EM), atomic force microscopy (AFM), super-resolution microscopy, nanoscale flow cytometry (nanoFACS), dynamic light scattering (DLS) and NTA are the most common.

- **EM** is a well-established technique that provides information regarding EV size, structure and shape. Three specific techniques are widely used for EV studies; TEM (Hu et al., 2020), cryo-TEM (Vall-Palomar et al., 2018) and scanning electron microscopy (SEM) (Singh et al., 2014). TEM uses beams of electrons that pass through the sample to produce secondary electrons that are subsequently collected to generate an amplified image of the sample. It provides information regarding the morphology and the size of EVs. However, it does not contribute with information on EV concentration. TEM needs samples to be fixed in glutaraldehyde and be dehydrated. That is, sample preparation for examination under TEM involves several steps that may affect EV morphology. Fixation and dehydration are also necessary in SEM. This technique uses a beam of scattered or reflected electrons that interact with atoms in the EV providing information only about the EV surface. One disadvantage of SEM with regard to TEM is its limit resolution. Cryo-TEM, instead, does not involve dehydration and fixation and, thus, preserves better the morphology and the structure of EVs. EM also allows to track specific protein markers in the lumen or the surface of EVs. This can be done with nanogold particles able to label a given antigen (Gurunathan et al., 2019).

- **AFM** is able to measure the vesicle size distribution and to analyze the protein content of the EV surface (Chopra et al., 2019; Chukhchin et al., 2020; Sharma et al., 2011). However, the limited use of AFM for EV characterization, compared to other techniques, is due to the
required immobilization conditions. That is, AFM needs EVs immobilized on a surface. Several types of surfaces have been used for EV characterization, such as muscovite mica, glass slides and silicon surfaces. These substrates allow the formation of electrostatic conditions for a strong binding between them and the EVs. Other possibility to keep EVs attached to the substrate are the antibody-coated surfaces (Parisse et al., 2017).

- **Super-resolution microscopy** is another alternative to conventional microscopy techniques. Some of the most common are the photoactivation localization microscopy (PALM) that uses photoactivatable fluorophores, and the direct stochastic optical reconstruction microscopy (dSTORM) which employs the photoswitching of single fluorophores. For EV characterization, PALM and dSTORM have already been used (Chen et al., 2016; Panagopoulou et al., 2020). The advantage of the super-resolution microscopy is the ability to image particles non-detectable by conventional microscopes. However, the sample preparation for EV characterization is challenging.

- **Flow cytometry** could be also interesting in the field of EV characterization. Flow cytometers are based on a laser beam with a specific wavelength which is directed to a suspension containing EVs. The more particles are present in the sample; the more light is scattered. However, conventional flow cytometers are not able to discriminate sEVs because they are smaller than the illumination wavelength. These size limitations make exosomes to escape from the detection by conventional flow cytometers (Stoner et al., 2016). High-resolution flow cytometers (nanoFACS) have been developed to eliminate boundaries of size discrimination that conventional flow cytometers have, conserving the ability to determine the antigen profile of different EV populations in an heterogeneous sample (Morales-Kastresana et al., 2017, 2019; Padda et al., 2019).
• **DLS** and **NTA** characterize EVs by measuring their size based on their Brownian (random) motion in a solution (Han et al., 2020; Mattera et al., 2020). That is, both techniques illuminate particles in the sample using a laser and relate their random movement with their hydrodynamic diameter when light is scattered. **NTA** tracks the movement of each particle, measuring their velocity and, subsequently, their size. However, **DLS** does not observe the particles but the variations in the scattered light intensity, which allows it to calculate the average particle size (Filipe et al., 2010). One of the major issues of both, DLS and NTA, techniques is that none of them can distinguish an EV from a non-EV particle since the latest also display Brownian movements (Carnino et al., 2019). In addition, NTA size and concentration measurements are less accurate when smaller particles are present in the sample. Protein aggregates and larger vesicles also interfere with the number of real small particles the NTA software can measure (Panagopoulou et al., 2020).

Some of these methods have been described to be useful to quantify EVs (*e.g.* NTA), others to characterize single vesicles (*e.g.* EM). However, they, as well as isolation methods, are not perfect. That is, several proteins, lipids or particles are isolated together with EVs but they are not really associated to them (*e.g.* soluble proteins, protein aggregates or lipoproteins) (Théry et al., 2018). The last step of EV characterization consists therefore in determining the recognition and the absence of specific antigens (proteins, receptors or lipids) in their membranes. Following the MISEV2018 guidelines, five different categories of antigens exist. The first category refers to proteins associated with the PM or endosomal membranes (*e.g.* tetraspanins CD63 or CD81). The second one is referred to cytosolic proteins with lipid or membrane protein-binding ability (*e.g.* Tsg101 or Alix). The third category relates to purity controls such as lipoproteins usually
co-isolated with EVs (e.g. albumin). The fourth group are proteins present in non-endosomal cellular compartments (e.g. nuclear or mitochondrial proteins). And the fifth category refers to soluble extracellular proteins (e.g. cytokines or growth factors). The presence or the absence of these antigens demonstrate the existence of a lipid bilayer in the material analyzed and the lack of contamination with biofluid- and subcellular compartments (other than endosomes)-derived material (Théry et al., 2018).

6.2. Aim of the study and experimental design

Based on evidence previously reported, the use of plasma sEVs might have a great potential in the clinical diagnosis of a disease. Therefore, in this chapter, before confirming their role as biomarker carriers, we aimed to separate and characterize our mouse and human-derived sEVs using some of the validated methodologies (in concrete, ultracentrifugation, NTA, super-resolution microscopy and EM) as above described.

To this purpose, we have collected blood from the 3xTg-AD and their wild type littermates (NTg) from 3 (T0) to 10 months of age (T7) to cover the early and the late stage of cognitive decline. We also assessed, in parallel to mouse, human plasma samples (clinical and socio-demographic characteristics of these subjects are shown in Table 9) derived from cognitively normal Ctrl, MCI and demented subjects (from now on, we will be referring to demented subjects as AD). SEVs were then isolated using an ultracentrifugation protocol and characterized using the NTA, SIM, TEM and Western blotting.

For their characterization, sEVs-containing pellets, obtained after applying the ultracentrifugation protocol described in chapter V with the same volume of human plasma (700
μL) and mouse plasma (30 μL) samples, were resuspended in 20 μL particle-free PBS 1X and lysed with 30 μL lysis buffer for Western blotting; in 20 μL particle-free PBS 1X for NTA (samples were diluted in more particle-free PBS 1X until the appropriate PF was obtained); in 50 μL 2% glutaraldehyde in particle-free PBS 1X for TEM; and in 500 μL 4% paraformaldehyde in particle-free PBS 1X for SIM.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl (N = 14)</th>
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<th>AD (N = 14)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>81.083 (7.521)</td>
<td>77.916 (4.01)</td>
<td>79.916 (8.401)</td>
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<tr>
<td>MMSE, mean (SD)</td>
<td>28.666 (1.027)</td>
<td>26.166 (1.724)</td>
<td>22.583 (2.841)</td>
<td>&lt; 0.0001†</td>
</tr>
</tbody>
</table>

†Welch’s ANOVA and χ² for categorical data.

Table 9. Chapter VI. Clinical and socio-demographic features of subjects. HIS, Hachinski ischemic score; SD, standard deviation; MMSE, mini-mental state examination.

We showed that our mouse and human sEVs-enriched preparations from plasma do not show significant differences in their concentration or particle parameters, they are positive for L1CAM and enriched in non-specific exosome markers.
6.3. Results

6.3.1. NTA analysis of mouse and human plasma-derived sEVs-enriched preparations showed no significant differences in the particle parameters or concentration profiles. 

![Figure 13](image.png)

**Figure 13. Size distribution profiles and concentration in plasma for NTg and 3xTg mice-derived sEVs.** Representative means of concentration profiles (particles/ml) of both, NTg (*shades of blue*) and 3xTg (*shades of orange*) mice were compared in the sample plot for each time point (T0 - T7; A - H). Each representation is the mean of, at least, NTA profiles of three mice. The particle concentration released by NTg and 3xTg in the initial plasma sample for each time point is represented (I) as mean±SD (N is at least three mice). Normal distribution was assessed by the D’Agostino-Pearson omnibus normality test. Two-way ANOVA test followed by Bonferroni’s multiple comparison test was used. P value < 0.05 was considered significant.
Concentration NTA profiles (particles/mL) were assessed by NTA for NTg (shades of blue) and 3xTg (shades of orange) for each time point analyzed (T0 - T7) (Figure 13 A - H). These NTA profiles for NTg and 3xTg and each time point are compared in the same plot and are the mean of at least three mice. Also, particle concentration (particles/mL) in the initial plasma sample volume for NTg (blue triangles) and 3xTg (orange circles) are compared in the same plot for each time point (mean±SD, at least 3 mice) (Figure 13 I). No significant differences were observed regarding the particle concentration in plasma.

Quantitative analyses of particle distribution parameters were also performed, indicated as mean±SD (at least three mice) (Figure 14). Differences were not observed when NTg with 3xTg mice were compared at different time points either for the mean diameter (Figure 14 A) or the D10 and D50 (Figure 14 B and C). However, a slight fluctuation was noticed in the D90 (Figure 14 D) (T0, t_{2.897}, df_{39}, *p = 0.0492; NTg mean = 237, 3xTg mean = 308.5) leading to a significant increase in T0 in the 3xTg compared to NTg mice.
Figure 14. Particle distribution parameters of plasma-purified sEVs for NTg and 3xTg mice. Mean particle diameter (A) and particle size distribution parameters (D-values, D10, D50 and D90 (B, C, D)) are represented for NTg (blue triangles) and 3xTg (orange circles). Each time point, for the quantitative variables studied, was assessed at least in three mice and summarized with mean±SD. Normal distribution was assessed by the D’Agostino-Pearson omnibus normality test. Two-way ANOVA test followed by multiple comparisons testing using Bonferroni’s test. P value < 0.05 was considered significant.

Human plasma-derived sEVs were also characterized using NTA (Figure 15). The size distribution profiles of sEVs derived from Ctrl (Figure 15 A), MCI (Figure 15 B) and AD (Figure 15 C) are represented as the median of five subjects. The mean diameter (Figure 15 E), D-values (Figure 15 F, G, H) and the particle concentration in the initial plasma sample volume (Figure 15 D) showed no significant differences.

Figure 15. Particle distribution and concentration parameters of Ctrl, MCI and AD plasma-derived sEVs. Representative NTA profiles for Ctrl (A), MCI (B) and AD (C) are showed as the mean of five subjects. The particle concentration released in the initial plasma
sample of Ctrl, MCI and AD subjects is also represented (D). For each group, mean particle diameter (E) and particle distribution parameters (D-values, D10, D50 and D90) (F - H, respectively) are also represented. These quantitative parameters were assessed in five subjects and represented in box-and-whisker plots which depict median, interquartile range (box) and range (error bars). Normal distribution and equality of variances were tested by the D’Agostino-Pearson omnibus normality test and the Brown-Forsythe test, respectively. Kruskal-Wallis test followed by Dunn’s multiple comparisons test (D10) or one-way ANOVA followed by Tukey’s multiple comparisons test were used respectively for non-parametric or parametric tests. P value < 0.05 was considered significant.

6.3.2. SEVs-enriched preparations showed positivity to common EV markers

After characterization with NTA, we aimed to identify some common antigens of EVs surface in our sEVs-enriched preparations.

SIM technique was performed for human plasma-derived sEVs positive for L1CAM (L1CAM+) of Ctrl, MCI and AD groups (Figure 16 A, B, C). SEVs (white dots) were identified and SIM resolution achieved (represented in the fast Fourier transform (FFT) radial plot profile) was established in a range from 130 to 160 nm. For Ctrl, MCI and AD groups, the maximum cut-off limit size was fixed at 190 nm. Only for MCI and AD subjects, smaller EVs (180 nm) were identified (Figure 16 D).

TEM was done with human and mouse (Figure 17 A and B, respectively) plasma-derived sEVs-enriched preparations. SEVs were not larger than 100 nm in size and conserved the characteristic round-shaped of exosomes.

The characterization of sEVs was completed with the study of specific EV and non-EV antigens (Figure 17 C). The plasma sample (mouse and human) was used to obtain the sEVs-depleted
pellet and the sEVs-enriched preparation in order to compare the antigen content of different vesicle populations. As shown, sEVs-enriched preparations had increased amounts of non-tissue specific markers (CD63), cell-specific markers (L1CAM) and cytosolic proteins recovered in EVs (Tsg101) for the same amount of protein extract.

Figure 16. Plasma-derived sEVs characterization by SIM. Human plasma-derived L1CAM+
sEVs were imaged using SIM technique (scale bar 2 µm). Resolution achieved was 138 nm for Ctrl- (A) and MCI- (B) and 160 nm for AD- (C) sEVs-enriched preparations as shown by the FFT radial plot profile. The medium sizes obtained for L1CAM+ sEVs were 190 nm (Ctrl) and 180 nm (MCI and AD) (D) and are represented as mean±SD, five single vesicles. Output with additional color-coding applied in lateral and orthogonal Fourier spectra is also shown (A, B, C).

As for EVs purity, protein levels of serum albumin and GM130 proteins were also measured (Figure 17 C). Mouse and human sEVs-enriched preparations were almost albumin-depleted. However, proteins bound to Golgi membranes (GM130) were not completely absent in human plasma sEVs-enriched preparations.

Figure 17. Plasma-derived sEVs characterization by TEM and Western blotting for common EV and non-EV antigens. Images are shown for human and mouse sEVs-enriched preparations using TEM (A and B, respectively) (scale bar 0.1 µm). Representative Western blots of mouse and human plasma preparations (sEVs-depleted or enriched preparations) showed the presence of non-tissue specific markers (CD63), cell-specific markers (L1CAM)
and cytosolic proteins recovered in EVs (Tsg101) (C). Major components of non-EVs co-isolated particles (albumin and GM130) are also shown (C). In human and mouse plasma, both sEVs-depleted pellet and sEVs-enriched preparation derived from the same sample. In the representative western blots, each lane is an individual plasma sample.

### 6.4. Discussion

In recent years, compelling evidence has proved that sEVs are potential carriers of AD disease-related biomolecules (Fiandaca et al., 2015; Goetzl et al., 2018). However, EV studies in the field of biomarker discovery should go hand-in-hand with a clear understanding of their limitations. These limitations are referred to available methods to isolate and characterize EVs. Since these methods are not well standardized, they do not produce the same outcome affecting reproducibility of results and, in short, keep EVs away from their potential useful role as clinical diagnostic tools in the disease progression (Ludwig et al., 2019).

If the available methods to isolate and characterize EVs represent a limitation, the unclear nomenclature and properties of EVs represent another one. For example, exosome size is not well defined. In fact, several researchers have described them as EVs comprised between 30 and 150 nm (Yang et al., 2020), while others accept every size less than 200 nm (Mathieu et al., 2019). Things get even more complicated when the term “exomere” was introduced to describe nanoparticles with diameters lower than 35 nm or when small exosomes (Exo-S) and large exosomes (Exo-L) with different biophysical properties, distinct cargo profile and distinct biological functions were identified (Zhang et al., 2018).

It is therefore clear that EV studies are complicated because of two main things. Firstly, their intrinsic nature and, secondly, the isolation and characterization methods that, even if exosome-
targeted, do not allow the complete separation and visualization of pure EV subtypes. To cope with this limitation, MISEV2018 guidelines (Théry et al., 2018) propose the terms “separation” and “concentration/enrichment” instead of isolation. That is, whenever is possible, contamination with other EV subtypes should be avoided. In addition, the enrichment of our sample with the EV subtype of interest with respect to another subgroup, must be tested with the presence of specific antigens. In short, before studying EV content also for biomarker discovery, we must be sure that what we have in our EV sample are mostly EVs.

Following this principle, we performed isolation and characterization of EVs-derived from mouse and human plasma samples and, as expected, NTA showed that in both human (Figure 15 E) and mouse (Figure 14 A) plasma samples, EVs separated using a conventional ultracentrifugation protocol (see chapter V for details) displayed diameters means below 200 nm. This confirmed that our EV samples did not contain only exosomes, but also ectosomes. This is also consistent with the fact that ultracentrifugation protocols are able to discard larger EVs but non-EV contaminants that pellet at the same high-speed spin than exosomes (B-Y. Chen et al., 2019). In addition, these non-EV contaminants also display Brownian motion (Carnino et al., 2019), and NTA has probably recognized them as part of our EV sample. The presence of particles with diameter lower than 200 nm and the likely presence of non-EV contaminant (such as soluble proteins or lipids in the biofluid) have led us to define our EV samples as sEVs-enriched preparations instead of pure exosomes. That is, they are enriched with smaller (< 200 nm) EVs (Théry et al., 2018), depleted of larger EVs and the presence of contaminants is quite likely reduced.

There is another consideration to be done with regard to NTA. This methodology is used to provide the global quantification of sEVs as proposed in MISEV2018 guidelines (Théry et al.,
2018). The EV source and the EV preparation must be quantified. As described with more
details in chapter V, the source of sEVs, the starting volume of biofluid and the corresponding
technical parameters were provided. SEVs in mice and human (Figure 13 I and 15 D,
respectively) were quantified for the starting plasma volume. However, since NTA cannot really
quantify EVs only, and the parameters it provides refer to particles and not vesicles, this global
quantification was not used to normalize the amount of proteins in our sEVs-enriched
preparations. In fact, the trend towards a decrease in the particle concentration of the sEVs-
enriched preparation (Figure 15 B) and in the particle concentration of the starting plasma
volume (Figure 15 D) for MCI with regard to Ctrl and AD subjects must be carefully
interpreted, since NTA detects every particle with Brownian motion, no matter if the particle is
an EV or not, suggesting that this decrease might not due to a real decrease in sEVs
concentration.

SIM and TEM were the two techniques chosen to visualize sEVs of our sEVs-enriched
preparations. Although SIM is innovative in the super-resolution microscopy field for EV
studies, it did not achieve an optimal resolution. In fact, it was limited to ~160 nm and only
larger L1CAM+ sEVs were identified in Ctrl, MCI and AD plasma samples (Figure 16 A, B,
C). This may suggest that SIM is not as suitable as dSTORM or PALM (Chen et al., 2016) for
the sEVs characterization and, thus, it requires improvements to be used for this purpose.

Due to the limit resolution obtained with SIM, TEM was done with human and mouse plasma-
derived sEVs-enriched preparations and sEVs with less than 100 nm in size were detected
(Figure 17 A, B).

As for sEVs enrichment efficacy of the ultracentrifugation method, there are several points that
need to be discussed. First of all, the selection of L1CAM as sEVs marker in SIM and Western
blotting. This cannot be separated from the choice of selecting the whole circulating population of sEVs in plasma rather than the NDEs fraction. NDEs show positivity for the transmembrane glycoprotein widely expressed in neural tissues, L1CAM. In fact, antibodies against L1CAM are used to isolate specific population of plasma sEVs derived from the brain (Fiandaca et al., 2015; Goetzl et al., 2015, 2018). However, as EV content change during the progression of the disease, EVs might also change the antigens they have in their membrane surface. This means that the target antigen (L1CAM in our case) may be “lost” or “gained” over the disease progression (Taylor and Shah, 2015). Additionally, L1CAM is not only expressed in the neural tissue where it has a role in axon guidance, cellular adhesion and migration (Hortsch, 1996) but it is also expressed in tumors (Raveh et al., 2009) and epithelial cells (Debiec et al., 1998). In other words, L1CAM expression not only depends on brain, it depends on the contribution of different tissues, being also present in plasma in its soluble form (Kiefel et al., 2012). In fact, the high L1CAM signal expression in sEVs-depleted pellets of the Western blot experiment (Figure 17 C) suggests the presence of L1CAM also in non-neural tissues. In addition, several proteins that have been proved to increase or decrease their concentration in NDEs derived from plasma samples of subjects suffering from AD are not even related with neurodegeneration, the progressive cognitive impairment, the disease severity or the disease progression. Instead, they reflect much better mechanisms that are also altered in other diseases, such as the lysosomal function (Goetzl et al., 2015). Therefore, it is difficult to assume that only NDEs content reflect neurodegeneration and if they do it, it is even more difficult to ensure that their content would also change depending on the ND (Pulliam et al., 2019). With this regard, Pulliam et al. identified some proteins not expressed in neurons which show high concentrations in NDEs, supporting the idea that since L1CAM is also expressed, although in low levels, outside the
brain, it might be also present in EVs from non-neural tissues (Pulliam et al., 2019). Furthermore, we should not forget that AD-related alterations are also reflected in peripheral tissues (François et al., 2014). Therefore, sEVs derived from other tissues may also have a role in AD as biomarker carriers. All these facts make us isolate circular general sEVs population instead of NDEs. L1CAM, instead, was used as a common general marker for sEVs in SIM and Western blot without purification of NDEs.

Tsg101 and CD63 were instead chosen to test the EVs enrichment efficacy of the ultracentrifugation protocol as non-tissue specific EV marker and as cytosolic protein recovered in EVs, respectively (Théry et al., 2018). CD63 and Tsg101 are non-specific markers for exosomes as they are both implicated in ectosome biogenesis. This supports the result showed for Tsg101 and CD63 in Western blotting (Figure 17 C) since these two proteins were also enriched in sEVs-depleted pellets where ectosomes might have co-isolated. CD63 in sEVs-enriched preparations from mouse was detected at higher molecular weight than in human sEVs-enriched preparations (Figure 17 C). This could be due to a different pattern of glycosylation in CD63 protein (already reported in the literature (Tominaga et al., 2014)) in the 3xTg and NTg mice with regard to human. Moreover, tetraspanins, such as CD63, CD9 or CD81, are not always present in all the exosomes or ectosomes (Crescitelli et al., 2013; Tauro et al., 2013). Instead, these antigens may be expressed only in a fraction of plasma-sEVs. This unpredictable presence of markers and the peripheral contribution to L1CAM are consistent with the increased L1CAM signal intensity for AD plasma-derived sEVs-enriched preparations (Figure 16 D) that could be due either to an increment of L1CAM+ sEVs secretion because of the disease stage or because the number of sEVs expressing L1CAM was higher than in Ctrl and MCI samples. Further analyses of plasma AD sEVs-enriched preparations are needed to verify or reject these
Chapter VI. Mouse and human plasma sEVs-enriched samples isolation and characterization | Soraya Moradi Bachiller

hypotheses.

The detection of albumin and GM130 proteins in plasma-derived sEVs-enriched preparations is another thing we need to discuss. As shown in Figure 17 C, the presence of albumin was reduced (in human plasma sEVs) and not detected (in mouse plasma sEVs) with regard to sEVs-depleted pellets. This support the efficacy of the ultracentrifugation protocol when discarding high-abundant proteins in plasma before the high-speed spin step to precipitate sEVs. However, the efficacy of our protocol was not optimal regarding GM130 since this is a protein localized at the Golgi apparatus and should not be present in sEVs. In fact, MISEV2018 guidelines (Théry et al., 2018) propose it as a negative marker “when claiming specific analysis of small EVs”. In human plasma-derived sEVs-enriched preparations we found it more enriched than in sEVs-depleted pellets that suggests the presence of contaminating non-endosomal vesicles in the sEVs-enriched preparation or the disruption of some non-endosomal vesicles whose components co-isolated with sEVs. Again, further analyses need to be done to confirm these hypotheses.
Chapter VII. Systems biology and its application in the field of biomarker discovery | Soraya Moradi Bachiller
CHAPTER VII

SYSTEMS BIOLOGY AND ITS APPLICATION IN THE FIELD OF BIOMARKER DISCOVERY

This chapter contains results included in the paper “Extracellular-vesicle ECSIT as possible biomarker for detection of early cognitive decline: in vivo evidence using Alzheimer’s disease transgenic mice and human plasma” (accepted for publication but under minor revisions).

7.1. When disease behaviour can be explained using networks

Humans are complex. Human diseases are complex too. In fact, malfunctions and defects in one single gene cannot explain them. Several participants and their interactions are, instead, involved in the development of a disease. The scientific discipline that model computationally and mathematically complex systems by studying every entity and interaction, is called systems biology. In other words, systems biology is the field of science that studies networks underlying complex systems with the aim of identifying properties that cannot be found when considering only individual entities. Systems biology studies the behaviour of complex systems (Pournoor et al., 2019).

As it is explained with more details in chapter II, 2.4.1., networks simplify real and complex systems by representing them as entities (nodes) connected by interactions (links). To create networks, different approaches exist. One can take advantage of the use of physical and biochemical interactions already available in the literature or the use of high-throughput experimental strategies to map the whole genome or proteome. Another possibility could be the
use of computational predictions based on available empirical data or on theoretical information. All these strategies have their own disadvantages and advantages. For example, when using experimental information from the literature, one should take into account the biases inherent in the published data (Safari-Alighiarloo et al., 2014; Vidal et al., 2011).

One of the most common type of biological networks are protein-protein interaction (PPI) networks (Safari-Alighiarloo et al., 2014), where nodes represent proteins and links represent physical or functional interactions between two proteins. As already explained in chapter II, 2.4., these interactions, that form the interactome, are specific and have a biological meaning. Moreover, they can be stable or transient interactions. And when mapping them through networks, complex systems and the corresponding phenotype derived from them, are easier to understand.

Experimentally, PPIs can be detected in the form of binary interactions (yeast two-hybrid system, protein fragment complementation assay, split-ubiquitin assay, mammalian protein-protein interaction trap or protein arrays) or in the form of associations within protein complexes (affinity purification followed by mass spectrometry (Huttlin et al., 2015) or co-fraction with mass spectrometry (Havugimana et al., 2012)). However, it should be kept in mind that networks produced with binary and protein-complex data show differences regarding their properties (Braun, 2012; Vidal et al., 2011).

As well as for most of real networks, PPI networks do not assume randomness. Instead, they are scale-free. In them, hubs hold the whole biological system together, they are more likely than less-connected nodes to confer lethality and bigger phenotypic outcomes, and are expressed in numerous tissues. Moreover, in the yeast interactome, it was demonstrated that two types of hubs exist — party hubs and date hubs (Figure 18) (Han et al., 2004). Party hubs connect nodes
within functional modules (sub-networks with a high number of links connecting their own nodes (Safari-Alighiarloo et al., 2014)) of the network. Date hubs, instead, connect different functional modules. The removal of date and party hubs produces different effects in the network, being the deletion of date hubs the most deleterious (Han et al., 2004). However, both party and date hubs are more likely to be associated with essential nodes (Jeong et al., 2001). Instead, less-connected nodes are much more associated with non-essential and, subsequently, with disease nodes (Goh et al., 2007), are expressed in a few tissues only and are responsible of diseases that can persist in the adulthood (Vidal et al., 2011). In short, in PPI networks, the degree of a protein is correlated to the essentiality of that given protein (Jeong et al., 2001; Raman et al., 2014).

**Figure 18. Date and party hubs.** In this schematic PPI network, two functional modules are represented, orange- and mustard-colored. Party hubs interact with nodes within the same functional module while date hubs connect different modules in the network. *Figure designed by Soraya Moradi Bachiller.*
All the characteristics cited above have been also demonstrated for real networks in human. In fact, the first proteome interaction map for humans also has scale-free properties (Stelzl et al., 2005). The human PPI network also shows a decreased clustering coefficient when the number of interactions per protein increases. In other words, human PPI network has hierarchical structure (that is, sub-networks are present). This means that human PPI network integrates scale-free property with a modular structure where less-connected nodes are part of highly clustered areas, and the communication between these highly clustered areas is maintained by hubs (Barabási and Oltvai, 2004).

Since several diseases are caused by mutations that affect protein binding sites, protein stability or protein folding, it is reasonable to think that existing PPIs could be destroyed and new PPIs could be also created. And this affects networks and leads to the basis of a given disease (Kann, 2007). In short, the interactome provided by PPI networks is essential to understand biological systems and their behaviour in healthy or disease conditions (X. Wang et al., 2012).

Following the systems biology and PPI network approach, Soler-López et al. studied AD network and demonstrated the highly interconnection of AD-related genes that work together in the same biological module, discovering new genes which might be involved in AD (Soler-López et al., 2011). The authors of this study performed matrix- and library-based yeast two-hybrid screens with 9 well-established AD-related genes (A2M, APOE, APP, BLMH, NOS3, PAXIP1, PLAU, PSEN1 and PSEN2) and specific candidate genes located in one of the three chromosome loci already associated to AD (7q36, 10q24 and 19p13.2) or derived from a human brain cDNA library. Hereafter, they generated an interaction set containing all the confirmed interactions, creating a PPI network which comprised 200 interactions among 8 AD seed genes and 66 candidates. The vast majority of the PPIs reported by them were novel and some were
also validated by co-immunoprecipitation and glutathione S-transferase-affinity binding methods. The 200 PPIs among 74 protein-coding genes were integrated in the network for the interactome of AD. This network showed scale-free property and hierarchical structure. Among the functional modules identified, one novel protein-coding AD-related gene candidate was identified, the evolutionarily conserved signaling intermediate in Toll pathway, *ECSIT*. This gene is located in the chromosomal region 19p13.2 and was present in modules of the network implicated in redox signaling and immune responses.

### 7.2. Aim of the study and experimental design

Since EVs are shed by all cells, released in several body fluids and carriers of specific cargoes with an important role in cell-to-cell communication, our aim here is to address the possible role of ECSIT as biomarker of cognitive impairment onset, in the 3xTg mouse model and in human subjects by focusing on its presence in plasma sEVs-enriched preparations to pursue a low-invasive biomarker discovery strategy.

To this purpose and as for chapter VI, we collected blood from the 3xTg mouse model of AD and their wild type littermates (NTg) from 3 (T0) to 10 months of age (T7). And also, from humans (Ctrl, MCI and demented (AD); clinical and socio-demographic characteristics for these subjects are shown in Table 10).

We separated sEVs from plasma using a conventional ultracentrifugation protocol. And we studied sEVs-ECSIT protein levels by Western blotting and ELISA and correlated these levels with the cognitive impairment of the human subjects and 3xTg mice.

In this chapter, we showed that plasma sEVs-ECSIT might be involved in neurodegeneration
and might serve as an early blood biomarker or a pharmacological target for cognitive decline in mouse and human.

<table>
<thead>
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<th></th>
<th>Ctrl (N = 43)</th>
<th>MCI (N = 44)</th>
<th>AD (N = 3)</th>
<th>P value</th>
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<td>Age, mean (SD)</td>
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<td>79.91 (4.094)</td>
<td>79.333 (2.357)</td>
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<tr>
<td>Sex, M/F, no.</td>
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<td>19/25</td>
<td>3/0</td>
<td>0.035*</td>
</tr>
<tr>
<td>Education, mean (SD)</td>
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<td>8.181 (3.15) (2.054)</td>
<td>7.666 (1.414)</td>
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<tr>
<td>HIS, mean (SD)</td>
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<td>1.975 (1.604)</td>
<td>2 (1.414)</td>
<td>0.251</td>
</tr>
<tr>
<td>MMSE, mean (SD)</td>
<td>28.122 (1.915)</td>
<td>25.636 (2.908)</td>
<td>18.333 (3.4)</td>
<td>0.005‡</td>
</tr>
</tbody>
</table>

‡Welch’s ANOVA and *Fisher’s exact test for categorical data.

Table 10. Chapter VII. Clinical and socio-demographic features of subjects. HIS, Hachinski ischemic score; SD, standard deviation; MMSE, mini-mental state examination.

In concrete, we demonstrated that ECSIT protein is associated with mouse and human plasma sEVs-enriched preparations. SEVs-enriched preparations from 3xTg-AD mice, MCI and AD subjects carry lower levels of ECSIT protein than NTg animals or Ctrl individuals. In the 3xTg-AD mouse model, the decrease in ECSIT protein is dependent on the genotype.

7.3. Results

7.3.1. 3xTg mouse model presents cognitive impairment from 5 months of age

One of the main clinical signs of dementia is the memory impairment. This deficit can
be recapitulated in mouse models, therefore, we started the *in vivo* study by mapping the memory impairment in the 3xTg and their wild type NTg mice from 3 – 10 months of age. The memory impairment was assessed using NORT for NTg and 3xTg mice. As shown in **Figure 19** (N = or > 6), no significant difference was detected between the D.I. of NTg and 3xTg either at 3 months (**A**) or 4 months of age (**B**). Instead, cognitive deficits in the 3xTg mice compared to the NTg started at 5 months (**C**) (t_{6,399}, df_{11}, ****p < 0.0001; NTg mean = 0.4014, 3xTg mean = -0.065). And it was maintained during the 6th (**D**) (t_{8,427}, df_{10}, ****p < 0.0001; NTg mean = 0.4240, 3xTg mean = 0.00053), the 7th (**E**) (t_{4,944}, df_{12}, ***p = 0.0003; NTg mean = 0.2989, 3xTg mean = -0.1753), the 8th (**F**) (t_{6,074}, df_{12}, ****p < 0.0001; NTg mean = 0.4325, 3xTg mean = -0.1753), the 9th (**G**) (t_{8,216}, df_{9,366}, ****p < 0.0001; NTg mean = 0.4205, 3xTg mean = -0.009686) and the 10th month (**H**) (t_{7,317}, df_{13}, ****p < 0.0001; NTg mean = 0.4885, 3xTg mean = -0.03883).

**Figure 19.** Cognitive deficit assessment for NTg and 3xTg mice from 3 – 10 months of age. Box and whiskers graphs depict median, interquartile range (box) and range (error bars) of the D.I. of NTg (*triangles, shades of blue*) and 3xTg (*circles, shades of orange*) mice from 3 months to 10 months. D’Agostino-Pearson and Shapiro Wilks tests were used to assess the normal
distribution and F test for the equality of variances. An unpaired t-test or Welch’s t-test (9 months) was applied. P value < 0.05 was considered significant. D.I., discrimination index.

7.3.2. ECSIT protein is present in mouse and human plasma sEVs-enriched fractions and co-localize with CD63 protein in mouse and human sEVs

Levels of ECSIT protein were measured in sEVs-enriched preparations by Western blotting in mouse (mean±SD, N = 3 mice) (Figure 20 A). In the 3xTg-AD, a trend towards lower levels of ECSIT protein was found compared to NTg mice for all time points analyzed. Moreover, the difference was significant for T1, T2, T4 and T7 (T1, t3.504, df32, *p = 0.011; NTg mean = 0.7078, 3xTg mean = 0.4597. T2, t4.243, df32, **p = 0.0014; NTg mean = 0.5855, 3xTg mean = 0.2850. T4, t4.973, df32, ***p = 0.0002; NTg mean = 0.7932, 3xTg mean = 0.4411. T7, t4.218, df32, **p = 0.0015; NTg mean = 0.7396, 3xTg mean = 0.4410).

In parallel, ECSIT levels were also measured in Ctrl, MCI and AD subjects (mean±SD, N = 3 subjects). And the difference between AD subjects with regard to Ctrl and MCI subjects (Figure 20 B) was also significant (Ctrl vs AD, q8.077, df6, **p = 0.003; Ctrl mean = 1.126, AD mean = 0.4825. MCI vs AD, q6.730, df6, **p = 0.0075; MCI mean = 1.019, AD mean = 0.4825).

Additionally, the concentration (ng/ml) of sEVs-L1CAM (18 Ctrl and MCI subjects) (Figure 20 C), sEVs-ECSIT (20/21 Ctrl and MCI subjects, respectively) (Figure 20 D) and plasma-ECSIT (13 Ctrl and 16 MCI subjects) (Figure 20 E) were measured by ELISA in Ctrl and MCI sEVs-enriched preparations and represented in box and whiskers plots. ELISA results indicated the same plasma-ECSIT proteins, lower for sEVs-ECSIT protein concentration in MCI compared to Ctrl (t2.330, df39, *p = 0.0251; Ctrl mean = 2.274, MCI mean = 1.567) and lower sEVs-L1CAM protein concentration (t2.736, df34, **p = 0.0098; Ctrl mean = 22.67, MCI mean =
26.48) in Ctrl compared to MCI subjects. Moreover, mouse (Figure 20 F) and human (Figure 20 G) sEVs showed positivity for ECSIT protein that co-localized with CD63 marker.

Figure 20. ECSIT protein in 3xTg and NTg mice and human plasma-derived sEVs-
**enriched preparations.** Representative Western blot showing the presence of ECSIT protein in sEVs-enriched preparations from the 3rd (T0) to the 10th (T7) month of age in 3xTg (orange circles) mice and NTg (blue triangles) (A). Each time point was assessed in three mice and is represented by mean±SD in the interleaved scatter plot with bars. The mean for the loading control (Ctrl in the western blot bands) (2-month-old-male NTg mouse, not shown) was set at 1.00 and used to normalize (to L1CAM protein) the values for individual samples (represented as the fold change). D’Agostino-Pearson omnibus test was used to assess the normal distribution. Two-way ANOVA was used followed by multiple comparisons using Bonferroni’s test. Western blot of ECSIT protein in sEVs-enriched preparations (normalized to L1CAM, arbitrary units) from human plasma (Ctrl, MCI and AD) is also shown (B). Each condition was assessed in triplicate and is represented in the scatter plot by mean±SD. Normal distribution and equality of variances were tested by the D’Agostino-Pearson omnibus normality test and the Brown-Forsythe test, respectively. One-way ANOVA was used, followed by Tukey’s multiple comparisons test. Concentrations (ng/mL) of sEVs-L1CAM (C) and sEVs-ECSIT (D) and plasma-ECSIT (E) are also shown and represented in box and whiskers plots with median, interquartile range (box) and range (error bars). Normal distribution and equality of variances were tested by the D’Agostino-Pearson omnibus normality test and F test. Unpaired t-test was used. Mouse (F) and human (G) plasma-sEVs positive for ECSIT (gold particle size = 10 nm) and CD63 (gold particle size = 6 nm) are also shown. P value < 0.05 was considered as significant.

### 7.4. Discussion

Two groups of gene products are found in PPI networks, essential and disease genes. Because of the larger number of links with other nodes, essential genes have a key role in the network. Less-connected nodes, instead, are positioned in the periphery of the network and are usually encoded by diseased genes (Barabási et al., 2011). In fact, the deletion of *APP* and *PSEN1* (both of them hubs in the PPI-AD network (Soler-López et al., 2011)) in mouse and C.
elegans models, leads to embryonic lethality which suggests they are essential in the interactome of these two organisms (Ewald and Li, 2012; Ewald et al., 2012; Shen et al., 1997; Wong et al., 1997). Attacking these nodes may destroy the network. However, pharmacological treatments are necessary, not to destroy the network, but to change the behaviour of its components. In this context, systems biology approach can help us to identify possible drug targets or biomarkers able to modify the disease progression by changing the behaviour of the network involved on it.

Also, thanks to this approach, ECSIT protein was identified as possibly involved in AD, due to its links to AD hubs, such as APOE, PS1 and PS2 (Soler-López et al., 2011). ECSIT is located on chromosome 19 (p13.2) and encodes a protein first described in 1999 by Koop and colleagues as an intermediate player in the activation of the nuclear factor-kappa B (NF-κB) in the mammalian TLR signaling pathway (Kopp et al., 1999). ECSIT protein also has an important role as an adapter cytoplasmic protein in innate immunity thanks to its interaction with the tumor necrosis factor receptor-associated factor 6 (TRAF6) and the mitogen-activated protein kinase kinase kinase 1 (MEKK-1) (Aderem and Ulevitch, 2000; Kopp et al., 1999; Soler-López et al., 2011). ECSIT interacts with the molecular chaperone nicotinamide adenine dinucleotide ubiquinone oxidoreductase complex assembly factor 1 (Carneiro et al., 2018; Vogel et al., 2007). It also acts as a cofactor for small mother against decapentaplegic (Smad) proteins 1 and 4, and it leads to the transcription of T-cell leukemia homeobox protein 2 (Vogel et al., 2007; Xiao et al., 2003). In addition, ECSIT, as a scaffold protein coupling TLR and bone morphogenetic protein (BMP) signaling pathways (Kopp et al., 1999; Moustakas and Heldin, 2003; Xiao et al., 2003), is able to localize to the nucleus or the mitochondria, serving as a crossroad between oxidative stress, mitochondrial dysfunction- and inflammation-related
damaging signals (Vogel et al., 2007).

In AD, all these pathways related to oxidative stress, inflammation and cellular processes are altered and are critical for the accumulation of Aβ peptides and, thus, for the initial stages of this disorder (Mattson, 2012). In fact, the presence of Aβ peptides in the brain allows TLRs to recognize them as danger signals, triggering the release of inflammatory factors that contribute to AD progression (Heneka et al., 2015).

As for inflammation, mitochondrial dysfunction is another feature of AD. The extremely high energy demand of neuronal cells makes mitochondria essential in the brain. In fact, a decrease in the amount of mitochondria is common in the AD-brain (Cenini and Voos, 2019). Therefore, inflammation, oxidative stress and mitochondrial dysfunction are frequent in AD and they all involve ECSIT, suggesting that this protein may participate in the pathology of AD.

However, the role of ECSIT in neuronal cells, the possible links of this protein with Aβ or tau or its involvement in cognitive decline, memory impairment or toxic protein accumulation has not yet been demonstrated.

In chapter VI, we confirmed the recovery of sEVs-enriched preparations depleted of larger EVs. This prompted us to investigate, in this chapter, the presence of ECSIT protein in a mouse model of AD and in human samples, with the aim of assessing its possible role as a biomarker in plasma sEVs-enriched preparations for the early diagnosis of cognitive decline.

In human and also in the 3xTg mouse model, ECSIT protein was detected in our plasma sEVs-enriched preparations. In 3xTg mice, sEVs-ECSIT level was lower than in the wild type NTg mice, from the T0 – T7 (Figure 20 A). This may suggest a genotype-dependent effect of transgene expression. However, ECSIT level did not correlate with cognitive impairment in
3xTg mice (it was detected at 5 months of age (Figure 19)). This may suggest that ECSIT is already altered at early stages, which is a positive aspect considering its possible application as biomarker.

In human plasma, sEVs-ECSIT was decreased in demented subjects and showed a trend to reduction in MCI subjects when the sEVs content was analyzed by Western blotting (Figure 20B). Using ELISA, sEVs-ECSIT reduction in MCI was instead significant with regard to Ctrl subjects (Figure 20D). Moreover, to demonstrate sEVs separation as preferential technique for ECSIT, we also assessed ECSIT concentration in plasma from Ctrl and MCI subjects (Figure 20E). Compared to sEVs-ECSIT concentration, plasma-ECSIT did not show significant difference between Ctrl and MCI subjects. This suggests that ECSIT might be a biomarker associated with sEVs rather than with the whole body fluid. However, in the MCI group (Figure 20E), we found two distant clusters which require further analyses to confirm ECSIT biomarker nature and to determine how ECSIT protein change in MCI subjects that progress differently in a longitudinal study.

In parallel, we also measured sEVs-L1CAM protein using ELISA (Figure 20C) to assess whether an apparently neurodegeneration unrelated protein changed between Ctrl and MCI subjects. We found an increase in the MCI group, whose biological meaning or relevance requires further investigation. In any case, this increase is opposite to sEVs-ECSIT protein which confirms that ECSIT protein decrease in MCI subjects is not due to a general trend to reduction of all biological material in MCI nanoparticles, as might happen because of their lower concentration (see Figure 15D of Chapter VI).

Regardless of these results, we confirmed the co-localization of ECSIT protein with CD63 within sEVs of both mouse (Figure 20F) and human (Figure 20G) samples. This suggests that
ECSIT protein is released and carried within sEVs.

These results support that ECSIT protein associated with sEVs may be a possible biomarker for dementia. As it is explained in chapter III, 3.7.1., several proteins have been described within sEVs in AD and it would be interesting to combine ECSIT with one of the already reported biomarkers and determine if that given combination improve the diagnostic relevance. For instance, one of those already described could be the ones reported by Fiandaca et al. in NDEs; P-S396-tau, P-T181-tau and Aβ(1-42). All of them showed higher concentrations in AD than in Ctrl subjects (Fiandaca et al., 2015). In other studies, levels of synaptic proteins in NDEs have also been found dysregulated in AD and MCI subjects. This is the case of Ng, synaptophysin, synaptotagmin and synaptopodin which were decreased in plasma NDEs of MCI subjects (Winston et al., 2018). Other presynaptic proteins are also decreased in plasma NDEs derived from subjects with dementia due to AD; this is the case of the neuronal pentraxin 2 and neurexin 2α, and their respective postsynaptic partners’ glutamate ionotropic receptor AMPA type subunit 4 and neuroligin 1 (Goetzl et al., 2018). Another protein whose concentration was also lower in serum NDEs of AD with regard to Ctrl subjects is the SNAP25 (Agliardi et al., 2019). And several others such as the cathepsin D and the lysosomal-associated membrane protein 1 are also able to distinguish AD from Ctrl subjects (Goetzl et al., 2015).

In this chapter, we provided some biochemical analysis that support a possible correlation between ECSIT levels in sEVs-enriched preparations and cognitive decline in 3xTg mouse model for AD, subjects with signs of early cognitive decline and demented patients, suggesting ECSIT protein as a marker of early cognitive impairment or as a target to monitor responses to pharmacological therapies.

One limitation of our study is that we were not able to focus the population on the amnesic
component only for our MCI samples. However, some times, a differential diagnosis of MCI is not possible, and considering this, a marker of MCI may also be useful.

Further analyses are needed to confirm the link of ECSIT and neurodegeneration and to find a reason why ECSIT protein is reduced in sEVs-enriched preparations of MCI or AD. One possible explanation may be that ECSIT is involved in some pathways that become activated during the progression of dementia, such as inflammation or oxidative stress (Soler-López et al., 2012). One can hypothesize that in normal conditions, ECSIT has a physiologic turnover that involves its externalization in sEVs. In pathological conditions, the activation of the pathways in which ECSIT is involved, may require the presence of this protein inside the cells (Soler-López et al., 2012). Its levels inside sEVs, therefore, decline. This explanation, however, does not fit with our findings in the 3xTg mouse model. However, in mice the APP(SW) transgene expression starts very soon and ECSIT sEV-directed pathway could be dysregulated earlier, while the cognitive decline is detectable later. One possible way to confirm the hypothesis of the role of ECSIT in neurodegeneration could be to study the levels of this protein in sEVs directly isolated from brain tissue. Or, if one wants to avoid the use of L1CAM (as commented in chapter VI), the isolation of sEVs from CSF may be another possibility. However, neither of these options was feasible for us.
Chapter VIII. SEV-TREM2 and NFL as possible biomarkers for early dementia | Soraya Moradi Bachiller
CHAPTER VIII

SEV-TREM2 AND NFL AS POSSIBLE BIOMARKERS FOR EARLY DEMENTIA

8.1. EV-proteins reflect Alzheimer’s disease progression

In the previous chapter, we proposed ECSIT protein as a possible sEV-associated biomarker in dementia. In the progression of dementia due to AD, several EV-associated proteins have been identified and evaluated as possible biomarkers of disease-related molecular players.

After the first two studies showing Aβ peptides and p-tau carried within exosomes (Rajendran et al., 2006; Saman et al., 2012), many other proteins, apart from those already cited in chapter III. 3.7.1 (Agliardi et al., 2019; Fiandaca et al., 2015; Goetzl et al., 2015, 2016a, 2016b, 2018; Jia et al., 2019; Winston et al., 2016, 2018, 2019), have been identified in association with sEVs in the context of AD and neurodegeneration.

For the cellular models, HEK293-APP Swe/Ind cells, which are cells overexpressing APP Swe/Ind constructs, were capable of enriching their culture medium with exosomes containing APP (Zheng et al., 2018). As well as for APP, cystatin C protein and neuronal pentraxin-1 are also secreted within mouse primary neurons-derived exosomes or within plasma EVs from the E4FAD mouse model, respectively (Ghidoni et al., 2011; Ma et al., 2018).

P-tau and Aβ peptides are obvious candidates to be searched inside sEVs, and they have been reported with higher levels in AD subjects when carried within plasma brain tissue or urinary EVs (Muraoka et al., 2020; Sun et al., 2020a, 2020b), but they are not the unique AD-related proteins that have been associated with sEVs. The low-density lipoprotein receptor-related...
protein 6, heat-shock factor-1, and RE1 silencing transcription factor are other examples. All of them showed lower levels in plasma-NDEs in AD compared to Ctrl subjects (Goetzl et al., 2015). The authors suggested that since all of these proteins mediate neuronal response against stress, a decreased level within plasma-NDEs might link the incapacity of neuronal cells to resist the neurotoxicity in the AD brain. Others, such as the lysosome-associated membrane protein-2, which has an important role in the autophagic-lysosomal system and thus, in the metabolism of Aβ peptides in AD, have been found increased in the plasma exosome fraction from AD compared to Ctrl subjects (Krishna et al., 2020). CD38, CMRF35-like molecule 1, CMRF35-like molecule 6 and sialic acid-binding Ig-like lectin 9, with a role in Aβ secretion, microglia activation and immune response, were instead down-regulated in plasma-EVs from MCI and AD subjects (Nielsen et al., 2020).

A relevant biochemical feature of NDs is the increase of advanced glycation end products, described also in AD. In fact, advanced glycation end products can be found in the hippocampus and parahippocampus of the AD subjects and, also, in aged brains (Takeda et al., 1996). Haddad et al. measured levels of advanced glycation products N-(1-carboxymethyl)-L-lysine and pentosidine in serum and also in serum-EVs. The authors showed that levels of EVs N-(1-carboxymethyl)-L-lysine were lower in the moderate stage of AD with regard to other AD stages, MCI or Ctrl subjects, while no differences were observed when N-(1-carboxymethyl)-L-lysine was studied in total serum (Haddad et al., 2019).

AD is also an inflammatory disease and, thus, the complement pathway play an important role in its progression (Morgan, 2018), and the complement proteins have been studied within plasma exosomes. Specifically, in ADEs from AD and Ctrl subjects, it has been shown that levels of C1q, C4b, C3d, factor B, factor D, Bb, C3b and C5b-C9 terminal complement complex
were significantly higher in AD. Since complement is pro-inflammatory and, when dysregulated, it is able to damage neurons and glia cells, higher levels of complement proteins within plasma ADEs may drive and contribute to the inflammation in AD, as suggested by the authors (Goetzl et al., 2018).

TAR DNA-binding protein 43 proteinopathy has also been described as a pathological feature possibly leading to comorbidity in AD. The role of this protein in neurodegeneration and cognitive decline is apparent in other neurological diseases, such as FTD or ALS. In plasma NDEs from AD subjects, TAR DNA-binding protein 43 was increased. However, these levels did not correlate with the severity of cognitive function, neuropsychiatric symptoms (motor disturbance, hyperactivity, apathy and psychosis) or differ between APOEε4 carriers or non-carriers (Zhang et al., 2020).

The just presented non-exhaustive list of proteins that have already been identified within EVs in the field of dementia is continuously expanding and other candidates are under investigation. This is the case of NFL and TREM2. These two proteins have an important role in neurodegeneration/axonal damage (Khalil et al., 2018) and inflammatory responses (Ulland and Colonna, 2018), respectively. They change over the AD progression in diverse body fluids, such as blood or CSF (Lashley et al., 2018; Lewczuk et al., 2018; Rauchmann et al., 2019; Suárez-Calvet et al., 2019), but to the best of our knowledge they have ever been reported as EV-associated proteins in AD.
8.2. Aim of the study and experimental design

NFL and TREM2 are implicated in several NDs, including AD. However, their presence in EVs derived from subjects suffering from dementia has not been elucidated yet.

In this chapter, our aim is to address the following three questions:

- Are NFL and TREM2 released within plasma sEVs-enriched preparations derived from AD, MCI subjects or the 3xTg-AD mice?

- NFL and TREM2 change their levels in blood and CSF (Lewczuk et al., 2018; Rauchmann et al., 2019; Suárez-Calvet et al., 2019). Do they change when released within sEVs-enriched preparations due to aging or neurodegeneration process?

- If they are released within sEVs-enriched preparations, are they specifically associated with sEVs as biomarkers?

As we did in chapter VII, we addressed these questions in the 3xTg-AD animal model and in human subjects and, also, focusing on the low-invasive biomarker discovery strategy using plasma-EVs.

To this purpose we have collected blood from 3xTg-AD transgenic mice and their wild type control strain (NTg) from 3 (T0) to 10 months of age (T7). In addition, plasma samples were used from Ctrl, MCI and AD subjects. The clinical and socio-demographic information for the subjects used in this study are shown in Table 11. We then separated sEVs from plasma as previously described in chapter V. Levels of sEVs-NFL and sEVs-TREM2 were measured using Western blotting and ELISA.
We showed that NFL and TREM2 proteins are detectable within plasma sEVs-enriched preparations. However, their presence within our sEVs samples does not seem to be specific for sEVs and their expression pattern does not always correlate with cognitive onset in mice.

### 8.3. Results

#### 8.3.1. TREM2 and NFL proteins are carried within mouse plasma sEVs-enriched preparations

We measured levels of TREM2 and NFL proteins in sEVs-enriched preparations by Western blotting in our mouse model (mean±SD, N = 3) (Figure 21). For NFL protein, we did not observe any significant change though at 6 months of age (T3) we noticed an increase and a slightly but non-significant difference between the 3xTg and the NTg mice. The rest of the

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Table 11. Chapter VIII. Clinical and socio-demographic features of subjects. HIS, Hachinski ischemic score; SD, standard deviation; MMSE, mini-mental state examination.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl (N = 20)</th>
<th>MCI (N = 20)</th>
<th>AD (N = 3)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, mean (SD)</strong></td>
<td>74.75 (6.82)</td>
<td>79.65 (4.114)</td>
<td>76.333 (3.681)</td>
<td>0.027*</td>
</tr>
<tr>
<td><strong>Sex, M/F, no.</strong></td>
<td>8/12</td>
<td>12/8</td>
<td>1/2</td>
<td>0.44</td>
</tr>
<tr>
<td><strong>Education, mean (SD)</strong></td>
<td>8.9 (4.011)</td>
<td>9.3 (3.974)</td>
<td>7 (2.16)</td>
<td>0.639</td>
</tr>
<tr>
<td><strong>HIS, mean (SD)</strong></td>
<td>0.684 (1.172)</td>
<td>2.3 (1.307)</td>
<td>1 (1.414)</td>
<td>0.0008*</td>
</tr>
<tr>
<td><strong>MMSE, mean (SD)</strong></td>
<td>28.3 (2.193)</td>
<td>26 (2.644)</td>
<td>22 (2.83)</td>
<td>0.012†</td>
</tr>
</tbody>
</table>

‡Welch’s ANOVA, Fisher’s exact test for categorical data and *One-way ANOVA
time points showed lower levels of NFL protein. Only at 8 months of age (T5) a trend towards increased levels of NFL protein was found for both the NTg and the 3xTg mice (Figure 21 A). For TREM2 protein, we were able to detect its soluble form with 25 kDa but we did not find any significant change between both groups of mice for any time point analyzed. However, and in a similar way to what showed for NFL protein, a clear upraising trend was found at T3, turning back to lower TREM2 protein levels for the rest of the time points (Figure 21 B).
Figure 21. NFL and TREM2 proteins in plasma-derived sEVs-enriched preparations from
8.3.2. TREM2 and NFL proteins are carried within human plasma sEVs-enriched preparations

In parallel, NFL and TREM2 protein levels were also measured in Ctrl, MCI and AD subjects (mean±SD, N = 3 subjects) using Western blotting. We noticed a clear trend towards the increase of TREM2 and NFL proteins in AD and MCI subjects with regard to the Ctrl group (Figure 22 A, B). Moreover, for NFL protein, a significant increase was detected between Ctrl and AD subjects (Ctrl vs AD, q<sub>5.107</sub>, df<sub>6</sub>, *p = 0.026; Ctrl mean = 0.659, AD mean = 1.164) (Figure 22 B).

The concentration (pg/ml) of TREM2 (Figure 22 C) and NFL (Figure 22 D) proteins were measured by ELISA in Ctrl and MCI sEVs-enriched preparations (box and whiskers plots, N = 19/20 subjects for TREM2 and NFL, respectively). ELISA results indicated no significant changes for TREM2 protein levels between Ctrl and MCI group. A significant change towards the increase of NFL protein levels (although this significant increase is only a variation in the picogram fraction) in MCI subjects (t<sub>2.144</sub>, df<sub>38</sub>, *p = 0.0385; Ctrl mean = 8.655, MCI mean =
8.657) was observed.

**Figure 22. NFL and TREM2 proteins in plasma-derived sEVs-enriched preparations from Ctrl, MCI and AD subjects.** Western blot of TREM2 (A) and NFL (B) proteins in sEVs-enriched preparations (normalized to L1CAM, arbitrary units) from human plasma (Ctrl, MCI and AD) are shown. Each condition was assessed in triplicate and represented in scatter plots as mean±SD. Normal distribution and equality of variances were tested by the D’Agostino-Pearson omnibus normality test and the Brown-Forsythe test, respectively. One-way ANOVA was used, followed by Tukey’s multiple comparisons test. Concentrations (pg/mL) of TREM2 (C) and NFL (D) in sEVs-enriched preparations are also shown and represented in box and whiskers plots with the respective medians, interquartile ranges (box) and ranges (error bars). Normal
distribution was assessed with the D’Agostino-Pearson omnibus normality test and equality of variances were tested by the F test. Unpaired t-test (NFL concentration) or Mann-Whitney test (TREM2 concentration) were used. P value < 0.05 was considered as significant.

8.4. Discussion

In this chapter, we investigated the presence of TREM2 and NFL in plasma sEVs isolated from the 3xTg mouse model for AD and human samples from Ctrl, MCI and AD subjects. Our general purpose was to assess TREM2 and NFL potential role as biomarkers in plasma sEVs-enriched preparations for the early diagnosis of dementia. Our specific aims, instead, were, firstly, to assess TREM2 and NFL presence in human and mice plasma sEVs-enriched preparations; secondly, to study if their protein levels changed within sEVs-enriched preparations (as they do in blood and CSF) due to the AD progression or due to the aging process; and thirdly, to assess whether they are specifically associated to sEVs as biomarkers.

For those purposes, it is necessary to remember the characterization of sEVs-enriched preparations (chapter VI) and to consider that these preparations, from the 3xTg-AD model and their matched control strain NTg, were obtained from the same animals that performed the NORT from the 3rd to the 10th month of age (T0 – T7) (chapter VII, Figure 19).

Both TREM2 and NFL proteins were detected in mouse plasma sEVs-enriched preparations. sEVs-NFL protein levels followed a similar expression pattern from T0 - T7, except for the T3 (6 months of age). At this time point, sEVs-NFL levels tended to increase in the 3xTg mice with regard to the NTg mice (Figure 21 A) and decreased again from the T4.

At T2 (5 months of age) the cognitive impairment becomes visible for the 3xTg mice (chapter VII, Figure 19) and it is the month after when we observed this non-significant increase in
sEVs-NFL protein levels in 3xTg mice. This suggests that NFL protein levels in sEVs-enriched preparations may follow the onset of cognitive deficits in the 3xTg mouse model, and contrary to what happened with mouse plasma sEVs-ECSIT concentration (chapter VII, Figure 20 A), this also suggests that sEVs-NFL is not altered at early stages of the AD in the 3xTg mice. It seems, therefore, that the application of sEVs-NFL as early diagnostic biomarker may be useless. A limitation of our study is, as what concerns this possibility, that it was not feasible for us to do the Aβ and tau histopathological studies with 3xTg-AD brain tissue. This makes us difficult to determine whether the increase of sEVs-NFL protein levels in the 6-month-old-3xTg mice is related to the onset of Aβ and tau pathology, even though Oddo et al. reported, in the 3xTg mouse model, the extracellular Aβ deposits at 6 months and the tau pathology at 12 months (Oddo et al., 2003).

The increase of NFL protein in the 3xTg mice plasma sEVs-enriched preparations was also found in human plasma sEVs-enriched preparations using Western blotting and ELISA. A significant increase and a trend towards the increase were detected in sEVs-NFL protein levels in AD and MCI subjects, respectively, assessed by Western blotting (Figure 22 B). The significantly increase in MCI with regard to Ctrl subjects, assessed using ELISA, might, hypothetically, suggest that sEVs-NFL protein levels correlate with the early stages of AD-related dementia in humans (Figure 22 D).

However, NFL, as a type of intermediate filament commonly present in axons, may be not specific for AD. In fact, several studies have reported increased levels of NFL protein either in blood or CSF from several neurological disorders, such as Parkinson’s disease (PD) (Lin et al., 2018), Down syndrome, amyotrophic lateral sclerosis, DLB, progressive supranuclear palsy, corticobasal syndrome (Delaby et al., 2020; Olsson et al., 2019) or AD (Mattsson et al., 2017).
The increased concentration of NFL reflects the neurodegeneration process, the onset of cognitive symptoms and the axonal damage (Zetterberg et al., 2016), suggesting that blood and CSF NFL (and, coherently, sEVs-NFL) have more prognostic than diagnostic value as biomarkers in humans. Its usefulness is then limited to the discrimination of two neurological disorders with different disease intensity and disease progression (Olsson et al., 2019).

Another matter concerning the use of NFL as biomarker in AD or non-AD dementias is the fact that NFL variations may be also part of the normal aging process. For example, Nyberg et al. showed that, at baseline and in a follow up study, plasma NFL protein levels increase as part of the neurodegeneration process in disease-free and aged brains (Nyberg et al., 2020). The same was shown by Idland et al. in CSF (Idland et al., 2017).

Moreover, several studies have reported that NFL protein levels predict either normal aging or other AD features (such as hippocampal atrophy) regardless of the Aβ pathology (Idland et al., 2017; Lim et al., 2020; Milà-Alomà et al., 2020). However, in what concerns this matter, other studies have observed the opposite. For example, Kang et al. reported a positive correlation (in AD and MCI subjects) for increased CSF/plasma NFL concentration and reduced grey matter density, but only in AD-vulnerable regions (precuneus/posterior cingulate cortex and hippocampus) showing positivity for Aβ (Kang et al., 2020).

Other studies have reported an association between NFL concentration in diverse body fluids and APP, PSEN1 or PSEN2 mutations. For example, Preische et al. showed increased CSF and serum NFL levels in pre-symptomatic familial AD mutation carriers (APP, PSEN1 or PSEN2). Moreover, serum NFL levels were able to discriminate the mutation carriers from non-carriers a decade before the onset of symptoms (Preische et al., 2019). In a longitudinal study, Quiroz et al. showed that plasma NFL increases with age and was able to discriminate carriers of PSEN1
E280A mutation and non-carriers when close to the clinical onset (Quiroz et al., 2020).

This link between NFL concentration, the healthy aging process, Aβ/tau pathology and mutations in APP, PSEN1 and PSEN2 should be further explored in our experiments with humans. For example, the association between sEVs-NFL levels with the healthy aging process could be tested by measuring sEVs-NFL concentration in aged Ctrl, MCI and AD subjects and comparing them with their respective younger partners. The link between sEVs-NFL levels and Aβ and tau pathology or the presence of APP, PSEN1 and PSEN2 mutations could be assessed by collecting more clinical data about the subjects enrolled or by dosing sEVs in AD mutation carriers.

With regard to the specificity of sEVs-NFL as biomarker, several studies have reported, in blood and CSF, increased NFL concentration that reflects and correlates with brain atrophy (Mattsson et al., 2016; Moore et al., 2020), grey matter volume loss (Dhiman et al., 2020), neuronal damage (Khalil et al., 2020; Zhou et al., 2017), white matter lesion volume (Schultz et al., 2020), cognitive performance (Aschenbrenner et al., 2020; Sugarman et al., 2020), NFT pathology (Ashton et al., 2019a; Lista et al., 2017) or brain hypometabolism (Mattsson et al., 2017). In human plasma sEVs-enriched preparations we also observed this trend towards the increase in MCI and a significant increase in AD subjects (Figure 22 B). In other words, it seems that NFL protein levels change in the same direction (it increases when AD progresses) either in body fluids (such as plasma and CSF) or sEVs-enriched preparations. This suggests that NFL protein may not be a biomarker specifically associated with sEVs.

TREM2 is a transmembrane receptor belonging to the immunoglobulin superfamily. It consists of an extracellular domain, an ectodomain, a transmembrane helix and a cytosolic tail (Peng et al., 2010). In AD pathogenesis, Aβ(1-42) oligomers can bind and activate TREM2 signaling
(Zhao et al., 2018; Zhong et al., 2018). It is expressed on the cell surface of microglia, macrophages, osteoclasts and dendritic cells, as well as bronchial epithelial cells, fibroblasts and lung adenocarcinoma cells. The cleavage of the membrane-spanning domain by the disintegrin and metalloproteinase domain-containing proteins 10 and 17 produces sTREM2 protein fragment (Bekris et al., 2018; Feuerbach et al., 2017; Wunderlich et al., 2013). TREM2 is responsible for the microglia responses to Aβ pathology and for the modulation of microglial activity in non-diseased brains (Filipello et al., 2018; Ulland et al., 2017).

SEVs-TREM2 expression followed a pattern similar to sEVs-NFL protein levels for the 3xTg mouse model (Figure 21B). In fact, the higher increase we observed also happened at 6 months of age (T3). However, sEVs-TREM2 protein levels increased both in the NTg and the 3xTg mice, without any significant difference between the two groups of animals, returning to lower levels from the T4. Moreover, in this case, there was not a sudden increase, as it happened for sEVs-NFL concentration at T3. For both NTg and 3xTg, we noticed a steady increase that became evident at T2 and T3. The fact that sEVs-TREM2 increases in both mice groups (NTg and 3xTg) suggests that this expression change does not reflect the cognitive impairment in the 3xTg mice but rather an age-associated shift in brain inflammatory proteins (Barrientos et al., 2015; Von Bernhardi et al., 2015). In other words, from the 3rd to the 6th month of age, NTg and 3xTg mice microglial cells reach their maturity and fate. During this period, there is an up-regulation of glial activation markers (Barrientos et al., 2015) regardless of the presence of cognitive impairment (VanGuilder et al., 2011). And thus, it seems plausible that sEVs-TREM2 concentration increases during the maturing age period while decreasing during the adult period (Hickman et al., 2013). These age-dependent changes for sEVs-TREM2 protein levels are consistent with other studies that have reported the association between TREM2 expression at
different developmental stages, showing that TREM2 is highly expressed in C57BL/6 mice up to the postnatal day 5 while it becomes undetectable after postnatal day 10 (Chertoff et al., 2013).

Instead, in human plasma sEVs-enriched preparations, TREM2 protein levels showed a trend towards the increase in MCI and AD with regard to Ctrl subjects (Figure 22 A). The same was observed with a more sensitive assay, where sEVs-TREM2 levels showed an increase in MCI compared to Ctrl subjects, although non-significant (Figure 22 C). Overall, as happened for sEVs-ECSIT (chapter VII, Figure 20 D) and sEVs-NFL (Figure 22 D) proteins, the expression change in TREM2 within sEVs-enriched preparations seems specific for a peculiar biochemical pathway and related to the aging or neurodegenerative processes.

This non-significant increase of TREM2 (assessed by Western blotting) and MCI (assessed by Western blotting and ELISA) compared to Ctrl subjects has already been reported in AD and, also, for other diseases such as neurological and psychiatric disorders (Mori et al., 2015) or PD (Wilson et al., 2020).

Supporting the idea that TREM2 is not a specific biomarker that reflects Aβ and tau deposition in AD brain, there are other studies such as one from Ohara et al. that followed up during 10 years how sTREM2 protein changes in people without dementia. At the end of the study, 300 out of 1349 subjects developed AD and other non-AD related dementias. All these subjects had higher sTREM2 levels in serum. This, and the previous cited studies, suggests that, as for sEVs-NFL protein, sEVs-sTREM2 is not a specific diagnostic biomarker for AD but rather a biomarker related to the risk of dementia (Ohara et al., 2019).

However, there are studies that reported CSF sTREM2 levels in AD subjects associated with
Aβ and tau pathology (Bekris et al., 2018). This correlates with other studies showing higher CSF sTREM2 levels in MCI and AD and a positive correlation with CSF t-tau and P-T181-tau (Heslegrave et al., 2016; Suárez-Calvet et al., 2016). This suggests that, in response to neuronal degeneration, microglia activation status also changes, and this can be reflected in TREM2 levels.

Previous studies showed an up-regulation of TREM2 in the frontal cortex of sporadic AD subjects as a late event that did not correlate with plaques, tangles or cognitive performance (Perez et al., 2017). Something similar was reported by Rauchmann et al. who showed that CSF sTREM2 is associated with markers of neurodegeneration and tau pathology while it was not related to amyloidosis. CSF sTREM2 increased over time with AD pathology in t-tau positive vs. negative individuals, while it was not related with cognition or other biomarker changes over time (Rauchmann et al., 2019). On the other hand, Ma et al. showed that a decrease in CSF sTREM2 levels are associated with Aβ pathology, while an increase in CSF sTREM2 is associated with tau pathology and neurodegeneration (Ma et al., 2020). This possible association with Aβ and tau pathology should be further explored in our studies as suggested for NFL protein.

Coming back to our analysis, the association between the aging process and sEVs-TREM2 protein levels in humans was difficult to establish, since it was not possible for us to compare sEVs-TREM2 concentration change in Ctrl, MCI and AD subjects using a longitudinal design. However, previous studies have shown that a little increase in CSF sTREM2 concentration of AD subjects was due to the aging process itself (Bekris et al., 2018). Something similar was observed by Henjum et al. They showed that there was a positive correlation between sTREM2 and aging among Ctrl subjects (Henjum et al., 2016).
Contrary to what happened for plasma sEVs-NFL concentration, our results for TREM2 levels in human plasma sEVs-enriched preparations do not correlate with what has been previously reported. Indeed, Liu et al. demonstrated that CSF sTREM2 levels significantly increase in MCI and AD subjects while sTREM2 in plasma did not change among these groups (D. Liu et al., 2018). The same was reported by Ashton and colleagues (Ashton et al., 2019b). Our results suggest a trend towards the increase, though non-significant, of TREM2 levels in MCI and AD plasma-sEVs (Figure 22 A). This hypothetical different pattern of TREM2 protein expression between plasma-sEVs and whole plasma suggests that TREM2 may be a candidate as specific biomarker for dementia risk specifically associated with sEVs. Further analyses are needed to confirm the advantage of studying TREM2 within sEVs with regard to the body fluid. Further studies are also needed to confirm the presence of TREM2 or sTREM2 inside sEVs and not only within sEVs-enriched preparations. One possible way to attempt this may be to study the co-localization of TREM2 and sTREM2 with CD63 inside sEVs, as we did for ECSIT.

In conclusion, our results support that both NFL and TREM2 are carried within sEVs-enriched preparations derived from the 3xTg mice and from MCI and AD subjects. We can also suggest that sEVs-NFL protein levels correlate with the cognitive impairment onset in the 3xTg mice, while sEVs-TREM2 might be more related to the age-associated changes of inflammatory markers and microglial cells maturity.

With regard to humans, both proteins in sEVs-enriched preparations showed a trend towards the increase in MCI and AD subjects. Controversial results have been published for both proteins and since we were not able to focus the population on the amnesic component only for the MCI samples, it was difficult for us to confirm whether expression changes of TREM2 and NFL perfectly reflect AD or non-AD dementias. And, finally, only TREM2/sTREM2 might be
specifically associated with sEVs. However, as previously said, further analyses are needed to confirm this hypothesis.

Since TREM2 and NFL proteins are not specific of AD but more related to the neurodegeneration process, it may be interesting to see if the combination of these two proteins with other possible biomarkers for AD (apart from Aβ and tau) improve their prognostic value as biomarkers in AD. This is something already reported by some groups. For example, Suárez-Calvet et al. identified higher CSF level of progranulin and sTREM2 only when there was underlying pathology (neuronal injury), allowing both of them to act as microglial activity markers which reflect the microglia activation during the disease or microglial changes during therapeutic interventions (Suárez-Calvet et al., 2018).
Chapter IX
CHAPTER IX

SEV-miRNAs AS POTENTIAL BIOMARKERS OF ALZHEIMER’S DISEASE PROGRESSION

9.1. EV-miRNAs reflect and influence Alzheimer’s disease progression

Protein changes, such as those happening with APP and tau protein, have been directly related to AD. However, miRNAs may also play an important role in the development of AD, since they control gene expression. This control relies on their ability to target mRNA complementary regions. The miRNA-mRNA binding results in the inhibition or degradation of the mRNA by the miRISC, as previously described in chapter III, 3.3.2.

In subjects suffering from AD, dysregulations of miRNAs moderate the expression of AD-related genes and, thus, they also reflect the phenotype derived from this disease. For example, in rat hippocampal neurons, miR-101 binds the 3'UTR of APP, reducing APP and Aβ load (Vilardo et al., 2010). MiR-186 suppresses BACE1 expression by targeting BACE1 mRNA and reduces Aβ load. The inhibition of endogenous miR-186, instead, increases BACE1 protein levels in neuronal cells (Kim et al., 2016). Another post-transcriptional regulator of APP is the miR-16, whose overexpression leads to reduced APP expression in vitro and in vivo (Liu et al., 2012). And the same happens with miR-384 that also suppresses mRNA and protein expression of both APP and BACE1 in SH-SY5Y cells (Liu et al., 2014b).

As for APP, miRNAs dysregulation also drives tau pathology. This is the case of miR-219. In a Drosophila model that produces human tau, the decrease of miR-219 was shown to exacerbate tau toxicity while its overexpression represses tau synthesis and decreases its toxic effects.
The expression of endogenous tau protein in human neuroblastoma cell lines is also controlled and repressed by the miR-34a, while the inhibition of miR-34 family leads to an increase of tau expression (Dickson et al., 2013).

Dysfunctionalities in the miRNA-processing machinery are critical for the development of brain atrophy, neurodegeneration and locomotor deficits. This suggests that miRNAs are also essential for the integrity and function of the central nervous system. This essential role of miRNAs has already been demonstrated as for the spatial and recognition memory formation (Hansen et al., 2016), neural progenitor cell proliferation (Bian et al., 2013), axonal growth of retinal ganglion cells (He et al., 2018), axonal development of cortical neurons (Dajas-Bailador et al., 2012) and neuronal differentiation (Makeyev et al., 2007), among others.

In 2008, Lawrie et al. provided the first evidence in humans that miRNAs are reliably detected in serum. The authors compared the tumor-associated miR-155 and miR-21 in serum from B-cell lymphoma patients and healthy controls, and showed that these two miRNAs could efficiently discriminate patients from the healthy control group (Lawrie et al., 2008). After this study, several authors have demonstrated that miRNA expression is also altered in subjects suffering from AD (Kenny et al., 2019a; Müller et al., 2016; Satoh et al., 2015).

MiRNAs are able to control the expression of AD-related genes, essential for the proper function of the central nervous system and to be detected in body fluids throughout the AD stages. Moreover, miRNAs have several advantages that make them likely good biomarkers. They are stable in circulation, they are resistant to RNAse digestion and to many extreme conditions, such as high temperature, extended storage at room temperature, boiling or extreme pH (Creemers et al., 2012). However, they also show some disadvantages since changes in their expression strongly depend on several factors such as the race, the gender or the biofluid in which a given
miRNA is being studied (e.g. CSF, whole blood, plasma or serum) (J.-J. Chen et al., 2019).

Endogenous miRNAs in the body fluids are protected, to avoid their degradation, by three mechanisms. MiRNAs can be associated to RBPs, lipoprotein complexes (e.g. high-density lipoprotein) and sEVs. The process by which miRNAs are targeted to bind RBPs, lipoprotein complexes or sEVs seems to be selective. Selective for the mechanism of extracellular transport but also for the miRNA, since cells may select miRNAs for their release and others for being retained (Creemers et al., 2012).

The release of free (associated with proteins) miRNAs in body fluids makes them to be more exposed to external conditions. To this regard, sEVs can avoid this problem. Moreover, sEVs can bypass the BBB, and, thus, brain-associated miRNAs can also cross it. That is, sEVs properties and characteristics allow sEV-miRNAs to reflect better the neurodegeneration process in every biofluid.

As previously described in chapter III, 3.7.1., several miRNAs have been shown to be released within EVs and to change their expression in AD. This opens up the possibility that sEV-miRNAs in AD may reflect neurodegeneration, influence the disease progression by affecting the translation process of targeted mRNAs, and characterizing the state of the disease. And, thus, they may contribute to the diagnosis of AD.

9.2. Aim of the study and experimental design

Compelling evidence has proved that EV-miRNAs have the potential to be a source of biomarkers for several diseases, such as AD. In the present chapter, we aimed to preliminarly explore expression levels of plasma sEVs-associated miRNAs of 4 Ctrl, 4 MCI and 4 AD
subjects (clinical and socio-demographic characteristics of subjects involved in this study are shown in Table 12) using RNA-seq. The preparation of the sEVs, the sEV-miRNAs, the libraries and the bioinformatics analyses performed and cited below are shown in details in chapter V.

<table>
<thead>
<tr>
<th></th>
<th>Ctrl (N = 4)</th>
<th>MCI (N = 4)</th>
<th>AD (N = 4)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>72.5 (1.5)</td>
<td>75.5 (4.715)</td>
<td>82.75 (7.324)</td>
<td>0.089</td>
</tr>
<tr>
<td>Sex, M/F, no.</td>
<td>2/2</td>
<td>2/2</td>
<td>2/2</td>
<td>N/A</td>
</tr>
<tr>
<td>Education, mean (SD)</td>
<td>11 (2.23)</td>
<td>9.75 (3.27)</td>
<td>7.25 (2.274)</td>
<td>0.259</td>
</tr>
<tr>
<td>HIS, mean (SD)</td>
<td>0 (0)</td>
<td>2 (1.22)</td>
<td>0.75 (1.293)</td>
<td>0.108</td>
</tr>
<tr>
<td>MMSE, mean (SD)</td>
<td>29.75 (0.43)</td>
<td>25.5 (2.69)</td>
<td>19.25 (3.766)</td>
<td>0.017 ‡</td>
</tr>
</tbody>
</table>

‡Welch’s ANOVA test.

Table 12. Chapter IX. Clinical and socio-demographic features of subjects. HIS, Hachinski ischemic score; SD, standard deviation; MMSE, mini-mental state examination.

Since we isolated the total population of miRNAs in sEVs-enriched preparations from plasma and not the miRNAs in NDEs-enriched preparations, two more analyses were done to select only sEVs-miRNAs that show a differential expression according to the diagnosis and the localization.

The first analysis aimed to predict the AD-associated mRNA targets for our plasma sEV-miRNAs. In other words, we aimed to correlate mRNA–miRNA pair expression over the AD
progression with the rationale of selecting and focusing only in sEV-miRNAs with a differential expression according to the diagnosis. For this purpose, we used two published GEO datasets (GSE63060 and GSE63061) which contain batch 1 (with 329 samples) and 2 (with 388 samples) of AD, MCI and Ctrl blood mRNA samples from the AddneuroMed Cohort (Lovestone et al., 2009). The GSE63060 and GSE63061 datasets contain AD subjects diagnosed using the CDR sum of boxes and MMSE scores. Ctrl and MCI subjects were tested using the CERAD battery test, and MMSE scores were also obtained for all of them (Sood et al., 2015).

The second analysis aimed to evaluate how the significant and DE sEV-miRNAs change their expression when free (associated with proteins) in serum. The rationale of this study was to select only dysregulated sEV-miRNAs that show differential expression according to the miRNA localization (in this case, in sEVs compared to serum). For this purpose, we took advantage of another cohort of subjects (GSE120584) with serum miRNA samples data (Table 13).

The GSE120584 dataset contains 1601 serum samples. 1021 subjects were AD subjects, 91 had VaD, 169 patients with DLB, 32 patients with MCI and 288 were Ctrl subjects. Some of them had cognitive complaints. However, they showed normal cognition when neuropsychological tests were done. All the Ctrl subjects had MMSE score > 23. Subjects suffering from AD and MCI were diagnosed with probable or possible AD. The probable AD subjects were used as AD group. The diagnosis was conducted using the neurological examination, neuropsychological tests (MMSE, AD assessment scale cognitive component-japanese version, logical memory I and II from the Wechsler memory scale, frontal assessment battery, Raven’s progressive matrices and geriatric depression scale), brain imaging (magnetic resonance imaging or computerized tomography), medical history and physical examination. Biomarkers in CSF were
not used for the diagnosis of dementia. For all the subjects, the MMSE score and the APOE genotype were obtained. In addition, subjects were > 60 years old (Shigemizu et al., 2019).

<table>
<thead>
<tr>
<th>GEO dataset</th>
<th>Platform</th>
<th>Matrix</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE120584</td>
<td>GPL21263</td>
<td>Human miRNA Serum (miRNAs)</td>
<td>1601</td>
</tr>
<tr>
<td>GSE63060 and GSE63061</td>
<td>GPL6947 and GPL10558</td>
<td>Illumina HumanHT-12 Expression beadchip</td>
<td>Blood (RNAs) 717</td>
</tr>
</tbody>
</table>

Table 13. Detailed information of GEO datasets used in this study.

GO analysis of DE miRNAs selected after the three analysis cited above, were performed with the aim to obtain a more comprehensive information of their function.

As we have already introduced, sEVs-specifically associated miRNAs exist because of the miRNA sorting mechanisms into exosomes (that are also specific), and their expression may change according to their localization. Determining which miRNAs are associated with sEVs as biomarkers of AD is essential and one of the most common unresolved question for several miRNAs (this will be covered in details in the discussion of this chapter). For this purpose, and since we are looking for biomarkers in early dementia, we decided to compare our DE sEV-miRNAs to already published data to determine which of them could be really sEV-associated biomarkers. To do so, a systematic search of literature in PubMed was conducted to retrieve articles discussing miRNAs in AD (for details chapter V). The direction of dysregulation (relative to Ctrl subjects in most of the cases) and the tissue/matrix (blood, plasma, serum, CSF and exosomes/NDEs/EVs) for the 225 DE miRNAs were listed and compared with DE miRNAs.
found in our plasma sEVs-enriched preparations.

We showed that from all the significant dysregulated sEV-miRNAs in plasma from AD subjects, only miR-15a-5p, miR-384 and miR-424-5p might be specifically released by sEVs. However, none of dysregulated sEV-miRNAs can predict or diagnose dementia in the early stage.

9.3. Results

9.3.1. RNA-seq identified significant DE miRNAs in AD group when compared to Ctrl subjects

MiRNAs isolated from plasma sEVs of 4 Ctrl, 4 MCI and 4 AD subjects were used to prepare the libraries for the RNA-seq as described in detail in the chapter V. Twelve nucleotides were obtained after the adapter trimming of single-end reads of 76 nucleotides. Reads were mapped to the miRBase database (alignment data is provided in the excel file “Alignment_data_RNAseq”). The average mapping resulted in the 9% of the reads. Then, the differential expression in the conditions AD vs Ctrl and MCI vs Ctrl was calculated using DESeq2 package (see excel files “diff-expres-DESeq2-ADvsCtrl_Plasma_sEVs” and “diff-expres-DESeq2-MCIvsCtrl_Plasma_sEVs”). The AD vs Ctrl contrast yielded 78 differentially and significantly expressed miRNAs with an adjusted p value (padj) < 0.1 and |log2FC| > 0.28. The MCI vs Ctrl contrast did not report any significant miRNA.

Among all detected dysregulated miRNAs, 18 miRNAs were found in the intersection of the top ranked (according to the padj) 100 miRNAs (Figure 23) of both AD/MCI vs Ctrl contrasts. The shared DE miRNAs are also listed in Figure 23.
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<table>
<thead>
<tr>
<th>TOP 100_miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td>8084, 4783-3p, 424-5p, 7704, 6804-3p, 3942-3p, 24-2-5p, 5571-5p, 623, 6786-3p, 4481, 633, 6877-3p, 504-3p, 3920, 887-3p, 497-5p, 523-3p</td>
</tr>
</tbody>
</table>

Figure 23. Venn diagrams depicting the unique and shared miRNAs of the intersection between the top 100 miRNAs that were up-regulated or down-regulated in AD vs Ctrl and MCI vs Ctrl contrasts. The first 100 miRNAs of each list (according to the padj) were compared and Venn diagram shows the number of identified miRNAs that are common (18) or unique (82) for the contrasts between AD/MCI and Ctrl groups. The table, instead, shows the IDs of shared miRNAs.

9.3.2. Plasma sEV-miRNAs matched with the corresponding blood mRNA target and did not perfectly fit with miRNA expression in serum

Dysregulated plasma sEV-miRNAs and blood transcripts (mRNAs) of the GSE63060 and GSE63061 datasets were correlated and paired. To get results as informative as possible considering the limited sample size, we decided to correlate the data when the following conditions met:

- MRNAs showed a significant and differential expression change over AD progression and so did sEVs-miRNAs with whom they matched.
- The expression changes in the mRNA had to be fulfilled by batches 1 and 2 (GSE63060
Moreover, the direction of the change in the expression of that given mRNA throughout AD stages was the same for batches 1 and 2.

Only one pattern of expression change in the mRNA was considered. Over the AD progression, the change occurred in the same direction. Therefore, from Ctrl to MCI and from MCI to AD, the direction of the change was the same. That is, if the gene expression increases in MCI with regard to Ctrl subjects, it also increases in the AD group, and vice versa.

And, finally, the miRNA-mRNA pairs occur when miRNA and mRNA expression change in the opposite direction, due to the inhibitory action of miRNAs over gene expression. In other words, miRNAatap R package paired the DE mRNAs of GSE63060 and GSE63061 (p value < 0.01) with their corresponding sEVs-miRNAs (padj < 0.1) (see excel file “diff-exprs-DESeq2-ADvsCtrl_Plasma_sEVs”) when they showed the opposite expression change throughout AD stages.

Then, the presence of significant and DE plasma sEV-miRNAs (all of them with their corresponding pairs mRNA target) was also evaluated in serum of 288 Ctrl, 32 MCI and 1021 AD subjects (obtained from the published GEO dataset GSE120584). Significant and steady miRNAs’ expression changes in serum throughout AD stages (Ctrl, MCI and AD) were identified (see excel file “GSE120584_gammaCtrl-MCI-AD_10bootstraps_miRNA_in serum”). MiRNAs whose presence was demonstrated in both matrices (sEVs-enriched preparations and serum) were classified in 3 groups (Table 14). The first was represented in blue color and
included miRNAs whose $|\gamma|$ value across Ctrl, MCI and AD groups in serum was $> 0.1$ (DE but not significant) and showed the opposite expression change in sEVs (that is, if that given miRNA was up-regulated in sEVs-enriched preparations, it was down-regulated when studied in serum, and vice versa). The second group was the green-colored one and showed miRNAs with a $|\gamma|$ value $> 0.1$ and with the same expression change in sEVs-enriched preparations than in serum. The last group, was marked in black color and represents miRNAs whose $|\gamma|$ value $< 0.1$ (and, thus, whose change in serum was not considered different across AD stages), while their change in sEVs-enriched preparations was shown to be up- or down-regulated (Table 14).

Finally, the information of these three analyses (miRNA expression in sEVs, miRNA expression in serum and mRNA expression in blood) was displayed in Figures 24 and 25. In Table 14 we showed miRNA expression in sEVs-enriched preparations (up- or down-regulation), the corresponding padj values for MCI vs Ctrl and AD vs Ctrl contrasts, and the miRNA expression gamma values in serum over AD stages (Ctrl, MCI and AD).
Table 14. DE plasma sEV- and serum- miRNAs in AD compared to Ctrl subjects. MiRNAs show here (first column) match the corresponding blood mRNA target of datasets GSE63060 and GSE63061. These miRNAs, in sEVs-enriched preparations, only reached the significance level (padj < 0.1) in the AD vs Ctrl contrast (second column) while significance was not achieved for them in the MCI vs Ctrl contrast (third column). The direction of the change in the expression for sEV-miRNAs in AD compared to the relative Ctrl subjects is also shown (fourth column). The Goodman and Kruskal's gamma coefficients (gamma mean values) across all the AD stages are indicated for miRNAs in serum (fifth column). MiRNAs were classified in three groups; group 1 in blue, group 2 in green and group 3 in black. Student’s t-test was used to detect DE miRNAs in sEVs-enriched preparations (fourth column). P values were calculated by DESeq2 and adjusted for multiple testing comparisons using the Benjamini–Hochberg method (second and third columns). UP, up-regulated; DW, down-regulated; positive gamma mean value means UP and a negative gamma mean value means DW in the last stage of the AD with regard to Ctrl subjects.

<table>
<thead>
<tr>
<th>miRNA ID</th>
<th>padj AD vs Ctrl</th>
<th>padj MCI vs Ctrl</th>
<th>sEV-miRNA’s expression change in AD vs Ctrl</th>
<th>miRNA’s gamma mean value in serum over AD stages</th>
</tr>
</thead>
<tbody>
<tr>
<td>384</td>
<td>0.004</td>
<td>0.835</td>
<td>UP</td>
<td>-0.126</td>
</tr>
<tr>
<td>3605-3p</td>
<td>0.009</td>
<td>0.880</td>
<td>DW</td>
<td>0.138</td>
</tr>
<tr>
<td>577</td>
<td>0.015</td>
<td>0.807</td>
<td>UP</td>
<td>-0.111</td>
</tr>
<tr>
<td>4312</td>
<td>0.075</td>
<td>0.940</td>
<td>UP</td>
<td>-0.107</td>
</tr>
<tr>
<td>633</td>
<td>0.076</td>
<td>0.750</td>
<td>UP</td>
<td>-0.115</td>
</tr>
<tr>
<td>100-3p</td>
<td>0.099</td>
<td>0.915</td>
<td>UP</td>
<td>-0.129</td>
</tr>
<tr>
<td>4756-5p</td>
<td>0.008</td>
<td>0.888</td>
<td>DW</td>
<td>-0.149</td>
</tr>
<tr>
<td>4695-5p</td>
<td>0.077</td>
<td>0.858</td>
<td>DW</td>
<td>-0.117</td>
</tr>
<tr>
<td>24-2-5p</td>
<td>0.032</td>
<td>0.750</td>
<td>UP</td>
<td>-0.091</td>
</tr>
<tr>
<td>4715-3p</td>
<td>0.034</td>
<td>0.867</td>
<td>DW</td>
<td>-0.091</td>
</tr>
<tr>
<td>27a-5p</td>
<td>0.046</td>
<td>0.807</td>
<td>UP</td>
<td>-0.037</td>
</tr>
<tr>
<td>2113</td>
<td>0.046</td>
<td>0.814</td>
<td>DW</td>
<td>0.075</td>
</tr>
<tr>
<td>4307</td>
<td>0.055</td>
<td>0.814</td>
<td>UP</td>
<td>0.002</td>
</tr>
<tr>
<td>1972</td>
<td>0.060</td>
<td>0.963</td>
<td>DW</td>
<td>-0.040</td>
</tr>
<tr>
<td>424-3p</td>
<td>0.005</td>
<td>0.865</td>
<td>DW</td>
<td>-0.089</td>
</tr>
<tr>
<td>4683</td>
<td>0.076</td>
<td>0.983</td>
<td>DW</td>
<td>-0.086</td>
</tr>
<tr>
<td>580-3p</td>
<td>0.032</td>
<td>0.882</td>
<td>UP</td>
<td>-0.060</td>
</tr>
</tbody>
</table>
Figure 24. Selected sEV-miRNAs with negative expression change throughout AD stages. Each graph represents, across Ctrl (violet shades), MCI (blue shades) and AD (yellow shades) subjects, the miRNA expression changes in plasma sEVs-enriched preparations (top of the graph), the miRNA expression change in serum (GSE120584) (middle of the graph) and the mRNA target in blood (GSE63060 and GSE63061) (bottom of the graph).
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Soraya Moradi Bachiller

A. hsa-miR-384
B. hsa-miR-384
C. hsa-miR-384
D. hsa-miR-24-2-5p
E. hsa-miR-577
F. hsa-miR-580-3p
G. hsa-miR-4312
H. hsa-miR-4312
I. hsa-miR-27a-5p
J. hsa-miR-4307
K. hsa-miR-633
L. hsa-miR-100-3p

CTRL  MCI  AD
Figure 25. Selected sEV-miRNAs with positive expression change throughout AD stages. Each graph represents, across Ctrl (violet shades), MCI (blue shades) and AD (yellow shades) subjects, the miRNA expression changes in plasma sEVs-enriched preparations (top of the graph), the miRNA expression change in serum (GSE120584) (middle of the graph) and the mRNA target in blood (GSE63060 and GSE63061) (bottom of the graph).

9.3.3. Gene ontology analysis explored the potential function and cellular compartment of significant DE sEV-miRNAs

To explore the potential biological process (BP), molecular function (MF) and cellular component (CC) of miRNAs from groups 1, 2 and 3 (Table 14) in the AD progression, we performed GO analysis of the most probable target genes of the DE sEVs-miRNAs. Significantly expressed (p value < 0.01, Kolomnogorov-Smirnov test) GO terms for each dysregulated sEV-miRNAs of Table 14 were identified. The BP analysis showed that the up-regulated sEVs-miRNAs were mostly enriched for phosphorylation (GO:0016310), positive regulation of hydrolase activity (GO:0051345), positive regulation of cellular processes (GO:0048522), drug metabolic processes (GO:0017144) and positive regulation of macromolecule biosynthetic processes (GO:0010557). The CC analysis showed that these miRNAs were significantly enriched for axon (GO:0030424) and vesicle lumen (GO:0031983), and the MF analysis showed that miRNAs were significantly enriched for chromatin binding (GO:0003682) and adenyl nucleotide binding (GO:0030554).

Meanwhile, for down-regulated sEVs miRNAs, cell-cell adhesion via PM adhesion molecules (GO:0098742), animal organ development (GO:0048513), negative regulation of cellular macromolecule biosynthetic process (GO:2000113), cell adhesion (GO:0007155), positive
regulation of transport (GO:0051050), regulation of transcription by RNA polymerase II (GO:0006357) and regulation of nucleobase-containing compound metabolic process (GO:0019219) in BP; bounding membrane of organelle (GO:0098588), protein-containing complex (GO:0032991) and integral component of PM (GO:0005887) in CC; RNA binding (GO:0003723), tubulin binding (GO:0015631) and transcription regulator activity (GO:0140110) in MF.

9.3.4. A plasma sEV-based 3-miRNA signature was able to distinguish AD from Ctrl subjects

We then performed multiple literature search to identify human body fluid-based miRNA biomarker for AD. Any report of differential miRNA expression in AD stages in CSF, blood and EVs was considered as long as the study was done in human subjects. All these studies, showed in Table 15, described the presence of dysregulated miRNAs in whole blood, serum, plasma, CSF, exosomes/EVs/NDEs.
<table>
<thead>
<tr>
<th>miRNA profile (previously reported in AD)</th>
<th>AD vs Ctrl</th>
<th>MCI vs Ctrl</th>
<th>Tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-26b-3p, miR-28-3p, miR-30c-5p, miR-148b-5p, miR-151a-3p, miR-186-5p, miR-425-5p, miR-550a-5p, miR-1468, miR-4781-3p, miR-5001-3p, miR-6513-3p</td>
<td>UP</td>
<td>NA</td>
<td>Blood</td>
<td>(Satoh et al., 2015)</td>
</tr>
<tr>
<td>let-7a-5p, let-7e-5p, let-7f-5p, let-7g-5p, miR-15a-5p, miR-17-3p, miR-29b-3p, miR-98-5p, miR-144-5p, miR-148a-3p, miR-502-3p, miR-660-5p, miR-1294, miR-3200-3p</td>
<td>DW</td>
<td>NA</td>
<td>Blood</td>
<td>(Satoh et al., 2015)</td>
</tr>
<tr>
<td>miR-107, miR-103a-3p</td>
<td>DW</td>
<td>NA</td>
<td>Blood</td>
<td>(Chang et al., 2017)</td>
</tr>
<tr>
<td>miR-151a-3p, miR-161, let-7d-3p, miR-5010-3p</td>
<td>UP</td>
<td>NA</td>
<td>Blood</td>
<td>(Leidinger et al., 2013)</td>
</tr>
<tr>
<td>let-7f-5p, miR-107, miR-103a-3p, miR-26b-5p, miR-532-5p</td>
<td>DW</td>
<td>NA</td>
<td>Blood</td>
<td>(Leidinger et al., 2013)</td>
</tr>
<tr>
<td>miR-146b-5p, miR-15b-5p</td>
<td>DW</td>
<td>NA</td>
<td>Blood</td>
<td>(Wu et al., 2020)</td>
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<tr>
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### miRNA profile (previously reported in AD)

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<th>MCI vs Ctrl</th>
<th>Tissue</th>
<th>Reference</th>
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### miRNA profile (previously reported in AD)

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<td>UP</td>
<td>Plasma</td>
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<td>Plasma</td>
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<td>Tissue</td>
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<td>miR-424-5p</td>
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<td>NA</td>
<td>Serum</td>
<td>(Burgos et al., 2014)</td>
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<td>(Tan et al., 2014a)</td>
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<td>(Tan et al., 2014b)</td>
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### miRNA profile (previously reported in AD)

<table>
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<th>miRNA profile</th>
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<th>Tissue</th>
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<tr>
<td>miR-26a-5p, miR-181c-3p, miR-126-5p, miR-22-3p, miR-148b-5p</td>
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<td>miR-29, miR-125b, miR-223</td>
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<td>Serum</td>
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</tr>
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<td>miR-210</td>
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<td>DW</td>
<td>Serum and CSF</td>
<td>(Zhu et al., 2015)</td>
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<td>miR-613</td>
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<td>NA</td>
<td>Serum EVs</td>
<td>(Li et al., 2020)</td>
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<td>miR-135a, miR-384</td>
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<td>NA</td>
<td>Serum exosomes</td>
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<td>DW</td>
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<td>Serum exosomes</td>
<td>(T. T. Yang et al., 2018)</td>
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</table>
### Table 15. MiRNAs previously reported as dysregulated in AD or MCI with regard to Ctrl subjects.

The miRNA profile already described in AD (first column), as well as the direction in the expression change for AD or MCI subjects compared to the Ctrl group (second and third columns, respectively), the tissue/matrix in which they were identified (fourth column) and the reference of each study (fifth column) is included in the table. *One study showed in this table (Ansari et al., 2019) did not included Ctrl subjects and miRNAs’ expression changes are reported as UP in the MCI vs Ctrl column though this up-regulation is referred to progressor MCI with regard to stable MCI. UP, up-regulated; DW, down-regulated; NA, not available.

<table>
<thead>
<tr>
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<th>AD vs Ctrl</th>
<th>MCI vs Ctrl</th>
<th>Tissue</th>
<th>Reference</th>
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<tbody>
<tr>
<td>miR-193b</td>
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<td>DW</td>
<td>Serum exosomes</td>
<td>(Liu et al., 2014a)</td>
</tr>
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<td>miR-361-5p, miR-30e-5p, miR-93-5p, miR-15a-5p, miR-143-3p, miR-335-5p, miR-106b-5p, miR-101-3p, miR-424-5p, miR-106a-5p, miR-18b-5p, miR-3065-5p, miR-20a-5p, miR-582-5p</td>
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<td>NA</td>
<td>Serum exosomes</td>
<td>(Cheng et al., 2015)</td>
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<td>miR-1306-5p, miR-342-3p, miR-15b-3p</td>
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<td>miR-223</td>
<td>DW</td>
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<td>Serum exosomes</td>
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<td>miR-9</td>
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<tr>
<td>miR-146a, miR-181a</td>
<td>NA</td>
<td>UP</td>
<td>Whole blood</td>
<td>(Ansari et al., 2019)</td>
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</table>
We were able to identify a total of 225 unique miRNAs previously reported in the literature. These 225 unique miRNAs have been found in different tissues/matrices as shown in Table 15. Some of the unique miRNAs have been identified in more than one matrix while no common miRNAs were found in blood, plasma/serum, CSF and exosomes/NDEs/EVs (Figure 26 A). For the up-regulated (Figure 26 B) and down-regulated (Figure 26 C) miRNAs in all the above-cited matrices, no common miRNAs were identified either.

Figure 26. Venn diagrams of all unique (A), up-regulated non-unique (B) and down-regulated non-unique (C) miRNAs with their corresponding distribution in 4 human
matrices. Blood, red; plasma/serum, pink; CSF, blue; exosomes/NDEs/EVs, grey. In A we represented 225 unique miRNAs already reported in the literature. In B and C, non-unique miRNAs reported as down- and/or up-regulated in a specific matrix are shown. To note, miRNAs in B and C are non-unique. That is, the sum between down- and up-regulated is 243, which means that from a total of 225 unique miRNAs, 18 of them have been reported as up- and down-regulated (let-7i-5p, miR-125b, miR-127-3p, miR-135a, miR-143-3p, miR-146a, miR-148b-5p, miR-15a-5p, miR-184, miR-206, miR-222, miR-223-3p, miR-23a-3p, miR-27a-3p, miR-29a, miR-29b, miR-384, miR-9. Therefore, they might have dual behaviour in different biological matrices.

We next compared these 225 miRNAs previously reported in the literature with what we found in our sEVs-enriched preparations. As shown in Table 16, sEVs-miRNAs 100-3p, 136-5p, 15a-5p, 34a-5p, 384 and 424-5p were able to distinguish AD from Ctrl subjects (padj < 0.1). Instead, sEVs-miRNAs 140-5p, 182-5p, 340-5p, 5010-3p, 605-5p and 424-5p showed a trend towards the increase in MCI subjects with regard to the Ctrl group (however, they did not reach significance). For down-regulated miRNAs in sEVs, 127-3p and 98-5p expression decreased in AD with regard to Ctrl subjects (padj < 0.1). MiRNAs 18b-5p, 27a-3p, 328-3p, 34c-3p, 532-5p, 613, 885-5p, 194-5p and 9-3p did not reach the significance though they showed a trend to down-regulation in MCI compared to Ctrl subjects.
## Chapter IX. SEV-miRNAs as potential biomarkers of Alzheimer’s disease progression | Soraya Moradi Bachiller

<table>
<thead>
<tr>
<th>Reported miRNA</th>
<th>AD vs Ctrl</th>
<th>MCI vs Ctrl</th>
<th>Tissue</th>
<th>Reference</th>
<th>Plasma sEV-miRNA</th>
<th>AD vs Ctrl</th>
<th>Log2F C</th>
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<th>Log2F C</th>
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<td>100-3p</td>
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<td>NA</td>
<td>CSF</td>
<td>(Lusardi et al., 2017)</td>
<td>140-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>UP</td>
<td>0.633</td>
<td>0.582</td>
</tr>
<tr>
<td>15a-5p</td>
<td>DW</td>
<td>NA</td>
<td>Blood</td>
<td>(Satoh et al., 2015)</td>
<td>15a-5p</td>
<td>UP</td>
<td>0.531</td>
<td>0.017</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15a-5p</td>
<td>UP</td>
<td>NA</td>
<td>CSF</td>
<td>(Sørensen et al., 2016)</td>
<td>15a-5p</td>
<td>UP</td>
<td>0.531</td>
<td>0.017</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>15a-5p</td>
<td>UP</td>
<td>NA</td>
<td>Serum exosomes</td>
<td>(Cheng et al., 2015)</td>
<td>15a-5p</td>
<td>UP</td>
<td>0.531</td>
<td>0.017</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>182-5p</td>
<td>DW</td>
<td>NA</td>
<td>Serum</td>
<td>(Burgos et al., 2014)</td>
<td>182-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>UP</td>
<td>0.496</td>
<td>0.750</td>
</tr>
<tr>
<td>340-5p</td>
<td>DW</td>
<td>NA</td>
<td>CSF</td>
<td>(Lusardi et al., 2017)</td>
<td>340-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>UP</td>
<td>0.322</td>
<td>0.750</td>
</tr>
<tr>
<td>34a-5p</td>
<td>DW</td>
<td>NA</td>
<td>Plasma</td>
<td>(Cosín-Tomás et al., 2017)</td>
<td>34a-5p</td>
<td>UP</td>
<td>0.460</td>
<td>0.032</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>384</td>
<td>DW</td>
<td>NA</td>
<td>CSF</td>
<td>(Liu et al., 2014b)</td>
<td>384</td>
<td>UP</td>
<td>0.802</td>
<td>0.004</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>384</td>
<td>DW</td>
<td>NA</td>
<td>Serum</td>
<td>(Liu et al., 2014b)</td>
<td>384</td>
<td>UP</td>
<td>0.802</td>
<td>0.004</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>384</td>
<td>UP</td>
<td>NA</td>
<td>Serum exosomes</td>
<td>(T. T. Yang et al., 2018)</td>
<td>384</td>
<td>UP</td>
<td>0.802</td>
<td>0.004</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>424-5p</td>
<td>UP</td>
<td>NA</td>
<td>Plasma NDEs</td>
<td>(Nie et al., 2020)</td>
<td>424-5p</td>
<td>UP</td>
<td>0.585</td>
<td>0.017</td>
<td>UP</td>
<td>0.463</td>
<td>0.665</td>
</tr>
<tr>
<td>424-5p</td>
<td>UP</td>
<td>NA</td>
<td>Serum EVs</td>
<td>(Li et al., 2020)</td>
<td>424-5p</td>
<td>UP</td>
<td>0.585</td>
<td>0.017</td>
<td>UP</td>
<td>0.463</td>
<td>0.665</td>
</tr>
<tr>
<td>424-5p</td>
<td>UP</td>
<td>NA</td>
<td>Serum exosomes</td>
<td>(Cheng et al., 2015)</td>
<td>424-5p</td>
<td>UP</td>
<td>0.585</td>
<td>0.017</td>
<td>UP</td>
<td>0.463</td>
<td>0.665</td>
</tr>
<tr>
<td>5010-3p</td>
<td>UP</td>
<td>NA</td>
<td>Blood</td>
<td>(Leidinger et al., 2013)</td>
<td>5010-3p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>UP</td>
<td>0.355</td>
<td>0.771</td>
</tr>
<tr>
<td>605-5p</td>
<td>DW</td>
<td>NA</td>
<td>CSF exosomes</td>
<td>(McKeever et al., 2018)</td>
<td>605-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>UP</td>
<td>0.568</td>
<td>0.582</td>
</tr>
<tr>
<td>127-3p</td>
<td>DW</td>
<td>NA</td>
<td>CSF</td>
<td>(Burgos et al., 2014)</td>
<td>127-3p</td>
<td>DW</td>
<td>0.400</td>
<td>0.046</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>127-3p</td>
<td>UP</td>
<td>NA</td>
<td>Serum</td>
<td>(Burgos et al., 2014)</td>
<td>127-3p</td>
<td>DW</td>
<td>0.400</td>
<td>0.046</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>18b-5p</td>
<td>UP</td>
<td>NA</td>
<td>Serum exosomes</td>
<td>(Cheng et al., 2015)</td>
<td>18b-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.723</td>
<td>0.750</td>
</tr>
</tbody>
</table>
Table 16. Previously reported dysregulated miRNAs are also plasma DE sEV-miRNAs. SEV-miRNAs deregulated in our plasma samples were compared with the 225 unique miRNAs reported in the literature in CSF, blood, plasma, serum, exosomes/NDEs/EVs. The first five columns of the table are referred to already described miRNAs in the literature (first column), its expression change in AD or MCI with regard to Ctrl subjects (second and third column, respectively), the tissue in which they were identified (fourth column) and the corresponding reference (fifth column). The second part of the table refers to plasma sEV-miRNAs (sixth column), their expression in AD or MCI compared to Ctrl subjects (seventh and tenth columns, respectively), the corresponding log2FC (log-ratio of the miRNA's expression value) (eighth and eleventh columns) and adjusted p value (ninth and twelfth columns). NA refers to a miRNA whose counts were zero and, therefore, no test was applied; UP (red), up-regulated, DW (green), down-regulated.

<table>
<thead>
<tr>
<th>Reported miRNA</th>
<th>AD vs Ctrl</th>
<th>MCI vs Ctrl</th>
<th>Tissue</th>
<th>Reference</th>
<th>Plasma sEV-miRNA</th>
<th>AD vs Ctrl</th>
<th>Log2FC</th>
<th>padj</th>
<th>MCI vs Ctrl</th>
<th>Log2FC</th>
<th>padj</th>
</tr>
</thead>
<tbody>
<tr>
<td>194-5p</td>
<td>DW</td>
<td>NA</td>
<td>Plasma</td>
<td>(Sørensen et al., 2016)</td>
<td>194-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.325</td>
<td>0.750</td>
</tr>
<tr>
<td>27a-3p</td>
<td>DW</td>
<td>NA</td>
<td>CSF</td>
<td>(Frigerio et al., 2013)</td>
<td>27a-3p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.595</td>
<td>0.750</td>
</tr>
<tr>
<td>27a-3p</td>
<td>UP</td>
<td>NA</td>
<td>CSF exosomes</td>
<td>(Jain et al., 2019)</td>
<td>27a-3p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.595</td>
<td>0.750</td>
</tr>
<tr>
<td>328-3p</td>
<td>DW</td>
<td>NA</td>
<td>CSF</td>
<td>(Lusardi et al., 2017)</td>
<td>328-3p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.453</td>
<td>0.750</td>
</tr>
<tr>
<td>34c-3p</td>
<td>UP</td>
<td>NA</td>
<td>CSF exosomes</td>
<td>(Jain et al., 2019)</td>
<td>34c-3p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.176</td>
<td>0.771</td>
</tr>
<tr>
<td>532-5p</td>
<td>DW</td>
<td>NA</td>
<td>Blood</td>
<td>(Leidinger et al., 2013)</td>
<td>532-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.313</td>
<td>0.750</td>
</tr>
<tr>
<td>613</td>
<td>UP</td>
<td>UP</td>
<td>CSF</td>
<td>(Li et al., 2016)</td>
<td>613</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.400</td>
<td>0.758</td>
</tr>
<tr>
<td>613</td>
<td>UP</td>
<td>UP</td>
<td>Serum</td>
<td>(Li et al., 2016)</td>
<td>613</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.400</td>
<td>0.758</td>
</tr>
<tr>
<td>885-5p</td>
<td>DW</td>
<td>NA</td>
<td>Serum</td>
<td>(Tan et al., 2014a)</td>
<td>885-5p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.343</td>
<td>0.750</td>
</tr>
<tr>
<td>9-3p</td>
<td>DW</td>
<td>NA</td>
<td>CSF</td>
<td>(Burgos et al., 2014)</td>
<td>9-3p</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>DW</td>
<td>0.382</td>
<td>0.750</td>
</tr>
<tr>
<td>98-5p</td>
<td>DW</td>
<td>NA</td>
<td>Serum</td>
<td>(Tan et al., 2014a)</td>
<td>98-5p</td>
<td>DW</td>
<td>0.318</td>
<td>0.051</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
From the shared miRNAs between what has already been reported in the literature and plasma sEV-miRNAs (Table 16), we decided to focus on the 11 DE miRNAs which showed up-regulation in AD or MCI stages with regard to Ctrl subjects. Among them, miRNAs 100-3p, 136-5p, 15a-5p, 34a-5p, 384 and 424-5p were able to distinguish AD from Ctrl subjects. The rest of them, miR-140-5p, 182-5p, 340-5p, 424-5p, 5010-3p and 605-5p were able to discriminate MCI from the Ctrl group.

Among these miRNAs, 3 of them; miR-15a-5p, miR-384 and miR-424-5p have been reported at least three times in the literature. MiR-15a-5p and miR-384 expression is down-regulated in blood and serum of AD subjects, respectively. Besides, the three of them reported a change in their expression towards the up-regulation (when AD progresses) in serum-exosomes (miR-15a-5p and 384) and, in serum-EVs, serum-exosomes and plasma-NDEs (miR-424-5p). Expression changes for these three miRNAs in EVs fit with our findings in plasma sEVs (as shown in Table 16) since we also showed that these three miRNAs were up-regulated in AD with regard to Ctrl subjects (|Log2FC| AD vs Ctrl contrast > 0.5 and padj < 0.1) in our plasma sEVs-enriched preparations.

9.3.5. Network analysis of DE miRNAs revealed more than 400 miRNAs-mRNAs interactions

We predicted the most probable target genes of the 11 significant DE and up-regulated sEV-miRNAs of Table 17 with miRNAtap. The gene targets for each miRNA are listed in the excel file “TABLE_11miRNAs-search-x-GeneTargets_440interactions”. For better visualization, miRNA-mRNA interaction network is presented in Figure 27. The network diagram resulted in 411 nodes (from which 11 are the miRNAs and the rest the targeted mRNAs Genes) and 440 links between paired miRNA-mRNA.
<table>
<thead>
<tr>
<th>HUB</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGO2</td>
<td>Short-interfering-RNA mediated gene signaling</td>
</tr>
<tr>
<td>ATXN7L2</td>
<td>Cognitive function/Lung cancer</td>
</tr>
<tr>
<td>AQP11</td>
<td>Transport of water, glycerol and hydrogen peroxide across membranes</td>
</tr>
<tr>
<td>AKT3</td>
<td>Signaling in response to insulin and growth factors as part of the kinase system</td>
</tr>
<tr>
<td>BTRC</td>
<td>Phosphorylation-dependent ubiquitination</td>
</tr>
<tr>
<td>C1orf21</td>
<td>Cell growth</td>
</tr>
<tr>
<td>CCNE1</td>
<td>Cell cycle</td>
</tr>
<tr>
<td>CDCA4</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>CFAP45</td>
<td>Cell proliferation, migration and invasion</td>
</tr>
<tr>
<td>EDA</td>
<td>Interaction ectoderm-mesoderm</td>
</tr>
<tr>
<td>EMC4</td>
<td>Cell proliferation</td>
</tr>
<tr>
<td>FGF2</td>
<td>Nervous system development</td>
</tr>
<tr>
<td>HIPK2</td>
<td>Functional response to stressors by affecting p53 activity</td>
</tr>
<tr>
<td>LUZP1</td>
<td>Neural tissue closure</td>
</tr>
<tr>
<td>NFIB</td>
<td>Brain development</td>
</tr>
<tr>
<td>NFIA</td>
<td>Gliogenesis</td>
</tr>
<tr>
<td>N4BP1</td>
<td>RNA binding</td>
</tr>
<tr>
<td>PUS3</td>
<td>Catalysing of the formation of tRNA pseudouridine</td>
</tr>
<tr>
<td>PRKACB</td>
<td>Signaling through cAMP as part of the kinase system</td>
</tr>
<tr>
<td>PPP2R2A</td>
<td>Negative control of cell growth and division</td>
</tr>
<tr>
<td>PAPPA</td>
<td>Metallopeptidase and endopeptidase activity</td>
</tr>
<tr>
<td>PHF19</td>
<td>Cell growth and cell proliferation</td>
</tr>
<tr>
<td>POU2F1</td>
<td>DNA binding</td>
</tr>
<tr>
<td>PTH</td>
<td>Regulation of blood calcium and phosphate levels</td>
</tr>
<tr>
<td>PTPN4</td>
<td>Cell growth and differentiation</td>
</tr>
<tr>
<td>RBM6</td>
<td>RNA binding</td>
</tr>
<tr>
<td>TMEM100</td>
<td>Vascular morphogenesis and arterial endothelium differentiation</td>
</tr>
<tr>
<td>TNRC6B</td>
<td>GW182 paralog/Translation silencing and mRNA degradation</td>
</tr>
<tr>
<td>TLK1</td>
<td>Regulation of chromatin assembly</td>
</tr>
<tr>
<td>UBE4B</td>
<td>Ubiquitination</td>
</tr>
<tr>
<td>UBN2</td>
<td>DNA binding</td>
</tr>
<tr>
<td>UNC80</td>
<td>Maintenance of membrane potentials in neurons</td>
</tr>
<tr>
<td>USP25</td>
<td>Deubiquitination</td>
</tr>
<tr>
<td>VAMP3</td>
<td>Docking and/or fusion of synaptic vesicles</td>
</tr>
<tr>
<td>WEE1</td>
<td>Negative regulator of entry into mitosis</td>
</tr>
<tr>
<td>ZBTB34</td>
<td>Transcriptional repressor</td>
</tr>
<tr>
<td>ZNF622</td>
<td>Embryonic development</td>
</tr>
</tbody>
</table>

**Table 17. Summary of characteristics of genes identified as hubs in the miRNA-mRNA interaction network.**

From the miRNA-mRNA network, we found interactions between all the miRNAs through their respective mRNA targets except for the miR-34a-5p. Among the targeted genes, we
found PRKACB, VAMP3, AGO2, PUS3, NFIB, NFIA, TNRC6B, PPP2R2A, HIPK2, ATXN7L2 and EDA as hubs in the interaction network though connecting only two or three miRNAs of the network. Instead, a higher number of hubs mRNAs targeted by miR-15a-5p and miR-424-5p were identified; AKT3, AQP11, BTRC, C1orf21, CCNE1, CDCA4, CFAP45, EMC4, FGF2, LUZP1, N4BP1, PAPPA, PHF19, POU2F1, PTH, PTPN4, TMEM100, RBM6, TLK1, UBE4B, UBN2, UNC80, USP25, WEE1, ZBTB34, ZNF622. These genes are listed and their functions are described in Table 17.
Figure 27. Interaction network of selected 11 up-regulated miRNAs and their most probable gene targets. In this map, hub genes and miRNAs, obtained using Cytoscape and aMatReader, are shown in darker colors. Edges indicate the interaction between nodes (miRNAs and mRNAs).
All the miRNAs are hubs in the network while the node color of genes is redder when they increase the number of significant interactions. The color of the node is different depending on the role; blue for miRNAs and shades of red for mRNAs.

9.4. Discussion

Increasing lines of evidence have shown that miRNAs play a critical role in several biological processes. It has been estimated that, at least, the 60% of human genes are regulated by miRNAs (Zhang and Wang, 2017). Many of these miRNAs are expressed in the brain (Martin et al., 2020; J. Zhao et al., 2020) and are responsible for the correct function and integrity of the central nervous system (Y. Cheng et al., 2018; De Luna et al., 2020).

In AD, an ideal biomarker should be able to diagnose the disease before clinical onset. In addition, its assessment should require minimally-invasive techniques and have the adequate reliability. In this context, several proteins have been detected in body fluids (such as CSF and blood) and have been proposed as biomarkers of AD progression (Cha et al., 2019; L. Cheng et al., 2015).

However, dysregulations of body fluids-associated miRNAs are not always consistent and no consensus has yet been reached. This difficulty to reach a common and unique dysregulated miRNA profile for subjects suffering from AD or MCI may be due to the discrepancies to diagnose AD in the enrolled subjects (different inclusion criteria used) (Alexandrov et al., 2012; Wu et al., 2020) or the different techniques utilized to detect and quantify these miRNAs (NGS or RT-qPCR) (Cheng et al., 2013; Cosín-Tomás et al., 2017; Satoh et al., 2015). In addition, miRNA expression also varies depending on the matrix or tissue where it is studied. For example, the miR-15a-5p is up-regulated in CSF (Sørensen et al., 2016) while down-regulated in blood (Satoh et al., 2015) of AD subjects. Also, if among body fluids, miRNA expression varies, it changes even more between body fluids and sEVs.
Therefore, though sEVs have been proposed as the ideal carriers for miRNAs (since they protect them better from the degradation and release them from brain to peripheral tissues), controversial results for sEVs- and body fluids-associated miRNAs studies have been found. However, these controversial results may be normal (as we discussed below with more details), since only miRNAs specifically associated with sEVs will show differential expression according to their localization.

As the main aim of this project was to find possible novel biomarkers of early dementia carried by plasma-sEVs, we performed next-generation RNA-seq analysis of sEVs-enriched preparations from a total of 12 subjects (4 Ctrl, 4 MCI and 4 AD) to determine, at the first attempt, the DE miRNAs between AD, MCI and Ctrl subjects. Only for the AD vs Ctrl contrast the statistically significance was reached (padj < 0.1). For the MCI vs Ctrl contrast, instead, no significance was achieved.

The statistical significance was established considering an padj < 0.1 and the |log2FC| > 0.28. Since log2FC measures how much the transcripts’ expression change, the higher this change is, the more reliable the differential expression of a miRNA between two conditions is. However, in our case, not many miRNAs achieved a high |log2FC| value (> 1) suggesting that more subjects should be enrolled in this study to confirm the reliability of our NGS results.

For a more in-depth analysis of our samples, we took advantage of three GEO datasets (GSE63060, GSE63061 and GSE120584) containing blood mRNA and serum miRNA data from Ctrl, MCI and AD subjects.

All the sEV-miRNAs that paired with their corresponding transcript gene target (GSE63060 and GSE63061), were also investigated for their levels in serum across the AD stages. As shown in Table 14, DE miRNAs, in serum and plasma-sEVs, followed three different
patterns of expression. On the one hand, group 1 (blue) and 3 (black), showed the opposite expression change in serum and sEVs. For example, miRNA-384 (group 1) was up-regulated in our plasma sEVs-enriched preparations while it was decreased in serum across AD stages. Something similar happens with miRNAs of group 3. For example, miR-580-3p showed up-regulation in sEVs-derived from AD subjects with regard to Ctrl. However, expression of this miRNA did not change in serum. Group 2, instead, showed the same expression change pattern in plasma-sEVs and in serum. These different patterns of expression in diverse matrices or tissues are consistent with what has been previously described in the literature. Liu et al. measured levels of miR-193b in peripheral blood, CSF and serum/CSF exosomes from Ctrl, MCI and AD subjects. The results indicated that serum exosome-associated miR-193b in AD and MCI was lower than in Ctrl subjects. However, this result was observed neither in blood nor CSF body fluids (Liu et al., 2014a). Others have compared miRNA levels in CSF and in the relative exosome-enriched fraction, and found the opposite pattern in the miRNA expression (Riancho et al., 2017).

The above described results suggest two different possible aspects. Firstly, miRNAs that change their expression in sEVs while remaining stable in serum, might reflect AD pathological progression earlier than other miRNAs. On the other hand, miRNAs showing opposite expression changes in sEVs-enriched preparations and in serum, may suggest that some miRNAs are specifically linked to and released within sEVs, being specific sEVs-associated biomarkers. This is consistent with the selective mechanisms of miRNAs sorting into sEVs (previously described in chapter III, 3.3.2.). Instead, miRNAs of group 2, that showed the same expression change pattern in sEVs-enriched preparations than in serum, could be not really associated with any of those matrices, are likely not sEVs-associated biomarkers and, in this case, there is no advantage in separating sEVs from serum to study them.
Apart from these different patterns observed, miRNAs from Table 14 also correlated with their corresponding dysregulated transcript targets in blood across AD stages (Figure 24 and 25). MiR-4715-3p, miR-2113, miR-1972, miR-424-3p, miR-4695-5p, miR-4756-5p, miR-3605-3p and miR-4683 paired with ANXA11, TYROBP, SNX11, LRP1, DMWD, NFAT, C4orf8, GPR108 and FRAT (Figure 24). For all those cases, sEVs-miRNA expression was down-regulated when AD progresses and, thus, their transcripts’ targets in blood were found up-regulated throughout AD stages. For the inverse expression pattern (miRNAs up-regulated in plasma-sEVs and gene targets down-regulated in blood), we found miR-384, miR-24-2-5p, miR-577, miR-580-3p, miR-4312, miR-27a-5p, miR-4307, miR-633 and miR-100-3p that matched with NIF3L, GTF2B, CHMP5, CCDC146, NEK1, TXN, FAM18B, PDCD4, BTF3, ZMYND11, CCDC58 and KPNA4 (Figure 25).

Some of the miRNA-transcript pairs showed interesting results. For example, the miR-2113 may act as the regulator of the transmembrane immune signaling adaptor TYROBP (TYROBP). This miRNA is down-regulated in sEVs across the AD stages, suggesting that his partner’s expression may be up-regulated in AD (as shown in Figure 24 B). This is consistent with previously results reported by Zhang et al. where TYROBP was identified as a binding partner of TREM2 in gene regulatory networks of post-mortem brain tissues of Ctrl and LOAD subjects. These two genes formed a microglia module that was up-regulated in LOAD whose overexpression results in AD-associated alterations (B. Zhang et al., 2013).

MiR-424-3p is also found down-regulated in plasma sEVs and did not correlate with levels of low density lipoprotein receptor-related protein 1 (LRP1) in blood that were up-regulated (Figure 24 D). This agrees with the study of Rauch et al. in which the authors demonstrated that LRP1 is implicated in the uptake of tau and its spread, both processes up-regulated in AD. Moreover, in neurons, the down-regulation of LRP1, reduces the propagation of tau (Rauch et al., 2020). Other studies have also reported that in human post-mortem samples,
lower levels of LRPI decreases Aβ deposition, suggesting that neuronal LRPI is essential for other proteins, such as APOE, for the accumulation of insoluble Aβ fibrils (Tachibana et al., 2019).

On his behalf, the nuclear factor of activated T-cells (NFAT) proteins are the link between Ca^{2+} and the gene regulatory machinery in astrocytes. In fact, NFATs are hyper-activated in NDs (Abdul et al., 2009) and have a critical role in the regulation of immune and inflammatory responses (Rojanathammanee et al., 2015). This is consistent with the increase of NFAT5 in blood and the possible role of miR-4756-5p as its regulator (Figure 24 F). However, miR-4756-5p (as shown in Table 14) decreased in plasma-sEVs and, also, in serum, which means that this miRNA may not be specifically associated with sEVs.

 Thioredoxin (TXN) is another important gene involved in AD pathogenesis and implicated in the cellular stress response. In fact, its decrease has already been demonstrated for amnestic MCI and AD subjects (Akterin et al., 2006; Di Domenico et al., 2010). This is consistent with our results and with the possible regulation of its expression by miR-580-3p (Figure 25 F).

Other genes, such as the programmed cell death 4 (PDCD4) has been shown to be significantly up-regulated in plasma of AD subjects, probably due to the miR-212 down-regulation (Wang and Chang, 2020). However, in plasma-sEVs, miR-4312 was up-regulated and, subsequently, it may down-regulate PDCD4 expression (Figure 25 H). This opposite result for PDCD4 gene expression and its miRNA target may be due to the subjects considered for the authors’ study (expression data from only one cohort of subjects) and ours (different expression datasets).

Curiously, GO terms for miRNAs listed in Table 14 showed that the up-regulated miRNAs were primarily localized in axons and vesicle lumen. This goes in hand with the idea that
sEVs-miRNAs separated from a peripheral tissue (in this case, plasma) also reflect what is released by neuronal cells.

In addition, up-regulated miRNAs were implicated in the modulation of the rate of chemical reactions or pathways involved in the formation of macromolecules. Since they are up-regulated (and due to their gene expression inhibitory effect), they might decrease the frequency of these reactions. This agrees with what previously reported by Kolisnyk et al. The authors of this study demonstrated that when acetylcholine is lost or not available (a clinical feature in AD subjects), neurons drastically increase their mRNAs encoding for specific amino acids essential in the brain. That is, changes in the RNA metabolism happens when the cholinergic system fails (Kolisnyk et al., 2017), and the up-regulation of some sEVs-miRNAs might block the translation of these mRNAs.

The down-regulated miRNAs, instead, were implicated in processes such as the cell-to cell adhesion. Cell-adhesion molecules have a critical role in AD pathogenesis as they are involved in synaptic transmission and plasticity, among others (Wennström and Nielsen, 2012). For example, their increased expression in the hippocampus reflects disease severity (Mikkonen, 1999).

Down-regulated miRNAs are also involved in negative regulation of cellular macromolecule biosynthetic processes. In other words, the same that happens with up-regulated miRNAs with the rate of chemical reactions; no increments in the molecule biogenesis might occur when AD progresses. They are also involved in the modulation of the rate of the transcription by RNA polymerase II, which may reflect the modulation of apoptotic processes in AD. For example, Padmanabhan et al. demonstrated that the inhibition of the RNA polymerase II protects neurons from undergoing apoptosis (Padmanabhan et al., 2015).

Overall, we found evidence that plasma sEV-miRNAs 2113, 424-3p, 580-3p, 4312 are most
likely to be related to AD pathogenesis since their probable gene targets are associated with AD. However, since miRNAs’ expression depends on their localization, understanding how they change in different matrices may help us to select better the body fluid where they need to be studied. With this regard, Table 15 showed miRNAs previously reported in blood, serum, plasma, CSF and EVs (exosomes, EVs or NDEs) in subjects suffering from AD. None miRNA was found in all the four matrices (Figure 26 A). Only some of them were up-regulated (Figure 26 B) or down-regulated (Figure 26 C) in at least two of them. This suggests the difficulties to detect a unique miRNA profile in all the body fluids able to reflect the progression of AD. However, it also suggests that miRNAs show a differential expression according to their localization, which represents an advantage in the biomarker discovery field.

Moreover, the comparison between DE sEVs-miRNAs and the literature showed just 22 previously reported miRNAs also identified by us (Table 16). We decided to select only up-regulated sEV-miRNAs in AD stages (miR-100-3p, miR-136-5p, miR-140-5p, miR-15a-5p, miR-182-5p, miR-340-5p, miR-34a-5p, miR-384, miR-424-5p, miR-5010-3p and miR-605-5p) because of their statistical significance in the AD vs Ctrl comparison (lower padj values). For these DE 11 sEVs-miRNAs, the miRNA-gene interaction network (Figure 27) was done. MiR-15a-5p and miR-424-5p showed the higher number of targeted and common genes. The corresponding function for these genes is listed in Table 17. Among them, FGF2 (with a protective role against cytotoxicity (X. Chen et al., 2020)), POU2F1 (down-regulated in AD and involved in immune and inflammatory responses (Taguchi et al., 2005)) and WEE1 (decreased in AD (Tomashevski et al., 2001)) participate in AD pathogenesis, and so miR-15a-5p and miR-424-5p might do. In fact, these two miRNAs are up-regulated in sEVs in AD with regard to Ctrl subjects and their gene targets (Figure 27) may be decreased in AD. This is consistent with the levels of POU2F1 and WEE1 previously detected in AD.
subjects. The down-regulation of \textit{FGF2} in AD may also agree with the Aβ-mediated cytotoxicity and the failure in brain detoxification mechanisms (X. Chen et al., 2020).

Moreover, miRNAs 15a-5p, 424-5p and 384 (Table 16) showed a clearly opposite expression pattern in sEVs with regard to the whole plasma. Curiously, two of these miRNAs (miR-15a-5p and miR-384) showed the opposite expression in serum and blood, respectively (Liu et al., 2014b; Satoh et al., 2015) than within our sEVs-enriched preparations. In addition, previously studies (Table 15) where miR-424-5p was measured in exosomes, EVs and NDEs (Cheng et al., 2015; Li et al., 2020; Nie et al., 2020) or where miR-384 was measured in exosomes (T. T. Yang et al., 2018), agree with the up-regulation found in our plasma-sEVs. This suggests that miR-15a-5p, miR-384 and miR-424-5p might be specifically associated with EVs since their expression did not change in the same manner in other tissues. In addition, the miR-424-5p was found as one of the best top-ranked DE in both AD vs Ctrl and MCI vs Ctrl contrast, though it did not reach significance in the MCI when compared to the Ctrl group (Figure 23).

MiR-384 was one of the miRNAs of the group 1 (blue, Table 14) in our plasma sEVs-enriched preparations. It was found to be significantly up-regulated in AD vs Ctrl contrast (padj = 0.004) in plasma-sEVs. It was also detected in serum where its gamma mean value (-0.126) indicated a slightly down-regulation throughout AD stages (Figure 25 A, B, C). It is also negatively correlated with \textit{NIF3L1}, \textit{GTF2B} and \textit{CHMP5} expression (Figure 25 A, B, C). And, it has already been shown that this miRNA targets the 3’ UTR \textit{APP} and \textit{BACE1}, which corroborate the link of this miRNA with the AD progression (Liu et al., 2014b).

In conclusion, the release of miRNAs does not always reflect the amount of these miRNAs in the parental cell (Pigati et al., 2010). Subsequently, the release of miRNAs in association with biofluids or sEVs is a selective process for the mechanism of miRNA sorting.
Therefore, it is essential to understand these mechanisms and the direction of the expression change for a miRNA to be able to associate it with a specific tissue (in our case, with sEVs) and to propose it as a biomarker for AD. Here, we found three miRNAs (miR-424-5p, miR-384 and miR-15a-5p) that probably associate with sEVs and which are able to discriminate AD from Ctrl subjects. However, one limitation of our study is the sample size that needs to be increased to confirm the results here presented. We were also unable to discriminate MCI from Ctrl subjects using sEV-miRNAs. One possible explanation for this is the sample size together with the great variability among MCI subjects, greater than that present among AD subjects. To reduce this variability, it is then important to focus the MCI population on the amnestic component only, something that we were not able to do so far. Another limitation of our study is that the results presented here derived from different cohorts of subjects (our human subjects and individuals of 3 different GEO datasets). It could be interesting, and even more informative, to correlate mRNA and miRNA data from the same cohort of subjects to get more reliable results.

As a final remark, when we compared our results with those available from the literature, we made every effort to include all the relevant publications, but we are not able to exclude a selection bias.
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CHAPTER X

BIOCHEMICAL INSIGHT OF ECSIT ROLE IN H4-SW CELLS, AN IN VITRO MODEL OF AMYLOID PRECURSOR PROTEIN OVEREXPRESSION

10.1. ECSIT links TLR and BMP signaling pathways

When the human TLR pathway and the activity of BMPs were described (Medzhitov et al., 1997; Urist, 1965), there was no evidence of connecting elements between these transduction pathways.

TLR pathway starts at the cell surface where TLRs can be triggered by both physiological and pathological activation (Morisato and Anderson, 1995; Takeda et al., 2003). The pathological activation of the TLR ectodomain by the pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) have been shown to recruit different adapters, such as the myeloid differentiation primary response protein 88, the TIR domain-containing adaptor protein, the TIR domain-containing adaptor protein inducing interferon-β and the TRIF-related adaptor molecule (Akira and Takeda, 2004; O’Neill et al., 2003).

DAMPs or PAMPs are firstly recognized by TLRs. Then, the recruitment of the adaptor protein to the TLR leads to the activation of the interleukin 1 receptor-associated protein kinases (IRAKs) which become auto phosphorylated as the result of the formation of the TLR-active adaptor protein-IRAKs complex. Auto phosphorylated IRAKs dissociate from the adaptor protein and then bind TRAF6 (Kopp and Medzhitov, 1999). The association between TRAF6 and IRAKs is completed with the mitogen-activated kinase kinase kinase 7 and its adaptors, the mitogen-activated kinase kinase kinase 7 binding proteins (Ninomiya-Tsuji et al., 1999). This association leads IRAK proteins undergoing proteosomal
degradation. Subsequently, TRAF6 is able to bind and activate the NF-κB inducing kinase (NIK) (Song et al., 1997). NIK activation is essential for the translocation of NF-κB to the nucleus. When DAMPs and PAMPs are not present extracellularly, NF-κB exists in the cytoplasm while interacting with modulatory proteins called IκBs that are inhibiting the nuclear entry of NF-κB (Hinz et al., 2012). For these inhibitory proteins to be detached from the NF-κB, IκBs need to be phosphorylated by the IκB kinases and degraded in the proteasome (Kanarek and Ben-Neriah, 2012). To shift into active state, IκB kinases are phosphorylated by NIK, leading to the nuclear entry of p65/p50 (the most common heterodimer of NF-κB transcription factor family (Oeckinghaus and Ghosh, 2009)). The nuclear entry of NF-κB p65/p50 subunits allows the transcription of genes implicated in the increase of innate immune and inflammatory responses (Ghosh and Karin, 2002).

TLRs can also activate the mitogen-activated protein kinases, such as MEKK1, and their downstream effectors (the Jun N-terminal kinase and the p38 mitogen-activated protein kinase) (Moustakas and Heldin, 2003), which are also capable of regulating immune and inflammatory gene expression (Arthur and Ley, 2013; Huang et al., 2009).

On their behalf, BMPs are part of the TGF-β family. BMP signaling pathway is critical for the cellular proliferation (Stewart et al., 2010), cellular differentiation (Kokabu et al., 2012; Luu et al., 2007) and embryonic development and patterning (Hemmati-Brivanlou and Thomsen, 1995). The BMP pathway mostly signals through the Smad proteins (Ramel and Hill, 2012), starting at the cell surface with two type I and two type II receptors, with serine/threonine kinase activity (Wrighton et al., 2009). When BMPs ligands bind to type I receptors, type II receptors (whose cytoplasmic domain is constitutively active) phosphorylate them on serine and threonine residues (Ramel and Hill, 2012; Wrighton et al., 2009). Then, they phosphorylate receptor-regulated Smads (R-Smads) in their C-terminal fragment SXS motif. Subsequently, R-Smad forms a heteroligomeric complex with the non-
phosphorylated Smad4. R-Smad-Smad4 complex dissociates from the receptors, translocates to the nucleus and regulates expression of BMP genes (Wrighton et al., 2009). Since Smads and BMPs allow the tissue homeostasis and the cellular proliferation and differentiation, the activated and nuclear R-Smad-Smad4 complex (which is phosphorylated in the SXS motif of the R-Smad) is strictly controlled by phosphatase-mediated dephosphorylation, ubiquitylation-dependent degradation and nuclear export (Xu et al., 2016).

In 1999 and 2003, two groups provided a new protein participant, a possible molecular link between TLR and BMP pathways (Figure 28).

Figure 28. ECSIT function in BMP and TLR signaling pathways. The activation of TLRs
on the cell surface make them to bind MyD88, which recruits TRAF6 and IRAK kinases. IRAK1 and TRAF6 interaction can 1) target and phosphorylate TAK1 and its regulators (TAB1 and TAB2) or 2) target ECSIT that recruits MEKK1. These two pathways will allow the activation of NF-κB (p50/p65) or MKKs, JNK and p38, respectively. In both cases, their translocation to the nucleus will lead to the transcription of pro-inflammatory genes. On the other hand, BMP pathway starts with the recognition of BMP by cell surface receptor I and II serine/threonine kinases. Receptor II phosphorylates and activates receptor I which phosphorylates R-Smad. R-Smad oligomerizes with non-phosphorylated Smad4, which can interact with ECSIT. R-Smad/Smad4 hetero-oligomer translocates to the nucleus and binds to the promoter of specific BMP target genes. ECSIT might therefore represent the crosstalk between TLR and BMP pathways, although the specific mechanism is unknown (question mark). Gray circles represent phosphorylation events. Figure designed by Soraya Moradi Bachiller and adapted from Moustakas and Heldin, 2003.

In 1999, Kopp et al. identified ECSIT as a cytoplasmic partner of TRAF6 that bridges TRAF6 to MEKK-1 in the TLR pathway (Figure 28). Moreover, the authors showed that a dominant negative mutant of ECSIT blocks MEKK-1 processing (from the full-length to the 80 kDa form) and the downstream activation of NF-κB, thus suggesting ECSIT as a critical mediator for the functionality of the TLR pathway in Drosophila and human cells (Kopp et al., 1999).

In 2003, Xiao et al. aimed to confirm the results obtained by Kopp et al. by creating a null mutant of ECSIT in mouse models. Surprisingly, the knockout mice died and showed the same prenatal phenotype than with the loss of BMP signaling (that is, impaired gastrulation and early embryonic tissue patterning). Moreover, they also demonstrated that ECSIT lost leads to the induction of teratomas in embryonic stem cells, a reduced cell proliferation, a poorly differentiated neural tissue, keratinized epithelium and adenoepithelium-like tissue. This suggested that ECSIT protein is essential for proliferation and differentiation of embryonic cells. In addition, Xiao et al. showed that ECSIT is expressed in three alternative
spliced forms: ECSIT1, ECSIT2 and ECSIT3. Among them, ECSIT2 is also capable of binding the R-Smad (Smad1 to Smad4), forming Smad1-Smad4 complex, translocating to the nucleus and binding to the promoter of specific BMP target genes (Figure 28). Moreover, and to corroborate the results found by Kopp et al., the knockdown of ECSIT inhibited NF-κB activity, suggesting that ECSIT is a mediator of the BMP signaling pathway as cofactor for Smad-Smad4 proteins and confirming its role in TLR signaling pathway (Xiao et al., 2003).

However, so far, the role of ECSIT protein in neurodegeneration and AD is still hypothetic (Soler-López et al., 2011).

10.2. Aim of the study and experimental design

In chapter VII, we showed that ECSIT protein is carried within plasma sEVs-enriched preparations showing lower levels in MCI and AD subjects and, in the 3xTg-AD mice (see chapter VII, Figure 20 A, D). In human samples, this decline seems to correlate to the early-onset cognitive deterioration, while in 3xTg-AD mice, ECSIT reduction from the 3rd to the 10th month of age might fit better with the basal APP(SW) transgene expression rather than with the onset of the cognitive decline (that begins later on, from the 5th month of age). In other words, in humans ECSIT might have a physiological turnover that also involves its incorporation in sEVs. In pathological conditions, ECSIT might be required inside the cell (and therefore, its levels inside sEVs decrease) due to its involvement in pathways activated during the progression of dementia (Soler-López et al., 2012). In 3xTg mice instead, ECSIT sEV-directed pathway might be dysregulated before the cognitive onset because of the APP(SW) transgene expression.

The above reported human evidence suggests some role for ECSIT in cognitive decline, and
the murine model indicated a possible influence of APP on ECSIT intracellular dynamics and sEVs externalization, but molecular insight to assess ECSIT involvement in molecular pathways important for AD was mandatory. A working hypothesis may be that considering the decline of ECSIT protein within sEVs-enriched preparations in pathological conditions (depicted in MCI/AD subjects and the 3xTg-AD mice), one could expect that ECSIT is required inside the cell as neuroprotective response (Soler-López et al., 2012).

Therefore, in this chapter, we aim to better characterize ECSIT biochemical features in a cellular model of human APP overexpression (H4-SW cells) (Colombo et al., 2009) and their derived sEVs, by assessing how ECSIT protein changes its subcellular localization and its incorporation within sEVs, and the possible regulation that ECSIT protein might exert over Aβ(1-42) generation.

To meet these purposes, we separated mitochondrial, nuclear, cytoplasmic and sEVs fractions from H4-SW cells and their control line, the H4-Native cells. We investigated the localization of ECSIT protein within each of these cellular fractions. Then, we down-regulated ECSIT with ECSIT-specific shRNA and studied the effect of ECSIT down-regulation on Aβ(1-42) release by H4-SW cells.

We showed that ECSIT protein changes its localization within the cell depending on the presence of APP(SW) because ECSIT protein increases within the nucleus of H4-SW. Moreover, ECSIT down-regulation decreases APP protein level in H4-SW cells and, also, Aβ(1-42) release in culture medium.
10.3. Results

10.3.1. NTA analysis of H4 cells-derived sEVs-enriched preparations showed no significant differences in the particle parameters or concentration profiles due to APP(SW) presence

H4-Native and H4-SW FBS-depleted conditioned media (CM) were collected after 48 h. In this setting ~80% of the cellular viability was conserved (Figure 29 A).

H4-Native and H4-SW CM-derived sEVs were then characterized using NTA. The size distribution profiles of sEVs derived from H4-Native cells (Figure 29 B) and H4-SW cells (Figure 29 C) are represented. The mean diameter (Figure 29 D), D-values (Figure 29 E, F, G) and the particle concentration in the initial CM volume (Figure 29 H) showed no significant differences between H4-Native and H4-SW cells.

Figure 29. Cell viability in serum-free medium, particle distribution and concentration parameters for H4-Native and H4-SW cells-derived sEVs. H4-Native and H4-SW cell viability 24, 48 and 72 h after FBS removal is represented as mean±SD (N = 6 replicates) (A). Representative NTA profiles (particles/mL) for H4-Native (B) and H4-SW (C) are showed as the mean of 3 cellular replicates. The mean particle diameter (D), the particle distribution parameters (D-values, D10, D50 and D90) (E-G, respectively) and the particle concentration in the initial CM volume (H) are also shown.
concentration in the initial CM sample (H) are also represented as mean±SD (N = 3 replicates) for H4-Native (blue triangles) and H4-SW cells (orange circles). Normal distribution was assessed by the Shapiro-Wilk test. Unpaired t-test was used. P value < 0.05 was considered as significant. CM, conditioned medium; H4-Nat, H4-Native cells; H4-SW, H4-SW cells.

10.3.2. SEVs-enriched preparations were positive to common EV markers and carried ECSIT protein

To complete the NTA characterization of H4 cells-derived sEVs-enriched preparations, we studied the specific EV marker (CD63) and markers for particles other than EVs (GM130) (Figure 30). With the aim to compare the marker enrichment in our sEVs with regard to the corresponding cell lysate, the same H4 cells were used to prepare both the cell lysate and the sEVs-enriched preparation. For the same amount of protein extract, sEVs-enriched preparations have increase amounts of the non-tissue specific marker CD63 (Figure 30 A).

Figure 30. H4 cells-derived sEVs characterization by Western blotting for common EV and non-EV markers and ECSIT protein. Representative Western blots of H4 cell preparations (cell lysates or sEVs-enriched preparations) showed the presence of non-tissue specific markers (CD63). Non-EVs co-isolated particles (GM130) are also shown (A). Both preparations (cell lysates and sEVs-enriched preparations) derived from the same sample. Densitometric analysis of ECSIT Western blot in sEVs-enriched preparations from H4 cells and in cell lysates normalized to CD63 (B) protein (arbitrary units) is also shown. Each
condition was done in triplicate in the scatter plot with bars by mean±SD. Normal distribution and equality of variances were tested by the Shapiro-Wilk normality test and the F test, respectively. Unpaired t-test or Welch’s t test were used. P value < 0.05 was considered as significant. H4-Nat, H4-Native cells; H4-SW, H4-SW cells; CL, cell lysate; sEVs, small extracellular vesicles.

Protein levels of GM130 (a peripheral membrane protein attached to the Golgi membrane) (Théry et al., 2018) were also measured to test sEVs purity. We found that our sEVs-enriched preparations were completely GM130-depleted (Figure 30 A).

As for ECSIT protein content, we detected lower levels (without reaching the significance) of ECSIT protein in H4-SW sEVs-enriched preparations when normalized with CD63 marker (Figure 30 B).

10.3.3. ECSIT protein localizes preferentially in the nucleus of H4-SW cells, while in H4-Native it is mainly cytoplasmic and mitochondrial

Cytoplasmic, mitochondrial and nuclear fractions were isolated from H4-Native and H4-SW cells. Representative Western blots for demonstrating the purity of each fraction are shown (Figure 31 A). Nuclear fraction is enriched in histone H3, while the voltage-dependent anion channel (VDAC) is increased in the mitochondrial fraction. The cytoplasmic preparation, instead, contains neither histone H3 nor VDAC (Figure 31 A).
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**A**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>ECSIT (~50 kDa)</th>
<th>VDAC (~30 kDa)</th>
<th>Vinculin (~130 kDa)</th>
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<tbody>
<tr>
<td>H4-Nat</td>
<td></td>
<td></td>
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<tr>
<td>H4-SW</td>
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**B**

Normalized ECSIT band intensity

- Cytoplasmic fraction
- H4-Nat
- H4-SW

**C**

Normalized ECSIT band intensity

- Mitochondrial fraction
- H4-Nat
- H4-SW

**D**

Normalized ECSIT band intensity

- Nuclear fraction
- H4-Nat
- H4-SW

**E**

Nuclei | ECSIT | Mitotracker | Merge

- H4-Native cells
- H4-SW cells
Figure 31. ECSIT protein presence within H4 cytoplasmic, nuclear and mitochondrial fractions. Representative Western blots of the three subcellular fractions separated from H4-Native cells (A). Representative Western blots and desitometric analyses for ECSIT protein levels within the cytoplasmic (B), the mitochondrial (C) and the nuclear fractions (D) are also reported for H4-Native and H4-SW cells. The relative intensity of ECSIT protein (arbitrary units) was normalized to vinculin, VDAC and H3, respectively. The four replicates for each condition were represented with box and whiskers plots depicting medians, interquartile ranges (box) and ranges (error bars). Representative immunofluorescence of ECSIT protein distribution within H4-Native and H4-SW cells (scale bar = 1 µm) is also shown (E). Normal distribution and equality of variances were tested by the Shapiro-Wilk normality test and the F test, respectively. Unpaired t-test or Welch’s t test were used. P value < 0.05 was considered as significant. H4-Nat, H4-Native cells; H4-SW, H4-SW cells.

The levels of ECSIT protein significantly increased in H4-SW cells compared with H4-Native cells only in the nuclear fraction \( (t_{3.918}, \text{df}_6, \ast\ast p = 0.0078; \text{H4-Native cells mean} = 0.4887, \text{H4-SW cells mean} = 1.093) \) (Figure 31 D). In the cytoplasmic (Figure 31 B) \( (t_{2.824}, \text{df}_6, \ast p = 0.030; \text{H4-Native cells mean} = 1.033, \text{H4-SW cells mean} = 0.8359) \) and mitochondrial fractions (Figure 31 C) \( (t_{5.621}, \text{df}_3.117, \text{Welch’s t test} \ast p = 0.01; \text{H4-Native cells mean} = 1.015, \text{H4-SW cells mean} = 0.6640) \), ECSIT protein levels significantly decreased in H4-SW cells compared to H4-Native cells.

The preferential nuclear distribution of ECSIT protein in H4-SW cells was also confirmed by immunofluorescence studies that showed ECSIT protein signal concentrated in the nuclear compartment (Figure 31 E).

10.3.4. ECSIT down-regulation decreases APP protein levels and Aβ(1-42) release by H4-SW cells

We next performed shRNA-driven down-regulation of ECSIT in H4-Native and SW
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cells. At basal level, we did not observe any significant change in ECSIT mRNA expression between H4-Native and H4-SW cells (Figure 32 A). After shRNA experiment, we detected a significant increase of ECSIT mRNA (t_{8.379}, df_{3}, **p = 0.0036; H4-SW (sh-negative) mean = 0.7514, H4-SW (sh-ECSIT) mean = 1.062) in H4-SW cells when infecting with the shRNA against ECSIT (sh-ECSIT) compared to the lentiviral scrambled sequence (sh-negative) (Figure 32 B).

Figure 32. shRNA-ECSIT in H4 cells and its effects on APP metabolism. ECSIT mRNA expression in H4-Native and H4-SW cells are shown in basal condition (A) and after
infection with shRNA lentiviral particles against ECSIT (sh-ECSIT). Scrambled sequences (sh-negative) are used as a control (B). The cytotoxicity of the scrambled sequence and sh-ECSIT for both cell types are tested by the LDH assay (C). Representative Western blotting and densitometric analyses for ECSIT and APP proteins (D) and Aβ(1-42) concentration (E) released by H4-Native and H4-SW cells after lentiviral infection are also shown. Each condition was assessed with 2 – 6 replicates and depicted in scatter plots with bars or box and whiskers plots with the respective medians, interquartile ranges (box) and ranges (error bars). Normal distribution and equality of variances were tested by the Shapiro-Wilk normality test and the F test, respectively. Unpaired t-test was used. P value < 0.05 was considered as significant. H4-Nat, H4-Native cells; H4-SW, H4-SW cells; sh-neg, sh-negative.

The scrambled sequence (sh-negative) did not differ from the sh-ECSIT concerning the ECSIT mRNA expression in H4-Native cells (Figure 32 B) although in both cell types the scrambled sequence increased the cytotoxicity by means of LDH release ($t_{4,299}$, $df_9$, **$p = 0.0020$; H4-Native (sh-ECSIT) mean = 0.2995, H4-Native (sh-negative) mean = 0.6923 and $t_{5.343}$, $df_{10}$, ***$p = 0.0003$; H4-SW (sh-ECSIT) mean = 0.09936, H4-SW (sh-negative) mean = 0.2615) (Figure 32 C).

Contrary to what happens with ECSIT mRNA expression, a significant decrease of ECSIT ($t_{5.561}$, $df_3$, *$p = 0.0115$; H4-SW (sh-ECSIT) mean = 0.616, H4-SW (sh-negative) mean = 1.242) and APP protein levels ($t_{5.041}$, $df_3$, *$p = 0.0150$; H4-SW (sh-ECSIT) mean = 0.2589, H4-SW (sh-negative) mean = 1.240) in H4-SW cells after lentiviral infection was observed (Figure 32 D).

Moreover, Aβ(1-42) concentration released in the cell culture medium by H4-SW cells after transfection of shRNA sequence against ECSIT was significantly reduced compared to its sh-negative control ($t_{5.710}$, $df_{10}$, ***$p = 0.0002$; H4-SW (sh-ECSIT) mean = 487.4, H4-SW (sh-negative) mean = 830.6) (Figure 32 E).
10.4. Discussion

ECSIT is a scaffold protein with an essential role in TLR and BMP signaling pathways in nuclear and cytoplasmic compartments (Kopp et al., 1999; Xiao et al., 2003). Moreover, several studies have demonstrated its ability to translocate to the mitochondrial compartment after losing its 49-amino acids N-terminal fragment, and interact with the mitochondrial complex I to the extent that its knockdown results in disturbed mitochondrial function (Vogel et al., 2007). However, it is not clear under which conditions ECSIT is targeted to the mitochondria, instead to other cellular compartments, where ECSIT may have an essential role controlling mROS production, mitophagy-dependent mitochondrial quality control and mitochondrial membrane potential (Carneiro et al., 2018).

In chapter VII, we hypothesized that the decrease of plasma sEVs-ECSIT in MCI/AD subjects (Figure 20 B, D) and 3xTg mice (Figure 20 A) might be explained by a different localization of ECSIT (as scaffold protein) depending on the cellular requirements. Here, we aimed to investigate the possible link of ECSIT protein with neurodegeneration by studying how ECSIT protein was localized in a cellular model for AD (H4 cells) and whether the overproduction of APP(SW) and downstream amyloid peptides in H4-SW cells affected this localization.

Therefore, we separated sEVs from 48 h-CM, a time point when neither H4-Native nor H4-SW cells showed a significant decrease in their viability (Figure 29 A), suggesting that sEVs release would not be affected by the stressful conditions that may occur when cell culture medium is FBS-depleted.

As we did for plasma-sEVs, the characterization of cellular sEVs-enriched preparations was also necessary. As shown in Figure 29, none of the parameters (mean diameter (D), D-values (E, F, G) or concentration (H)) showed significant differences between H4-Native and H4-
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SW cells, suggesting that any subsequent difference in sEVs protein content was independent from these structural elements. For what concerns the presence of EV and non-EV markers in sEVs (Figure 30 A), we observed an increased amount of CD63 protein levels in H4 cells-derived sEVs-enriched preparations with regard to the cell lysate, suggesting an enrichment of this EV marker in the sEVs. Conversely to what shown for our plasma sEVs, we here demonstrated that our cellular sEVs-enriched preparations were devoid of contamination by non-endosomal vesicles since GM130 marker was not detected (Figure 30 A).

In addition, in H4-SW cells, we observed a trend towards the decrease of ECSIT protein inside sEVs-enriched preparations (Figure 30 B).

Then, we separated mitochondrial, nuclear and cytoplasmic fractions from H4 cells. Together with the slight decreased amount of ECSIT within sEVs, H4-SW cells also showed lower levels of this protein in mitochondrial and cytoplasmic compartments (Figure 31 B, C), while it was increased within nuclei (Figure 31 D). This specific localization for ECSIT protein in the nuclei was also detectable by immunofluoresce studies (Figure 31 E) and suggests that the activation of TLR signaling pathway that ends up with the nuclear entry of ECSIT-p65/p50 complex (Mi Wi et al., 2015) might not be the only nuclear function for ECSIT that might be also involved in APP expression.

To further test this hypothesis in H4-SW cells, we evaluated Aβ(1-42) concentration release when down-regulating ECSIT. ECSIT mRNA basal expression did not show significant difference between H4-Native and H4-SW cells (Figure 32 A). When we tried to down-regulate it by shRNA, we expected to see a decrease. However, in H4-SW cells we detected the opposite, a significant increase in ECSIT mRNA expression (Figure 32 B) while nothing happened for H4-Native cells. Instead, we identified lower levels of ECSIT protein when
H4-SW cells were infected with shRNA against *ECSIT* (Figure 32 D).

Regarding this result and bearing in mind that shRNA have two different mechanisms of action (they can associate with target mRNAs and block their translation or cleave and degrade them) (Wang et al., 2011), one could expect that shRNA against *ECSIT* blocks the target mRNA without reducing mRNA expression while affecting protein translation (Alemán et al., 2007).

The increase in *ECSIT* mRNA expression (Figure 32 B) may be explained supposing that, in H4-SW cells, the shRNA is preventing ECSIT protein translation but, at the same time, there is a high demand of ECSIT protein in the nucleus. Therefore, the mRNA expression increase in H4-SW cells infected with the shRNA against *ECSIT* might be a cellular response trying to compensate ECSIT protein shortage. Instead, for H4-Native cells we did not observe any change in ECSIT protein or mRNA expression (Figure 32 D). This might suggest that ECSIT protein and mRNA levels depend on the amount of APP within the cells.

However, considering the result for the LDH release assay (Figure 32 C) where a higher toxicity is showed for the H4-Native cells with the scrambled RNA (sh-negative) than with the shRNA for ECSIT, one may speculate that the amount of cells, at the end of the experiment, was lower with the scrambled RNA. Therefore, levels of ECSIT mRNA and protein in H4-Native with sh-negative control may be underestimated.

In H4-SW cells, the decrease of ECSIT protein level was correlated with a decrease of APP protein when the infection was done with shRNA against ECSIT (Figure 32 D). As a result of *ECSIT* down-regulation, H4-SW cells decreased Aβ(1-42) release in the cell culture medium (Figure 32 E). The decrease of APP protein levels after ECSIT down-regulation might be explained with a possible role of ECSIT as a cofactor for *APP* transcription, as it does with Smads proteins in the BMP signaling pathway (Xiao et al., 2003) (Figure 32 E).
However, we need further experiment to confirm this possibility.

There are several open questions in this study. The link between ECSIT and APP needs to be better clarified. It might be that ECSIT in the nucleus acts as a cofactor (Xiao et al., 2003) for APP transcription, directly or indirectly by interacting with p65/p50 (Snow and Albensi, 2016), so when it is absent, APP mRNA cannot be produced and, therefore, APP and Aβ(1-42) protein levels will decrease (Figure 32 D, E). To confirm any of these hypotheses, further analyses are needed, for example, by measuring APP mRNA expression after shRNA experiments.

Therefore, ECSIT is not only a point of intersection for inflammation responses and mitochondrial function (Soler-López et al., 2012). ECSIT may have an important role in the nucleus in the context of AD pathologic mechanisms (Mi Wi et al., 2015), since under APP permanent overexpression (H4-SW cells), ECSIT protein increases its localization in the nuclear compartment. Moreover, this supports a decrease in the release of this protein in human (MCI and AD subjects) and mouse (3xTg-AD) plasma-sEVs (chapter VII).
Chapter XI

Chapter XI. Overall discussion and concluding remarks

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The current health crisis caused by COVID-19 has demonstrated how essential and important are science and scientific research (Janowitz and Tuveson, 2020). Any vaccine or cure need time to be developed and to reach the society. And, in some cases, even more than 100 years are not sufficient to find an effective treatment able to prevent the disease progression.

This is the case of AD. This ND is one of the most common cause of dementia in the elderly population (Gale et al., 2018). There are several hypotheses that have been implemented to explain AD pathologic mechanisms. Just to summarize a non-exhaustive list, the cholinergic hypothesis consider AD as a failure in the cholinergic system (Davies and Maloney, 1976). The tau hypothesis supports the intracellular accumulation of NFTs, which are formed by the aggregates of hyperphosphorylated tau able to spread from one cell to another (Frost et al., 2009). The mitochondrial cascade hypothesis instead supports the idea that mitochondrial dysfunction and damage affects APP processing in the sporadic AD and thus, it is the mitochondrial function the trigger factor of the amyloid cascade (Swerdlow and Khan, 2004). Another one is the inflammatory hypothesis in which Aβ is considered the trigger factor that activates microglial cells. This activation allows microglia to migrate to the plaques to phagocytose and process Aβ. However, this occasional activation becomes prolonged and the continuous release of pro-inflammatory factors by activated microglia exacerbates neuroinflammation and damages neurons (Kinney et al., 2018). However, among all these hypotheses and others that are not discussed here (Liu et al., 2019), the amyloid hypothesis is the most supported one. In fact, this hypothesis has sustained most of the scientific research made, firstly, to understand how AD begins and progresses and,
secondly, to develop more than the 20% of the clinical trials until now (Liu et al., 2019).

The core of this hypothesis comes from the idea that the Aβ(1-42) peptide is the triggering factor which causes and initiates AD pathogenesis, which has been also supported by genetic studies showing that autosomal-dominant mutations of APP and mutations in PSEN1 or PSEN2 (Citron et al., 1992; Scheuner et al., 1996). Aβ(1-42) forms deposits (Jarrett and Lansbury, 1993; Jarrett et al., 1993) that will trigger the formation of pathological tau (Bloom, 2014), synapse dysfunction (Palop and Mucke, 2010) and inflammation (Heneka et al., 2015) leading to the neurodegenerative process characteristic of AD dementia. Moreover, in the AD continuum, low levels of Aβ(1-42) in CSF (Parnetti et al., 2012) have already been shown in the preclinical phase of AD (before the symptoms onset) (Gale et al., 2018). In line with these facts, it seems that Aβ peptides, and the downstream products derived from their aggregation, are good biomarkers for the early diagnosis of AD, also at peripheral level, where in fact an intense research was done (Fandos et al., 2017; Nabers et al., 2018; M. J. Wang et al., 2017; Zetterberg and Burnham, 2019).

But clinical trials with drug therapy targeting amyloid systematically failed (Oxford et al., 2020), and amyloid-positive normal subjects exist (Mintun et al., 2006; Villedragne et al., 2011). The amyloid hypothesis’ linear structure has been recently criticized and, therefore, the potential and reasonable therapeutic target that amyloid has been since the discovery of AD becomes less obvious (Mullane and Williams, 2020; Kametani and Hasegawa, 2018). Maybe amyloid is not the best pharmacological target or maybe the anti-amyloid approach only works with some populations with genetic subtypes of AD dementia.

Another important still unsolved issue, despite intensive research with Aβ peptides as biomarkers of disease, is misdiagnosis. This misdiagnosis results in false positives; subjects with altered amyloid parameters but that remain cognitively normal are instead classified within the early stage of the disease when their membership to the preclinical AD group may
not be real (Sperling et al., 2011). This is of course a pivotal limitation to be avoided for clinical trial’s success.

The early diagnose and the misclassification are difficult to achieve with cognitive assessments only. Therefore, their combination with biomarkers is needed for the inclusion/exclusion criteria of the clinical trial enrollment, for the drug’s safety testing, to follow up the drug’s treatment effect and, in essence, for the early diagnosis of the disease (Cavedo et al., 2014). With this regard, several sources of biomarkers have been used. CSF is the most used for NDs because of its proximity to the brain. However, blood and its derivatives are more accessible, less costly and easier to obtain in the routine clinical practice (population screening or evaluation of the disease progression with or without a drug treatment) where repeated measurements of the biomarker are necessary (Gabelli, 2020). Its current disadvantage compared to CSF is its lower specificity of brain pathology (Aluise et al., 2008).

EVs instead may overcome the lower specificity of blood, plasma and serum biomarkers. They contain biological material that change according to the cellular and environmental conditions (Akuma et al., 2019). They provide an accurate diagnosis in early stages before symptoms onset (Kapogiannis et al., 2019), and since they can cross the BBB (Alvarez-Erviti et al., 2011) and naturally arrived in blood, they permit the development of a low-invasive biomarker discovery strategy. Moreover, EVs are less complex compared to blood (Boukouris and Mathivanan, 2015).

Though several advantages surrounded the isolation of sEVs compared to the whole biofluid in the field of biomarkers, there are also several disadvantages that need to be considered. SEVs separation and characterization requires time, current techniques do not allow to overcome similar physical properties between EV subtypes and EV markers (necessary, for example, to act as housekeeping genes in Western blotting) are not equally present in
different EV subtypes or within the same population of EVs. All these factors make quite complex the searching for new biomarkers. In addition, the use of sEVs as the source of biomarkers makes sense only if that given biomarker is differentially expressed according to the localization. In other words, for each biomarker studied within sEVs, the levels of that given biomarker in the source from which sEVs derived should be also provided to confirm its specific association with sEVs.

In this thesis, we investigated proteins and miRNAs associated with sEVs for their application in the early diagnosis of AD dementia.

SEVs-miRNAs in blood have drawn much attention since its packaging within exosomes is a selective process with specific loading mechanisms involved (Guduric-Fuchs et al., 2012). Moreover, according to Chevillet et al., only the 2.5% of the extracellular miRNA content of plasma is associated with the exosomal fraction (Chevillet et al., 2014), suggesting that only some miRNAs are functional in this type of cellular communication. The field of sEVs-protein biomarkers has been more studied. Instead, there is not a consensus or a reliable panel of miRNA biomarkers for AD or MCI stage of the disease, maybe due to the different sensitivity of methods used. In addition, there is not an agreement about the source from which miRNA should be separated. Here, we isolated miRNAs from plasma sEVs and compared their expression levels with those in serum to determine whether they are specifically enriched in sEVs. Moreover, in our study, we only considered miRNAs with a reported AD-associated mRNA target over which they exert an inhibitory effect. Although we were not able to identify any miRNA significantly and DE in MCI condition, we showed three miRNAs, miR-15-5p, miR-384 and miR-425-5p as possible sEVs-associated biomarkers for the AD dementia stage. Moreover, we also identified 3 down-regulated (miR-424-3p, miR-2113 and miR-4756-5p) and 2 up-regulated (miR-4312 and miR-580-3p) miRNAs in plasma sEVs that might also act as sEVs biomarkers for AD due to the control
they have over the expression of some AD-related genes.

With regard to proteins, ECSIT, NFL and TREM2 were further investigated as possible sEVs-associated biomarkers.

TREM2 and NFL proteins have been widely studied in AD and neither one nor the other has been definitively described as specific AD-related biomarkers. However, it has not been demonstrated whether their study within sEVs increases their correlation with cognitive decline onset in AD. Moreover, they have not been studied as sEVs-associated biomarkers. To this respect, we have contributed to show that they were both carried within mouse and plasma sEVs. However, measuring them within sEVs did not provide any significant advantage with regard to TREM2 or NFL in plasma.

On his behalf, ECSIT protein was first related to AD by using networks (Soler-López et al., 2011). However, since then, nobody has studied this protein and its link with neurodegeneration, cognitive decline or AD-related proteins. That prompted us to investigate ECSIT protein within human and mouse plasma sEVs. In our study, we demonstrated that ECSIT protein shows lower levels within sEVs derived from MCI and AD subjects and also from the 3xTg mice compared to their respective controls. This decrease was likely associated with the release of this protein within sEVs rather than in plasma. In the 3xTg mouse model, lower ECSIT protein levels were not purely linked with the cognitive deficit onset. In humans, since we did not isolate NDEs, ECSIT association with neurodegeneration was far from being confirmed. This led us to study ECSIT association with APP and Aβ(1-42) peptide and whether the overproduction of these two could affect the localization of ECSIT protein among different cellular compartments and sEVs.

We demonstrated than in H4-SW cells, ECSIT protein accumulates in the nucleus, suggesting that under AD pathologic mechanisms, this protein has an important function in
this compartment. And thus, it makes sense a reduced amount of ECSIT within sEVs in pathological conditions such as dementia or its early stages.

To conclude, our study showed several limitations and unanswered questions that have been widely addressed in each chapter and that we hope to answer in the future. We contributed to the field of biomarker discovery by demonstrating that miRNAs, ECSIT, NFL and TREM2 proteins deserve to be further studied alone or in combination with other biomarkers for the early diagnosis or progression of AD. Further studies are also required to demonstrate that EVs are better than any other biological matrix to isolate biomarkers in the field of AD. For sure, better universal nomenclature, separation and characterization methodologies are the basis for the EV field to move forward.
Chapter XII
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FUTURE PERSPECTIVES

In light of the results presented in this thesis, we would continue working to answer the open questions and trying to overcome the limitations of our studies.

Firstly, we will continue exploring the role of ECSIT in neurodegeneration. We will do it by studying the effects of ECSIT down-regulation in another cellular model of AD and elucidating the possible role of ECSIT as a cofactor of $APP$.

Secondly, TREM2 and NFL may not be specifically associated with sEVs. In addition, neither TREM2 nor NFL are specific for AD brain pathology. Therefore, proteomic analyses of plasma sEVs may be a reasonable strategy to discover new candidate biomarkers inside sEVs. Of course, the dysregulation of these possible candidates will be also assessed in plasma, to assure their association with sEVs.

And lastly, we will increase the number of subjects in our study with miRNAs to confirm the dysregulated sEVs-miRNAs we detected in AD subjects and, maybe, to discover some of them in MCI group. Moreover, the validation of miRNA candidates will be also assessed by real-time quantification.
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