**Universidade de Aveiro**
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**Maria José Cardoso Ferreira**

# Identificação de novos candidatos a biomarcadores para a doença de Alzheimer por análise bioinformática

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Professora Doutora Ana Gabriela Henriques, Professora Auxiliar do Departamento de Ciências Médicas da Universidade de Aveiro

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“If they are not laughing, you are not dreaming big enough.”

To all the dreamers out there.

**o júri**

presidente

Professor Doutor Ramiro Daniel Carvalho de Almeida
Professor Auxiliar, Universidade de Aveiro

Professora Doutora Raquel Monteiro Marques da Silva
Professora Auxiliar, Universidade Católica Portuguesa - Viseu

Professora Doutora Ana Gabriela da Silva Cavaleiro Henriques
Professora Auxiliar, Universidade de Aveiro

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**palavras-chave** Placas senis, tranças neurofibrilares, bioinformática, biomarcadores, doença de Alzheimer

**resumo** A doença de Alzheimer (DA) é a forma de demência mais comum em todo o mundo, caracterizada sobretudo pelo aparecimento de placas senis (SPs) e tranças neurofibrilares (NFTs) no cérebro de pacientes. Estes dois depósitos são as principais características histopatológicas da DA e, embora sejam caracterizados por componentes principais, como fibrilas amilóides nas SPs e proteína Tau hiperfosforilada nas NFTs, a composição molecular destas lesões ainda não está totalmente desvendada. Neste trabalho, procedeu-se a uma análise bioinformática dos proteomas das SPs e das NFTs obtidos por revisão da literatura. Obtiveram-se 836 proteínas para as SPs e 623 proteínas para as NFTs, sendo que 374, representam o proteoma comum. Análise funcional (Gene Ontology) dos proteomas associados a cada característica histopatológica, permitiu identificar os eventos moleculares subjacentes à formação destas lesões. Adicionalmente, a análise das proteínas comuns aos proteomas permitiu desvendar vias que ligam ambos os eventos histopatológicos e identificar novos alvos moleculares putativos para diagnóstico de DA ou intervenção terapêutica.

**keywords** Senile plaques, neurofibrillary tangles, bioinformatics, biomarkers, Alzheimer’s disease

**abstract** Alzheimer's disease (AD) is the most common form of dementia worldwide, above all characterized by the emergence of senile plaques (SPs) and neurofibrillary tangles (NFTs) in the patients' brains. These two deposits are the main histopathological hallmarks of AD, and even though these are characterized by main components, like amyloid fibrils in SPs and hyperphosphorylated Tau protein in NFTs, the molecular composition of these lesions is not yet fully understood. In this work, a bioinformatics analysis of the SPs and NFTs proteomes obtained by literature review was carried out. 836 proteins were obtained for SPs and 623 proteins for NFTs, with 374 representing the common proteome. Functional analysis (Gene Ontology) of the proteomes associated with each histopathological characteristic, allowed to identify the molecular events underlying the formation of these lesions. Additionally, the analysis of proteins common to the proteomes allowed to unravel pathways that link both histopathological events and identify putative molecular targets for AD diagnostic or therapeutic intervention.

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# Abbreviations

| | |
|---|---|
| AA | Antibiotics and Antimycotics |
| ALB | Albumin |
| AD | Alzheimer's Disease |
| APP | Amyloid Precursor Protein |
| Aβ | Amyloid-beta peptide |
| APOE | Apolipoprotein E |
| APP-CTFα | APP Carboxy-terminal Fragment of α-secretase processing |
| APP-CTFβ | APP Carboxy-terminal Fragment of β-secretase processing |
| AICD | APP intracellular domain |
| AEP | Asparagine endopeptidase |
| BACE1/2 | Beta-secretase 1 or 2 |
| BCA | Bicinchoninic acid |
| CaMK-II | Ca2+/calmodulin-dependent protein kinase II |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| CDR | Clinical dementia rating |
| Cdk5 | Cyclin-dependent kinase 5 |
| dH2O | distilled H2O |
| EOAD | Early Onset AD |
| ESCRT | Endosomal Sorting Complex Required for Transport |
| ELISA | Enzyme-linked immunosorbent assay |
| EV | Extracellular Vesicle |
| FAD | Familial Alzheimer's Disease |
| FBS | Fetal Bovine Serum |

| | |
|---|---|
| GABA | Gamma-aminobutyric acid |
| APH-1 | Gamma-secretase subunit APH-1 |
| GO | Gene Ontology |
| GFAP | Glial fibrillary acidic protein |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GSK3 | Glycogen synthase kinase 3 |
| GTPase | Guanosine triphosphatase |
| ILV | Intraluminal vesicle |
| L1CAM | L1 cell adhesion molecule |
| LOAD | Late Onset AD |
| LB | Loading Buffer |
| mRNA | Messenger ribonucleic acid |
| MAP | Microtubule associated protein |
| MAPT | Microtubule associated protein tau |
| MARK | Microtubule-affinity regulating kinase |
| MEM | Minimum Essential Media |
| MTBD | Microtubule-binding repeat domain |
| MCI | Mild cognitive impairment |
| MMSE | Mini-Mental State Examination |
| MAPK | Mitogen-activated protein kinase |
| MAPK1 | Mitogen-activated protein kinase 1 |
| MAPK3 | Mitogen-activated protein kinase 3 |
| MVB | Multivesicular body |
| NFT | Neurofibrillary Tangles |
| PHF | Paired helical filaments |

| | |
|---|---|
| PD | Parkinson’s Disease |
| PTEN | Phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase |
| p-Tau | Phosphorylated Tau |
| PET | Positron emission tomography |
| PS | Presenilin |
| PS1 | Presenilin 1 |
| PS2 | Presenilin 2 |
| PEN-2 | Presenilin enhancer 2 |
| PEN2 | Presenilin enhancer protein 2 |
| PDPK | Proline-directed protein kinases |
| PK | Protein Kinase |
| PKC | Protein kinase c |
| PP | Protein Phosphatase |
| PP2A | Protein phosphatase 2A |
| PP2B | Protein phosphatase 2B |
| PP5 | Protein Phosphatase 5 |
| PP1 | Protein Phosphatase 1 |
| RIPA | Radioimmunoprecipitation assay |
| ROS | Reactive oxygen species |
| RNA | Ribonucleic Acid |
| SP | Senile Plaques |
| PPP3CA | Serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform |
| PPP3CB | Serine/threonine-protein phosphatase 2B catalytic subunit beta isoform |

| | |
|---|---|
| SDS-PAGE | Sodium dodecyl sulphate–polyacrylamide gel electrophoresis |
| sAPPα | Soluble amyloid precursor protein alpha |
| sAPPβ | Soluble amyloid precursor protein beta |
| SCD | Subjective cognitive decline |
| SN | Substantia nigra |
| SVE | Synaptic Vesicle Endocytosis |
| SYNJ1 | Synaptojanin-1 |
| SNAP25 | Synaptosomal-associated protein 25 |
| T-Tau | Total tau |
| TBS | Tris-buffered saline |
| TBS-T | Tris-buffered saline + Tween |
| UA | University of Aveiro |

# Chapter 1. Introduction

# 1. Alzheimer's disease

## 1.1. Through time and space

Alzheimer's disease (AD) is a degenerative, progressive, and incurable brain disorder leading to progressive changes in cognition and memory. It impacts the individual capability to live independently due to memory loss, behavior perturbations and disorientation at speech and visuospatial levels, ultimately leading to death in the upcoming years (Alzheimer's Association, 2019; Soria Lopez, González and Léger, 2019). On average, people with Alzheimer's disease live between three and 11 years after diagnosis, with exceptions surviving 20 years or more. The degree of impairment at diagnosis can affect life quality and expectancy (Jack *et al.*, 2010).

This disease was first described by Alois Alzheimer in 1906 (Alzheimer A. *et al.*, 1995), but only in the mid-1980s AD subsequently evolved into a more specific neuropathology, when the two main molecular identities of the disease where identified, the beta amyloid (Aβ) peptide found in senile plaques (SPs) and hyperphosphorylated Tau protein found in neurofibrillary tangles (NFTs) (Glenner and Wong, 1984b, 1984a; Grundke-Iqbal *et al.*, 1986; Kosik, Joachim and Selkoe, 1986), as discussed below.

According to the World Health Organization, nearly 50 million people live with dementia worldwide, with over 10 million new diagnoses every year. By 2050, it is estimated that approximately 152 million people will be affected by some sort of dementia (World Health Organization, 2020). With the prevalence of AD increasing with age, this disorder is considered the most common type of dementia, accounting for more than 50% of the cases (Cacace, Sleegers and Van Broeckhoven, 2016; Alzheimer's Association, 2019; Deture and Dickson, 2019; Zvěřová, 2019). Women are the majority affected, regardless of age and ethnicity, covering nearly two-thirds of the total number, mainly because of their longer life expectancy and potentially due to biological or genetic variations or even different life style experiences (Chêne *et al.*, 2015; Alzheimer's Association, 2016; Scheyer *et al.*, 2018).

Even though, the majority of AD cases manifests later in life, changes at the molecular and histological levels in the brain, begin decades prior to clinical symptoms manifestations. By the time that Alzheimer's is clinically diagnosed, neuronal loss and neuropathologic lesions already occur in different brain regions like the hippocampus, cerebral cortex, and amygdala, all regions of the brain that play major roles in memory, cognition, and behavior (DeKosky and Marek, 2003; Henriques *et al.*, 2015).

## 1.2. Brain lesions, Alzheimer's disease hallmarks

In many neurodegenerative disorders, is becoming clear that a common pathological process surpasses through generation of proteinaceous aggregates (Ballatore, Lee and Trojanowski, 2007). The pathophysiology of AD is still subject of some controversy, but, regardless of that, the main brain hallmarks of the disease are, concordantly, extracellular deposition of Aβ peptides in the form of SPs (Deture and Dickson, 2019), and NFTs (Figure 1) as a consequence of Tau protein hyperphosphorylation (Hyman *et al.*, 2012) but also synaptic and neuron loss (Lane, Hardy and Schott, 2018). In addition to these features, neuropil threads, dystrophic neurites, associated astrogliosis, microglial activation, and cerebral amyloid angiopathy frequently coexists (Serrano-Pozo *et al.*, 2011).

Senile plaques are the outcome of the anomalous processing of amyloid precursor protein (APP) that renders in Aβ production, that subsequently aggregates and deposits in the brain. SPs are extracellular accumulations predominantly composed of abnormally folded Aβ with 40 or 42 amino acids (Aβ40 and Aβ42), being Aβ42 more abundant within plaques due to the higher level of fibrillization and insolubility (Lane, Hardy and Schott, 2018). The two main pathways by which APP can be processed are the amyloidogenic and the non-amyloidogenic pathways (da Cruz e Silva et al. 2004). In the non-amyloidogenic pathway, APP is cleaved by α-secretases within the Aβ domain producing the large soluble ectodomain sAPPα (soluble amyloid precursor protein alpha) and the carboxy-terminal fragment APP-CTFα. This last one can be additionally cleaved into the P3 fragment, which

cannot form stable oligomeric intermediates as Aβ does, and the APP intracellular domain (AICD) (Dulin *et al.*, 2008; Gupta and Goyal, 2016). In the amyloidogenic pathway, APP is cleaved by β- and γ-secretases resulting in the release of soluble secreted APPβ (sAPPβ) and the Aβ peptide with unpredictable length, ranging from 37 to 43 amino acids. Although regulation of these pathways is barely understood, there is evidence that overproduction or reduced clearance of Aβ, normally soluble, induces it to accumulate into neurotoxic oligomers, and eventually into highly regular amyloid fibrils of mostly Aβ40 and Aβ42, which form the plaques visible in the disease brains (Selkoe, 2004; Thal *et al.*, 2006; Gupta and Goyal, 2016; Deture and Dickson, 2019). Nonetheless, besides Aβ, many other proteins can be found deposited into SPs. These are molecules involved in several processes dysregulated in AD pathology as neuroinflammation (Domingues, da Cruz e Silva and Henriques, 2017), apoptosis (Moujalled, Strasser and Liddell, 2021) and abnormal APP processing (Zhang and Song, 2013).

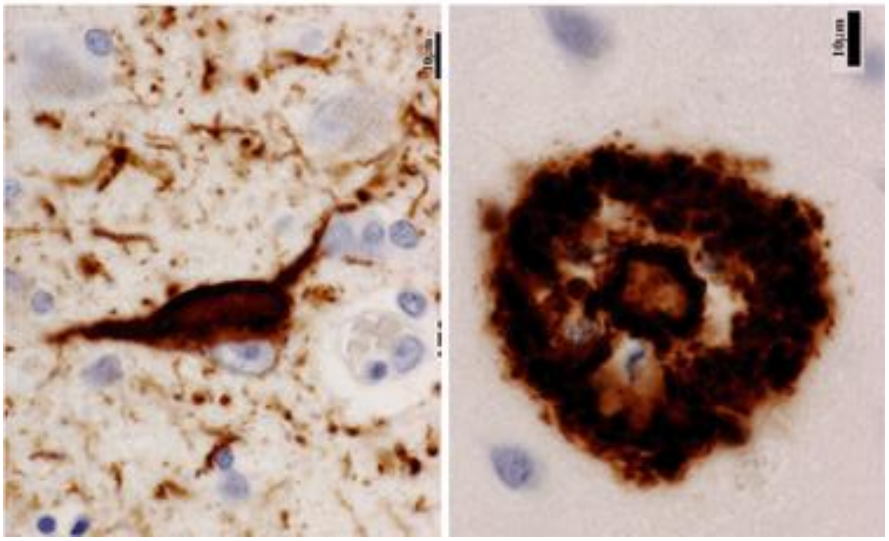


**Figure 1. AD brain sections' microphotographs** showing intracellular neurofibrillary tangles (right) and neuritic plaque (left). Adapted from Sengoku, 2020.

Neurofibrillary tangles are mostly intracellular structures that mainly cause neurons degeneration (Fukutani *et al.*, 1995; Avila, 2010). The molecular nature of the paired helical filaments (PHF) that form NFTs lies behind its major constituent, the microtubule-associated protein Tau (Goedert *et al.*, 1988; Chen, 2018). Tau is a microtubule-associated protein whose function is to maintain the microtubule assembly and the stabilization of

microtubule polymers, and phosphorylation and dephosphorylation are key events in these processes. Post-translational modification of this protein can have pathological consequences, provoking tauopathies-related disorders. Hyperphosphorylated Tau can occur by activation of Tau kinases such as cyclin-dependent kinase 5 (Cdk5), glycogen synthase kinase-3b and protein kinase A (GSK-3$\beta/\alpha$), and/or inhibition of Tau phosphatases like protein phosphatase 2A, or protein phosphatase 1. Tau hyperphosphorylation leads to the loss of its ability to stabilize microtubules, decreasing Tau affinity for them (Avila, 2010; Deture and Dickson, 2019; Lee, Mankhong and Kang, 2019; Brunello *et al.*, 2020).

Since Tau was first classified as a phospho-protein, till now there are known at least 85 phosphorylation sites, mostly serines and threonines (Oliveira *et al.*, 2017; Brunello *et al.*, 2020). The advanced hyperphosphorylation and aggregation of Tau in AD brains is linked to neuron degeneration and consequent synapse loss, major neuropathological findings in the brains of individuals with AD, that occur at specific brain areas such as the entorhinal cortex and the hippocampus (Scheff *et al.*, 2006; Sheng, Sabatini and Südhof, 2012); impacting the individual normal brain function and capabilities, as mentioned above. Likewise, besides Tau, other proteins can be found in association with NFTs. Despite both hallmarks are present in other neurological diseases, the presence of both SPs and NFTs in the human brain is typical in AD (Tiwari *et al.*, 2019).

## 1.3. Disease Evolution

Many are the speculations, but there is no certainty on how AD starts. However, nowadays, there is already some kind of chronology for the different events that occur alongside the disease' evolution. Alzheimer's disease develops along a timeline that begins with a long and asymptomatic preclinical phase (10–20 years), evolves into mild cognitive impairment (MCI), and culminates in clinical dementia diagnosis (Jack *et al.*, 2010).

Referring to cognitive impairment, it starts with a subtle decline, subjective cognitive decline (SCD), in the preclinical AD phase. This is a state in which a subjectively apparent decline in cognition appears undetected through neuropsychological tests, and,

is affected by numerous conditions such as aging, psychiatric conditions, personality alterations, neurologic and medical disorders, substance use, and medication, making it difficult to identify individuals with preclinical AD. Tends to occur at the late phase of preclinical AD and has been proposed as a pre-MCI stage (Jessen *et al.*, 2014). However, subjective decline in cognition is unspecific, which is a risk factor for mild cognitive impairment and disease development (Reisberg *et al.*, 2010).

Mild cognitive impairment is known as the middle stage before clinical diagnosis of AD. It refers to individuals who have some cognitive impairment but without sufficient severity to be considered dementia (Petersen, 2004). The severity of cognitive deficiency in the MCI phase of AD differs from the initial appearance of memory dysfunction to widespread dysfunction in other cognitive domains (Jack *et al.*, 2010). Synaptic dysfunction and loss of synapses themselves, due to biochemical and molecular dysregulation is indeed a disease early event (Oliveira *et al.*, 2017; Kent, Spires-Jones and Durrant, 2020).

## 1.4. Forms of Alzheimer's disease

There are two major forms of AD, the early onset AD (EOAD) also known as Familial Alzheimer's Disease (FAD) and the late onset AD (LOAD), mainly distinguished by the age when symptoms start. Age is the most sustained risk factor associated with AD (Carr *et al.*, 1997; Cacace, Sleegers and Van Broeckhoven, 2016). Approximately 10% of all AD diagnoses are EOAD. Early onset AD can be caused by very rare autosomal dominants gene mutations in three different genes: APP (located on chromosome 21); presenilin 1 (located on chromosome 14) and presenilin 2 (located on chromosome 1) (Cacace, Sleegers and Van Broeckhoven, 2016). Heritability also plays a role in this disorder, particularly for early onset cases (Wingo *et al.*, 2012), but a large number of genetically unexplained EOAD patients indicate that maybe additional causal genes remain to be identified (Cacace, Sleegers and Van Broeckhoven, 2016). Further, late onset AD is known to be sporadic and occurs by a mix of genetic and epigenetic factors, environment associated, and the contribution of genetic risks like APOE4 (Cacace, Sleegers and Van Broeckhoven, 2016;

Lane, Hardy and Schott, 2018; Deture and Dickson, 2019). In fact, a person carrying one copy of the ε4 allele have a two- to fivefold increase in relative risk of AD compared to non-carriers (Chouraki and Seshadri, 2014; Alzheimer's Association, 2016). Nonetheless, inheriting the ε4 form of the APOE gene is not guarantee that an individual will eventually develop AD since it is not sufficient neither necessary to cause the disease.

According to the statistics, up to half of the AD cases are also potentially attributed to modifiable risk factors such as diabetes, hypertension, smoking, obesity, and cognitive and physical inactivity. As such, evidence from an epidemiological perspective, suggests that healthy lifestyle habits can have a protective role against AD (Barnes and Yaffe, 2011; Crous-Bou *et al.*, 2017).

## 2. Imbalanced phosphorylation and AD

Post-translational modifications like protein phosphorylation and ubiquitination are in the spotlight of abnormal cellular processes that occur in AD. Depending on the equilibrium between protein kinase (PK) and protein phosphatase (PP) activities, signaling cascades need to be precisely controlled by active reversible protein phosphorylation. In AD there are several proteins that present abnormal phosphorylation states, responsible for physiological dysfunctions, and resulting in formation of both histopathological disease hallmarks, which are correlated to abnormal phosphorylation events (da Cruz e Silva and da Cruz e Silva 2003; Oliveira et al. 2017).

NFTs mainly result from hyperphosphorylated Tau, whereas the different Aβ peptides at SPs' core can be as results of phosphorylation state of APP and abnormal APP processing (Rebelo *et al.*, 2007b, 2007a; Vieira *et al.*, 2009). Furthermore, in postmortem analysis of AD brains it was registered a decreased level of PPs opposed to an increased in PKs, reuniting the perfect conditions for proteins hyperphosphorylation (da Cruz e Silva and da Cruz e Silva 2003; Gong et al. 2006; Oliveira et al. 2017; Rebelo et al. 2007).

### 2.1. Tau hyperphosphorylation

Microtubule-associated protein Tau was first discovered by Weingarten in the 70's (Weingarten *et al.*, 1975), but only more than a decade later was brought to the spotlight when found to make up PHF in AD brain (Lee *et al.*, 1991; Gong *et al.*, 2006).

Tau protein is encoded by the microtubule associated protein Tau (MAPT) gene, which in humans is located on the chromosome 17. Is composed of 16 exons which give rise to six different splicing isoforms, with the length depending on alternative splicing exons 2, 3 and 10 ranging from 352 to 441 amino acids (aa). Splicing isoforms of Tau contain either three (3R) or four (4R) microtubule-binding repeat domains (MTBDs), which affects both their microtubule-binding affinity (4R > 3R) and their tendency for aggregation. Tau is characterized by the existence of different functional domains. For example, the N-terminal projection domain consists of residues 1–150 (of the longest isoform) and is responsible for regulating microtubule binding even though it does not engage in the physical interaction.

When phosphorylation occurs within this type of domains, the Tau-microtubule binding is diminished (Ballatore, Lee and Trojanowski, 2007; Martin, Latypova and Terro, 2011; Matsumoto *et al.*, 2015; Brunello *et al.*, 2020; Xia, Prokop and Giasson, 2021).

Furthermore, the largest form of Tau is known to date to have at least 85 phosphorylation sites in different residues, more than 90% being serines and threonines and only 5% tyrosines. For each Tau phosphorylation site there's one or more protein kinase directed to action. There are classes where Tau kinases are distributed: protein kinases PDPKs (proline-directed protein kinases), protein kinases non-PDPKs and protein kinases specific for tyrosines. GSK-3 (glycogen synthase kinase-3), CDK5 (cyclin-dependent kinase-5) and MAPKs (mitogen-activated protein kinases) like MAPK-1 (mitogen-activated protein kinase 1) are examples of protein kinases PDPKs, while microtubule-affinity regulating kinase (MARK), Ca2+/calmodulin-dependent protein kinase II (CaMK-II) and protein kinase C (PKC) are kinases from the non-PDKs group (Avila *et al.*, 2004; Metcalfe and Figueiredo-Pereira, 2010; Martin, Latypova and Terro, 2011; Oliveira *et al.*, 2017).

Even though different kinases participate in Tau modification, emerging evidence points that GSK-3 plays an important role in regulating Tau phosphorylation under normal (physiological) or pathological (non-physiological) conditions and, has also been proposed to function as a molecular linkage between Aβ and Tau in AD pathogenesis (Avila *et al.*, 2004; Hernández *et al.*, 2010; Sayas and Ávila, 2021).

When there is a dysregulation in Tau phosphorylation state, passing to a hyperphosphorylation state, Tau sequesters normal Tau and other microtubule associated proteins (MAPs), resulting in microtubule destabilization and polymerization. Microtubules are important elements of the cytoskeleton, and thus compromise of its normal function will automatically affect neuronal axonal transport and, consequently, synapses and neurotransmission become impaired (Oliveira *et al.*, 2017; Kent, Spires-Jones and Durrant, 2020).

Also, the self-assembly of the hyperphosphorylated form of Tau starts a snowball effect, forming PHFs, and this β-sheet conformation structures posteriorly form larger

structures such as NFTs, affecting profoundly signaling cascades, leading to more abnormal phosphorylation events which potentiates the formation of more NFTs. This ultimately result in neuronal breakdown as mentioned above (Roland and Jacobsen, 2009; Martin, Latypova and Terro, 2011).

## 2.2. APP and the consequences of phosphorylation

APP is a transmembrane glycoprotein encoded by a single gene located on chromosome 21. There are many isoforms described, however, three isoforms, prevail in most tissues: APP695, APP751, and APP770, being APP695 the predominant form in neuronal tissues (da Cruz e Silva and Da Cruz e Silva 2003).

As mentioned before, APP can be processed by two main pathways, a non-amyloidogenic and an amyloidogenic pathway, although a more recent pathway has been described (Willem *et al.*, 2015). In the non-amyloidogenic pathway (Figure 2), APP is cleaved by an α-secretase (which can be a zinc metalloprotease) within the Aβ domain, resulting in the release of sAPPα and formation of a membrane anchored α C-terminal fragment (C83). Posteriorly, this fragment is then cleaved by γ-secretase complex, originating a non-toxic p3 peptide and the APP intracellular domain (AICD) polypeptide fragment (Edbauer *et al.*, 2003; Sheng, Sabatini and Südhof, 2012; Gupta and Goyal, 2016).

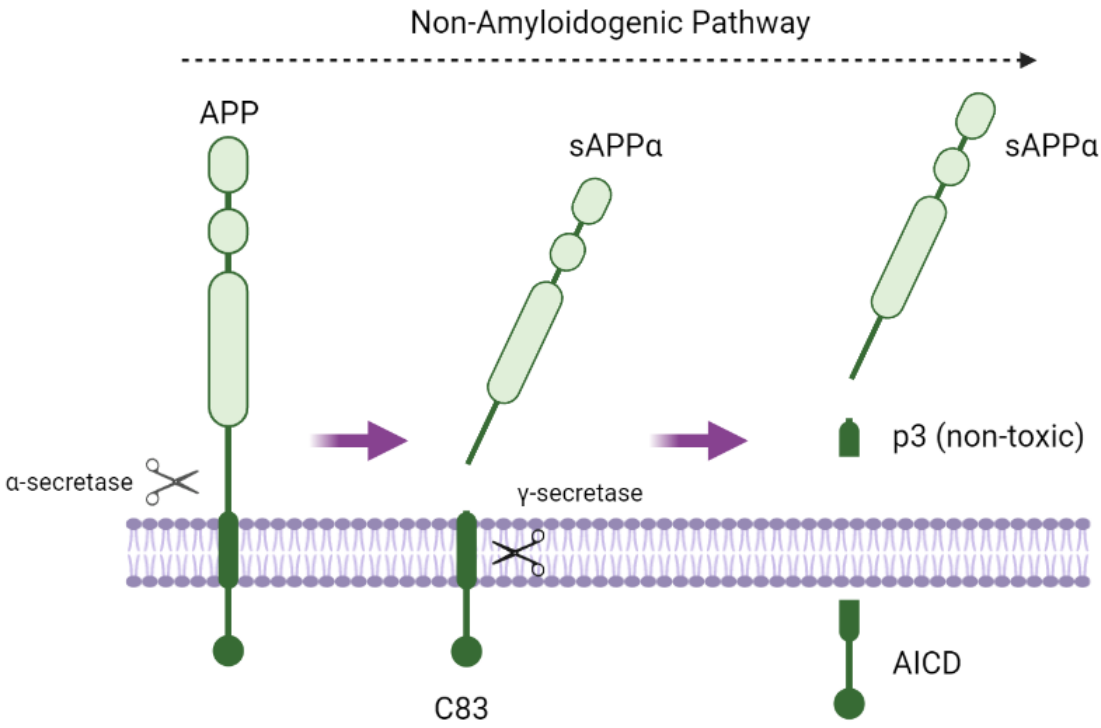



**Figure 2. Non-amyloidogenic Pathway of APP processing.** APP is cleaved by α-secretase originating sAPPα and αCTF (C83). Then, the C83 fragment is cleaved by γ-secretase producing p3 and AICD. Created with BioRender.com.

In the amyloidogenic way (Figure 3), APP is first cleaved by a β-secretase such as beta-secretase 1 or 2 (BACE1, BACE2) instead of an α-secretase, resulting in sAPPβ and a β C-terminal fragment (C99). This last one is then cleaved by a γ-secretase complex giving rise to AICD fragment and the Aβ peptide (Zhang and Song, 2013; Gupta and Goyal, 2016).

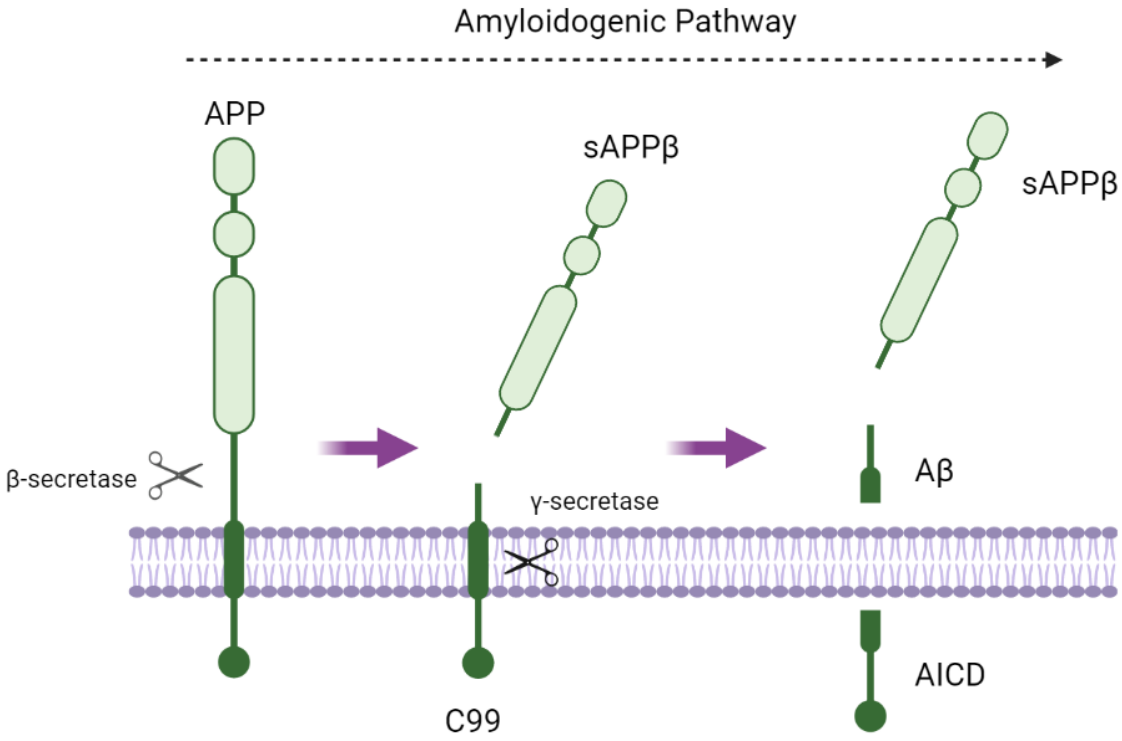



**Figure 3. Amyloidogenic Pathway of APP processing**. APP is cleaved by β-secretase generating sAPPβ and βCTF (C99). γ-secretase cleaves C99 fragment in AICD and in Aβ peptide. Created with BioRender.com.

There are many sites already described where APP can be phosphorylated, in both domains, extracellular and intracellular. In neuronal cells, APP695 can be phosphorylated at serine, threonine, and tyrosine residues (Zambrano *et al.*, 2001; Lee *et al.*, 2003). Even though it is not clear the biological importance of some of these phosphorylation events, many can already be associated with different physiological processes like phosphorylation at Y687 which is relevant for APP endocytosis and subsequent Aβ production or T668 phosphorylation important to regulation of APP binding to other proteins (Oishi *et al.*, 1997; Lee *et al.*, 2003; Rebelo *et al.*, 2007b, 2007a; Barbagallo *et al.*, 2011).

Through other proteins phosphorylation linked to APP cleavage or processing, there is a lot that can influence the signal cascades and contribute to AD pathophysiology. For example, the γ-secretase complex, formed by at least four different proteins: Presenilins (PS), Nicastrin, APH-1 (Gamma-secretase subunit APH-1), and PEN-2 (Presenilin enhancer 2) proteins that link with each other to form an active complex. There are two PS

homologous well known by the role of their mutations in EOAD, being those PS1 and PS2, as previously mentioned (De Strooper, 2003; Edbauer *et al.*, 2003; Verdile, Gandy and Martins, 2007; Sheng, Sabatini and Südhof, 2012). For instance, known phosphorylation process of PS1 by diverse kinases, works as a regulator in different processes such as apoptosis progression. Furthermore, studies have shown that PS1 can stimulate PI3K/AKT signaling, with the effect of suppressing GSK-3 mediated Tau hyperphosphorylation typical in AD. Apparently EOAD mutations PS1-dependent PI3K/Akt activation is inhibited, having the opposite effect of boosting GSK-3 mediated Tau phosphorylation (Baki *et al.*, 2004; Sayas and Ávila, 2021).

AD association with abnormal phosphatases activities is likewise evident in this neurodegenerative disease (Gong *et al.*, 1993). Different phosphatases in the human brain such as PP2A, PP5, PP1, and PP2B have also been shown to regulate Tau phosphorylation, with different levels of accountability, being PP2A the major Tau phosphatase. Curiously PP2A is also correlated with Aβ peptides production and PP1 is a post-synaptic marker correlated with loss of synapses in AD (Gong *et al.*, 1993; Liu *et al.*, 2005). Furthermore, PP inhibition was also shown to impact APP processing (Da Cruz Silva *et al.*, 1995).

This reflects the complex cascade of events and molecular players involved in this pathology and the impact that abnormal phosphorylation can have on disease progression.

# 3. Exosomes

## 3.1. Exosomes' biogenesis

Exosomes, a subset of extracellular vesicles (EVs) of endosomal origin, were initially described as waste material, condensed in small lipid vesicles, captured from the cytoplasm of maturing reticulocytes (Johnstone *et al.*, 1987). Nowadays, the designation is attributed to small, secreted organelles of ∼40 to ∼160 nm in diameter, with a single-membrane and enriched in a variety of constituents of the cells that secrete these, like proteins, lipids, nucleic acids, amino acids, and metabolites, displaying molecular heterogeneity. Exosome cargos embody a diverse source of normal or pathological biomarkers that apprehend a cell's metabolic state at a specific moment (Arraud *et al.*, 2014; Pegtel and Gould, 2019; Kalluri and LeBleu, 2020; Gleason *et al.*, 2021). These nanovesicles are currently recognized to be involved in various physiological and pathological processes, with essential roles in intercellular communications, even though most processes are still not understood. Besides exosomes, there are other two main subtypes of EVs which are microvesicles and apoptotic bodies, both considered to be larger than 100 nm. The production of EVs by cells appears to vary according to cellular origin, metabolic status, and environment of the cells (Konoshenko *et al.*, 2018). This leads to secretion of a heterogeneous population of exosomes with distinct sizes and composition/cargo (Colombo *et al.*, 2013).

Exosome's formation occurs in a process involving double invagination of the plasma membrane and maturation to intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). ILVs are then secreted as exosomes via MVB fusion to the plasma membrane and exocytosis. Alternatively, MVBs can also fuse with lysosomes or autophagosomes to be degraded, and in this case there is no exosome liberation (Van Niel, D'Angelo and Raposo, 2018; Kalluri and LeBleu, 2020).

Notably, the mechanisms of MVB trafficking and fusion with the cell membrane are regulated by several Rab guanosine triphosphatase (Rab GTPase) proteins and are synchronized with cytoskeletal and molecular activities (Ostrowski *et al.*, 2010). Rab11, Rab27, and Rab35 all appear to be involved in exosome release, since selective inactivation of each one partially impacted this pathway (Hsu *et al.*, 2010).

Diverse are the elements involved in exosome's biogenesis. Endosomal Sorting Complex Required for Transport (ESCRT) is the most common studied mechanism implicated in MVBs formation, which is composed of approximately thirty proteins that assemble into four known complexes, all with complementary but, distinct roles in the exosomes' biogenesis process (Hanson and Cashikar, 2012; Colombo, Raposo and Théry, 2014).

The four complexes that compose the ESCRT complex are ESCRT-0, -I, -II and -III. ESCRT-0 complex binds to transmembrane proteins that are ubiquitinated and thus destined for degradation. ESCRT-I and ESCRT-II are both tetramers involved in budding formation, sequester of ubiquitinated proteins, and seem to be necessary for exosome secretion, and ESCRT-III complex is engaged in scission of ILV (Hurley, 2010; Colombo *et al.*, 2013). Despite the ESCRT process, there is evidence that exosome biogenesis possibly occur via independent mechanisms, mainly mediated by lipids and tetraspanins (Trajkovic *et al.*, 2008; van Niel *et al.*, 2011; Soares Martins *et al.*, 2021).

## 3.2. Exosomes in Alzheimer's disease

The precise function of exosomes in the brain is not fully understood, nonetheless, most cell types in the central nervous system (CNS) release exosomes, including astrocytes, microglia, oligodendrocytes, and neurons (Gleason *et al.*, 2021). There are different neurological processes described, where exosomes play different roles. For instance, exosomes-mediated communication between oligodendrocytes and neurons are relevant for myelination and axons survival, support neuronal metabolism and can display neuroprotective roles. The last being demonstrated by the fact that neurons treated with oligodendroglial exosomes were less sensitive to oxidative stress or starvation. Furthermore, the secretion of exosomes from oligodendrocytes can be stimulated by glutamate release, being posteriorly endocytosed by neurons (Frühbeis, Fröhlich, Kuo and Krämer-Albers, 2013; Frühbeis, Fröhlich, Kuo, Amphornrat, *et al.*, 2013). Also, there is emerging evidence to suggest that the shedding of neuronal EVs at the synapses could be functionally relevant for plasticity-associated processes, and, possibly that in the regulation of synaptic plasticity, EVs from neurons can trigger synaptic pruning by microglia (Bahrini *et al.*, 2015; Koniusz *et al.*, 2016).

The transfer mechanism of cytotoxic proteins between nerve cells in AD remains unclear; however, recent studies have shown that nanoscale extracellular vesicles (exosomes) originating from cells may play important roles in the process. In fact, not only a wide range of proteins implicated in neuronal function have been identified in exosomes, but also exosomes can carry proteins related to AD pathogenesis. After the development of the considered first stages in AD, Aβ peptides may be secreted to extracellular space associated with exosomes, while most Tau proteins released into extracellular fluids are cut-off mid-region Tau, lacking the tail ends that mediate aggregation. In this latter event, there is evidence that the transmission of full-length Tau through the exosomes is possibly the main vector of spread of abnormal Tau (Kanmert *et al.*, 2015; Wagshal *et al.*, 2015; Guix *et al.*, 2018; Jiang *et al.*, 2019).

Further, it was also reported that exosomes can spread both Aβ and Tau protein by endosomal pathway and axonal transport (Polanco *et al.*, 2018). On the other hand, in the

vicinity of small SPs and large diffuse plaques, from brains of post-mortem AD patients, it was observed an enrichment of the exosome marker Alix, suggesting a correlation between Aβ release into exosomes, plaques formation and the progression of the disease (Rajendran *et al.*, 2006). Besides Aβ peptides and Tau, amyloid precursor protein (APP), APP C-terminal fragments and amyloid intracellular domain are some of the components associated with Alzheimer's pathology that have already been found in exosomes, thus increasing the interest on these EVs in the disease context (Soares Martins *et al.*, 2021). Although, there are mixed feelings in relation to the range of exosomes roles from neuroprotection to neurotoxicity in AD, there is no doubt that EVs may lead the way in providing a platform for putative biomarkers for disease diagnosis and or therapeutic strategies.

## 3.3. Exosome's potential in AD diagnostics

The exosomal potential to unravel disease mechanisms, or to be used as a source of biomarkers for therapy and/or diagnosis, is being explored, in the field of AD, where these nanovesicles appear recently to have a relevant role in disease pathogenesis. However, alterations in EV activity and/or content can be a feature of other pathologies, as cancer (Ogorevc, Kralj-Iglic and Veranic, 2013). By harnessing the capability of EVs to transfer their contents into target cells it may also be possible to convert these nanovesicles into vehicles for the delivery of therapeutic proteins, RNA molecules and drugs. (Lee, Mankhong and Kang, 2019; Kalluri and LeBleu, 2020; Soares Martins *et al.*, 2021).

So far, concerning the diagnosis of AD, the measurement of the Aβ1-42/1-40, total Tau (T-Tau) and phosphorylated Tau (p-Tau) 181 triplet in the cerebrospinal fluid (CSF), associated with PET scan and cognitive tests are nowadays the most accurate form of diagnose. Even though this combination is highly accurate, their broad implementation is restricted by high cost, limited accessibility, and invasiveness, sometimes reducing the diagnosis to only cognitive tests (Blennow and Zetterberg, 2009). Even now there is much to understand concerning exosomes' biogenesis, secretion, and functions, some of the biomarkers measured in CSF have already been found in exosomes (Figure 4), which may open the door to diagnostic methods surpassing the limitations that are faced nowadays (Soares Martins *et al.*, 2021).

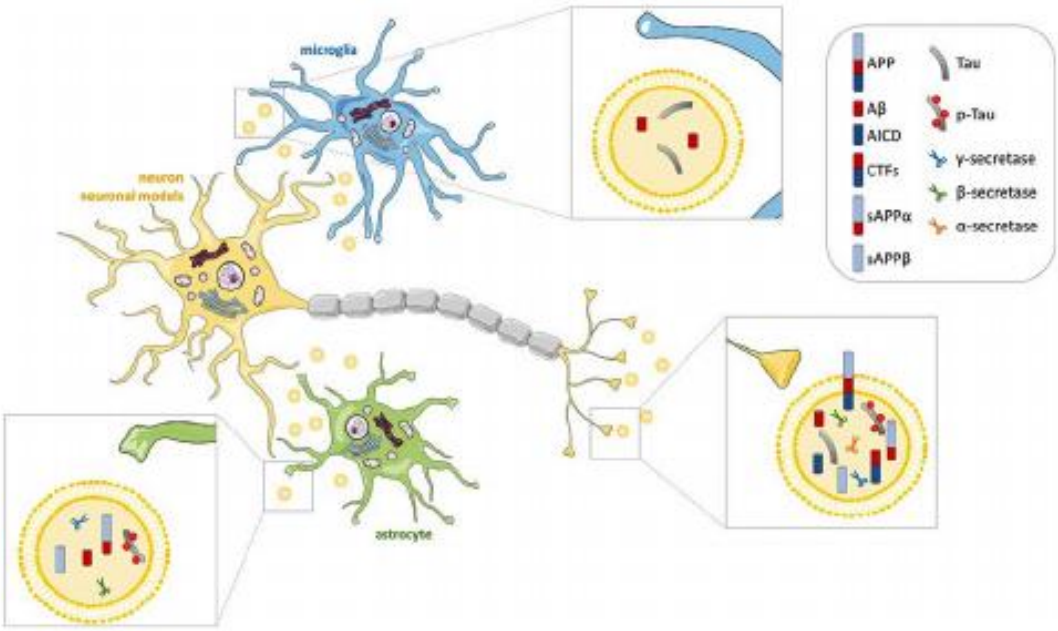



**Figure 4. AD-related molecules in exosomes secreted from different nervous system cells.** APP: amyloid precursor protein; Aβ: amyloid beta peptide; AICD: APP intracellular domain; CTF: carboxy-terminal fragment; sAPP α/β: soluble amyloid precursor protein α/β; p-Tau: phosphorylated Tau. From Soares Martins *et al.*, 2021.

## Aims

With the population getting older, dementia as become a daily life challenge. The misleading diagnosis, the few options for treatment for several neurological conditions, and the lack of understanding of the mechanisms underlying disease progression promotes the intensive search for novel molecular targets for AD diagnosis and/or treatment. With billions spend every year in research and patients caring, intensive research has been directed into biomarkers that can assist in early disease and differential diagnosis.

In this context, the main goal of this thesis was to identify novel putative exosomal biomarker candidates for AD. To achieve this main goal the following specific aims were set:

**To characterize the molecular proteomes of the two well-known hallmarks of AD, the SPs and NFTs**. A bioinformatic analysis was carried out allowing to enlighten the different groups of proteins, the pathways, and processes most affected in both lesions.

**To analyze the common SPs and NFTs proteome and link it to phosphorylation**. This is relevant since AD pathology and development involves abnormal phosphorylation events, where kinases or phosphatases could represent new targets.

**To validate putative candidates arising from the *in silico* analysis in human samples**. Nowadays the diagnosis through CSF biomarkers, is consider an expensive and invasive procedure, leading the search for biomarkers in peripheric fluids, like blood or urine, an urgent necessity. Since several proteins cannot pass though the blood brain barrier, exosomes analyses are a promising tool, as these nanovesicles can carry a disease molecular fingerprint.

The data obtained was subsequently presented in two chapters: literature review and bioinformatic analysis to identify putative biomarkers in Chapter 2 and validation of biomarker candidates in Chapter 3.

# Chapter 2. Bioinformatic Analysis for Identification of Novel Putative Targets for AD

# 1. Materials and methods

## 1.1. Senile Plaques and Neurofibrillary Tangles' proteomes

An extensive literature overview was carried out to identify both SPs and NFTs proteomes, recurring to Pubmed data base (https://PubMed.ncbi.nlm.nih.gov/). Articles published till 2020 (included) were consider. A set of keywords was used for each AD hallmark, as indicated in the flowchart (Figure 5).


**Figure 5**. **Literature Overview Flowchart**. Criteria implemented to obtain the final list of proteins present in both senile plaques and neurofibrillary tangles are indicated. The research was carried on PubMed database.

As indicated in the flowchart, a set of criteria was applied to identify the SPs and NFTs proteome, being identified a total of 86 and 63 articles, respectively. Some articles contributed for both SPs and NFTs proteomes, while others appeared specifically for each AD hallmark when running the literature search with each set of keywords.

After filtering the relevant articles, the information of the proteins was systematized and organized by: “Uniprot ID”, “Protein name”, “Gene Name”, “Method of identification”, if was by “proteomics or peptidomics”, “Brain tissue/area” where the protein was isolated, and the reference of the article from where the information was retrieved. Only proteins isolated from human brains, from patients diagnosed with Alzheimer's disease, were considered. The “Uniprot ID”, “Protein name”, “Gene Name” information was completed in accordance with Uniprot database (https://www.uniprot.org/).

## 1.2. Proteomes’ Gene Ontology Analysis (GO)

Gene Ontology (GO) analyses were conducted for SPs and NFT’s proteomes, and, also for the proteins in common to both structures, using ClueGO v2.5.8 and CluePedia v.1.5.8, plugins/apps from Cytoscape v3.8.2. Cytoscape is an open-source software platform designed for large-scale network analysis and visualization. Between many features, it allows the visualization of biological pathways and molecular interaction networks, and also has available a range of apps and plugins for different analysis (Shannon *et al.*, 2003).

On the ClueGO panel were defined the following variables: analysis mode “Functional Analysis”, organism “Homo Sapiens”, visual style “groups”, ontology, network specificity “medium+” and advanced statistical options, such as statistical test “Enrichment/Depletion (Two-sided hypergeometric test)” and *pV* correction “Bonferroni step down”, as proposed by (Trindade *et al.*, 2019).

For the analysis of both SPs and NFTs proteomes, the top 10 most relevant processes (significantly different and most representative) were presented with the respective genes associated.

## 1.3. Network Creation and Analysis

The protein-protein interaction networks were conducted using STRING online database (version 11.0; https://string-db.org/). “Gene Name” lists were submitted to STRING, and the resulting network information was loaded on Cytoscape software. The networks were analyzed through the “Network Analyzer” allowing to identify the betweenness centrality of the different nodes, and by adjusting different characteristics to that feature, the central nodes stood out.

## 1.4. Additional information collected from distinct databases.

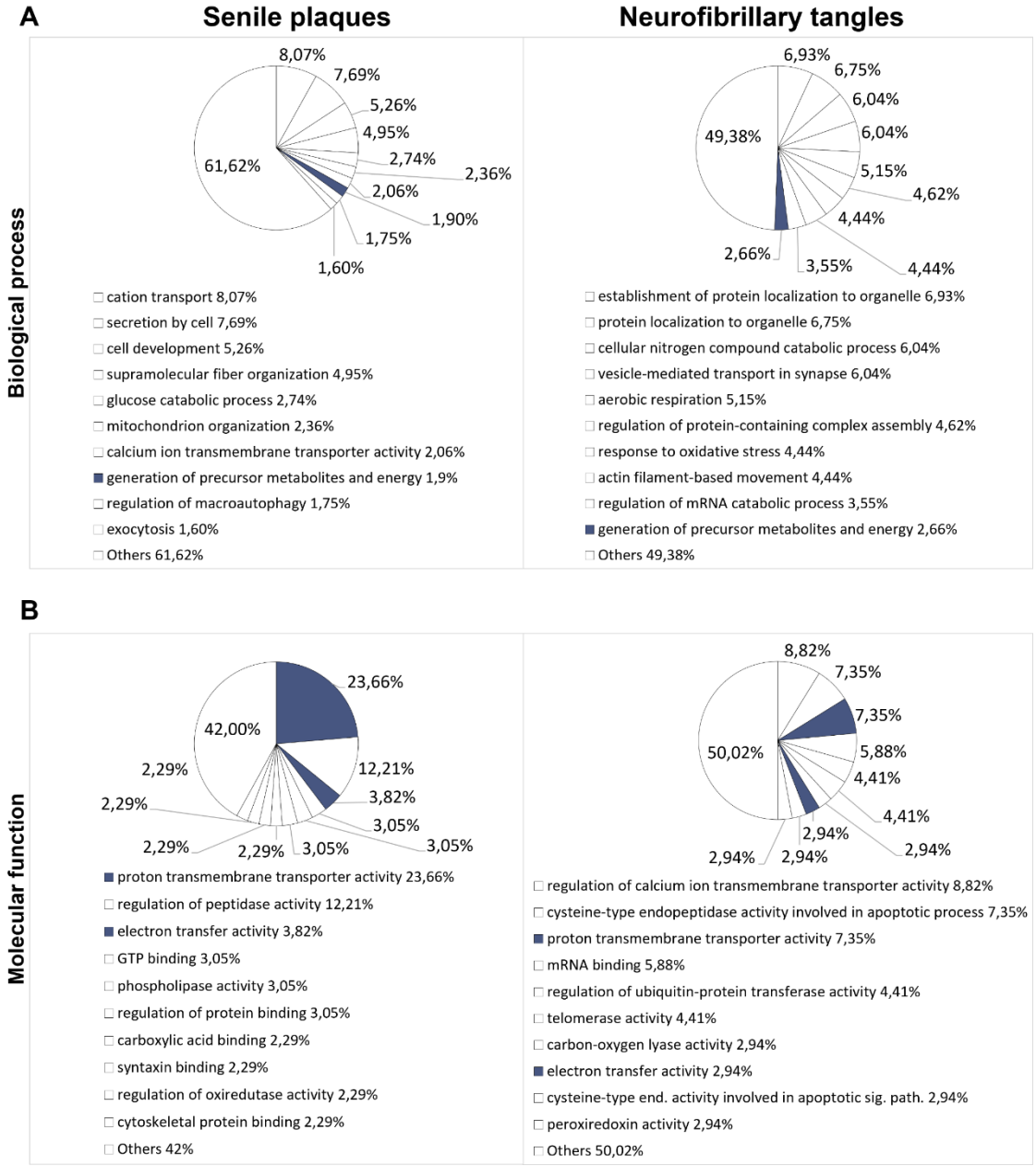
A Disgenet list of genes associated with AD was imported from https://www.disgenet.org/ on the 15$^{th}$ of October 2020, using “Alzheimer’s Disease” as key word. In addition, the list of Human Phosphatases was downloaded from http://hupho.uniroma2.it/ on the 19$^{th}$ of November 2020 and the Human kinases list was retrieved from http://www.kinhub.org/ on the 2$^{nd}$ of February 2020.

These lists were used to identify relevant targets for AD by overlap with the common SPs and NFTs proteomes.

# 2. Results

## 2.1. Gene Ontology analysis of SPs and NFTs proteomes

From the literature search conducted using Pubmed it was possible to associate 836 proteins (gene names) to the SPs proteome and 623 to the NFTs proteome (Supplementary material 1 & 2). The characterization of each proteome by Gene Ontology (GO) analysis was accomplished at different levels, including Biological Process, Molecular Function and Cellular Component. The top 10 terms obtained for each case are presented in Figure 6A, B and C.



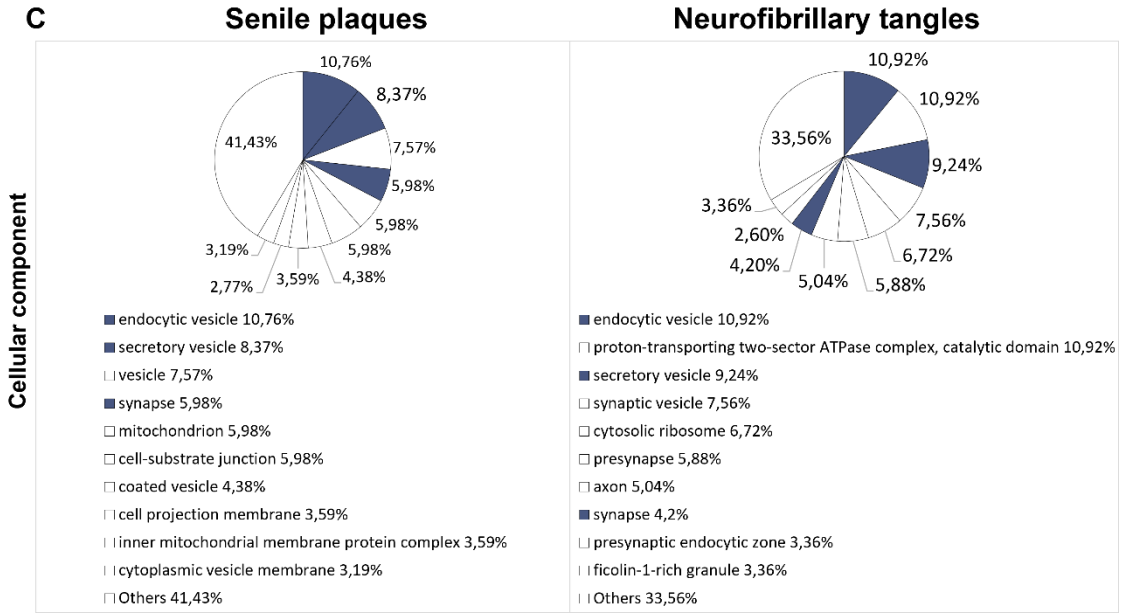



**Figure 6. Senile Plaques and Neurofibrillary tangles proteome's Gene Ontology analysis**. **A.** Biological Process; **B.** Molecular Function; **C.** Cellular Component. On the left side are the characterization of SPs proteome and on the right side are the NFT's. At a darker color are highlighted the terms in common to both proteomes.

At the biological process level, and looking at the top 10, few were the similarities between both proteomes, supporting that these are indeed very distinct. There was only one common term "generation of precursor metabolites and energy". For SPs, the top 3 processes were "cation transport", "secretion by cell" and "cell development", while for NFTs the top 3 were "establishment of protein localization organelle", "protein localization to organelle" and "cellular nitrogen compound catabolic process".

For the molecular function, two terms were found in common to both proteomes, being one of them "protein transmembrane transport activity", and the other "electron transfer activity" in both cases at the top 3 functions. Nevertheless, there was a lot of protein activity regulation and binding processes associated with both proteomes.

At the cellular component level, both proteomes presented an association with "endocytic vesicle", "secretory vesicle" and "synapse", being the first two, present in both proteomes at the top 3 components. Even though both proteomes evidence a strong relation with vesicle related terms, the SPs proteins also related with mitochondria, while

NFTs proteome associated with synapse related terms, among others. In general, the leading biological process, molecular function, and cellular component showed differences for the two structure's proteomes characterization, however, resemblances could also be detected for both proteomes

## 2.2. Characterization of the SPs and NFTs common proteomes

Overlapping of the SPs and NFTs proteomes revealed a total of 374 proteins (genes names) in common (Figure 7).

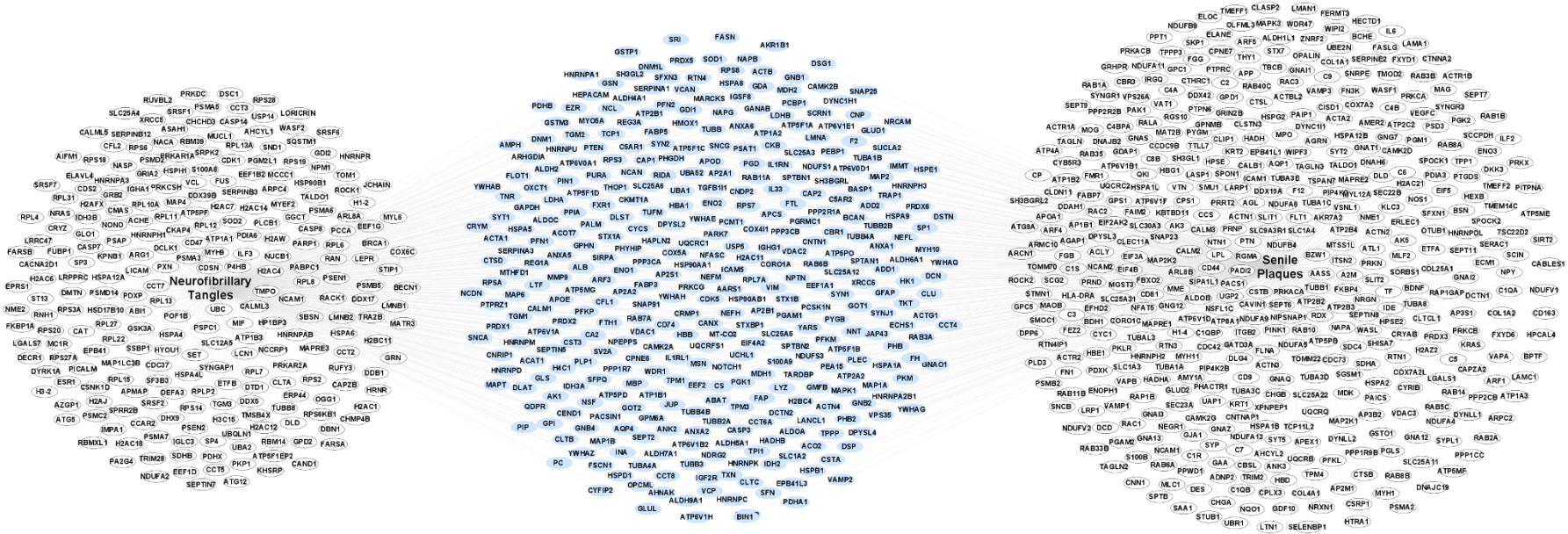



**Figure 7. Senile Plaques and Neurofibrillary Tangles common proteome**. On each side are the proteins found in the literature overview that appear only associated to each lesion. At a blue color are the gene names of the proteins common to both lesions, representing a total of 374 proteins. Network created on Cytoscape v3.8.2.

For this common SPs and NFTs proteome, Gene Ontology analyses at the biological process level (Figure 8) and for the Reactome pathways associated (Figure 9) were carried out. The top 10 processes or pathways were identified. Regarding the biological processes, several interesting terms came up related to various processes like “vesicle mediated transport in synapse”, “mitochondrial membrane organization”, “glucose catabolic process” and “regulation of intrinsic apoptotic signaling pathway”.

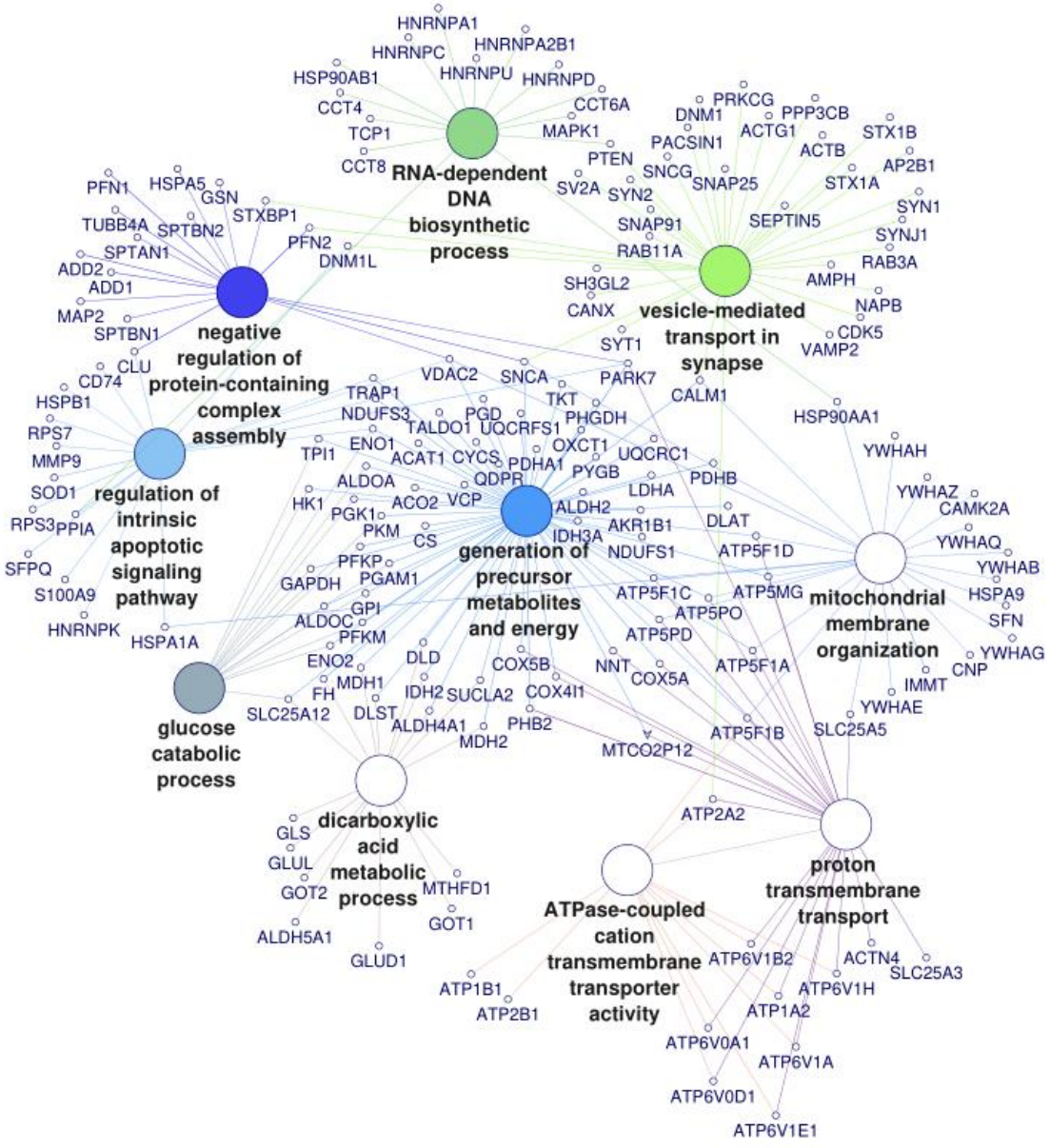



**Figure 8. Top 10 Biological Processes associated to the common SPs and NFTs proteome**. Network created on Cytoscape v3.8.2. with ClueGO v2.5.8 + CluePedia v.1.5.8 plugins. Genes linked to each process are indicated in blue. Only pathways with $p \leq 0.05$ were considered.

Among the main pathways linked to this common proteome, the ones with more significance were “L1CAM interaction” and “Recycling Pathway of L1”. Nonetheless, other relevant pathways also arose, like “GABA synthesis release, receptor and degradation”, “Neurotransmitter receptors and postsynaptic signal transmission” and “chaperone mediated autophagy” (Figure 9).

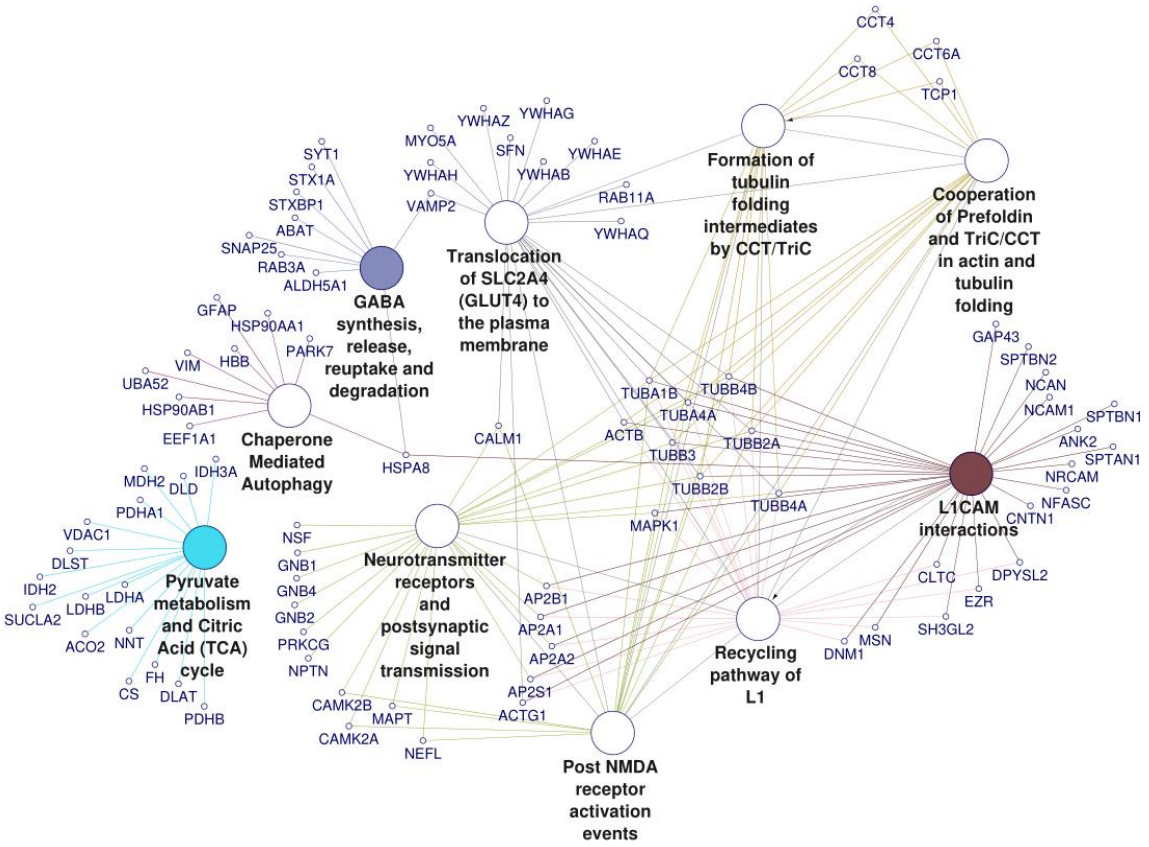



**Figure 9. Top 10 Reactome Pathways associated to SPs and NFTs common proteome**. Network created on Cytoscape v3.8.2. with ClueGO v2.5.8 + CluePedia v.1.5.8 plugins. Genes linked to each pathway is indicated in blue. Only pathways with $p \leq 0.05$ were considered.

## 2.3. Identification of putative phosphotargets relevant for AD

To identify putative targets relevant to AD two distinct approaches were considered. The first included the overlap of the SPs and NFTs common proteome with a list of genes associated with AD imported from the Disgenet database, and a subsequent network construction of these proteins interactions. This analysis resulted in a set of 59 proteins (gene names) identified (Figure 10).

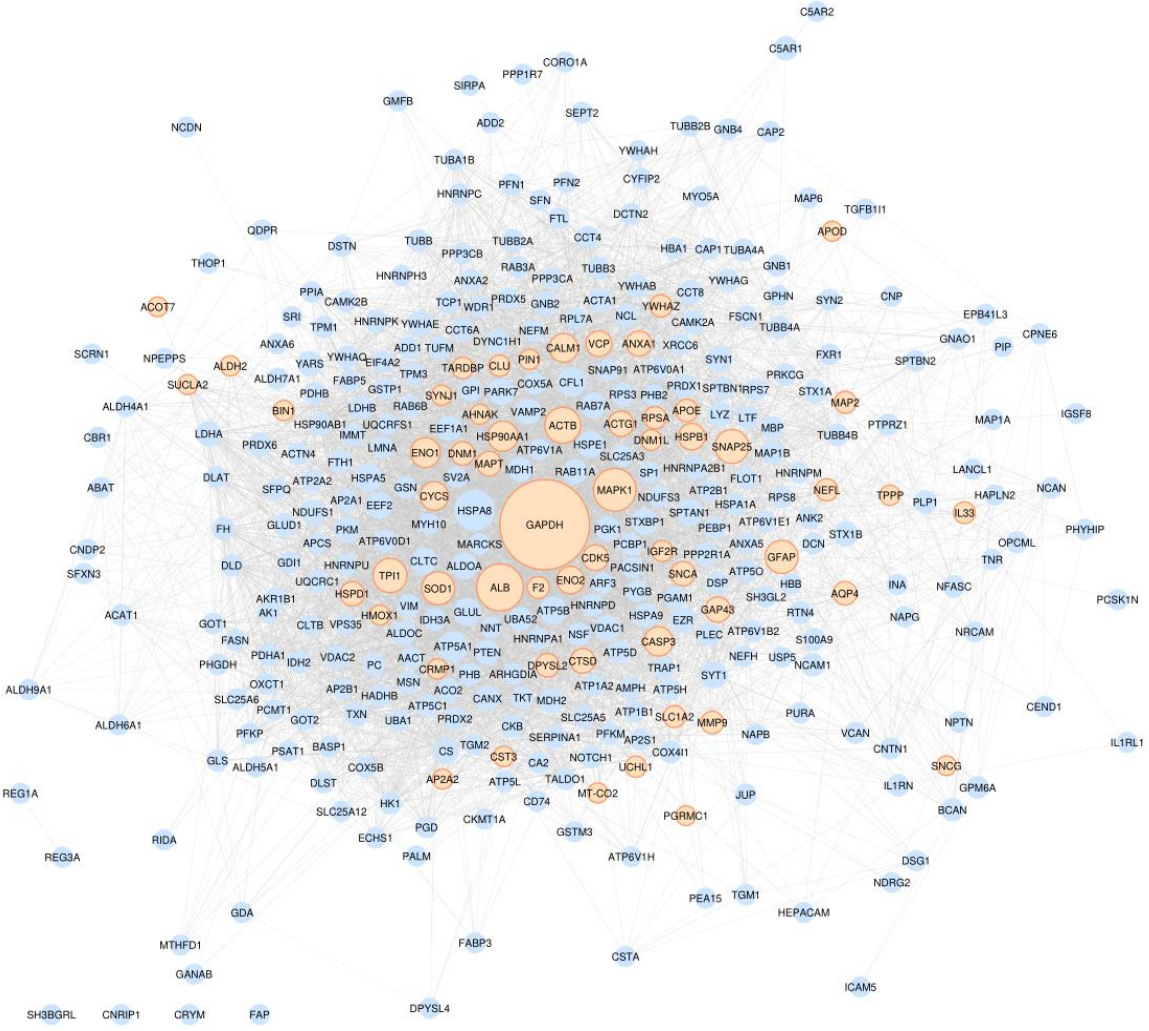



**Figure 10. Overlap of SPs and NFTS common proteome with AD Disgenet list**. Network of the common proteins between SPs and NFTs proteomes that overlapped with the list of genes linked to AD. The genes that overlap with the Disgenet list are represented in orange. From Disgenet, only proteins with score ≥ 0.1 are indicated This interactome was created on STRING and then imported to Cytoscape v3.8.2 for network analysis.

The main node of this network was GAPDH, which codes for Glyceraldehyde-3-phosphate dehydrogenase. Other central nodes identified included MAPK1 (Mitogen-activated protein kinase 1), ALB (Albumin), SNAP25 (Synaptosomal- associated protein 25) and GFAP (Glial fibrillary acidic protein).

In parallel, due to the relevance of abnormal phosphorylation events for AD, the SPs and NFTs proteomes were also overlapped with a list of human kinases and phosphatases (Figure 11).

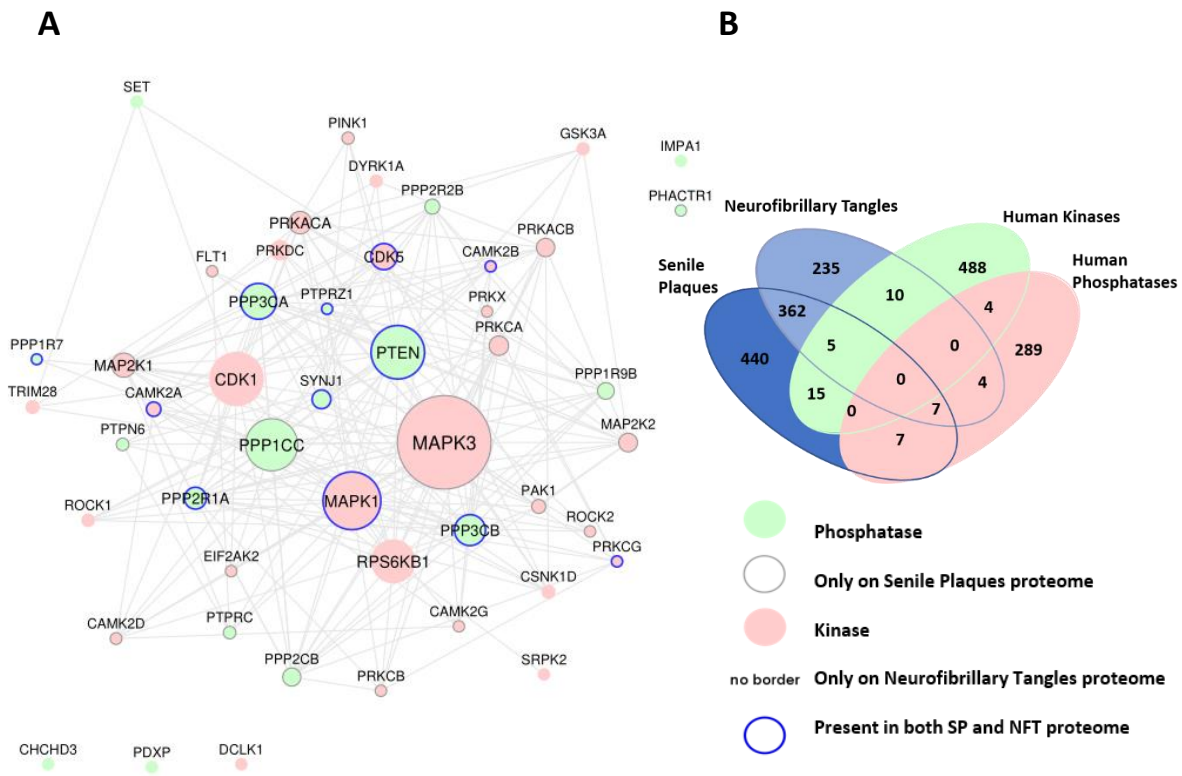



**Figure 11. Identification of AD relevant phosphotargets from the common SPs and NFTs proteome. A.** Network obtained for the phosphatases (green) and kinases (pink) present in SPs (grey contour), NFTs (no contour) or both proteomes (blue contour). This interactome was created on STRING and then imported to Cytoscape v3.8.2. **B.** Venn diagram representative of the proteins in figure A.

This analysis allowed to identify 30 kinases and 18 phosphatases, from which 5 kinases and 7 phosphatases were common to both proteomes. MAPK1 appeared as a central node common to both proteomes. Another kinase common to both SPs and NFTs was CDK5 (Cyclin-dependent-like kinase 5). Likewise, several common phosphatases were also identified, namely PTEN, PPP3CB and PPP3CA.

The approaches employed allowed to identify a set of proteins that could constitute possible biomarker candidates for AD diagnosis. Common to both approaches were 3 molecular targets, namely MAPK1, SYNJ1 (Synaptojanin-1) and CDK5, making them ideal candidates for validation in human samples.

Our focus centers on the study of these candidates on exosomes and thus the presence of these proteins on exosomes from peripheric fluids was addressed recurring to previous exosomes lists achieved from a literature search carried out by the group (Martins et. al. 2021).

**Table 1. Presence of putative biomarker candidates identified in exosomes from peripheral biofluids.** Information regarding the presence of MAPK1, CDK5 and SYNJ1 in exosomes from peripheric fluids, from Exosomal proteomes from (Martins *et al.*, 2021) was collected. "Yes" means that the gene name was found in the data related to the presence in exosomes of the respective peripheric fluid. "NO" means the opposite.

| Gene Name / Fluid | CSF | Serum | Plasma |
|---|---|---|---|
| **MAPK1** | Yes | No | No |
| **CDK5** | No | No | No |
| **SYNJ1** | No | No | Yes |

From these 3 putative candidates, CDK5 was the only protein thus far not reported in exosomes. Thus, the kinase MAPK-1 and the phosphatase SYNJ-1 were the two candidates selected for subsequent validation in human samples.

# Chapter 3. Phosphotargets validation in serum-derived exosomes from AD cases

# 1. Materials and methods

## 1.1. Human Samples

Human samples were obtained from participants of the primary care based-cohort (pcb-cohort) previously established by the group (Rosa *et al.*, 2017). This cohort includes controls and individuals with dementia, characterized by cognitive testing such as clinical dementia rating (CDR) and Mini-Mental State Examination (MMSE). The study was approved by the Ethics Committee (Comissão de Ética para a Saúde da ARS Centro, protocol No. 012804-04.04.2012) and by the National Committee for Data Protection (CNPD No. 369/2012). The regional pcb-cohort includes 9 individuals, clinically diagnosed as AD cases (mean age 78.67±5.07) and 9 age- and sex-matched controls (mean age 77.56±4.83). All participants gave written informed consent.

## 1.2. Exosomes Isolation from human serum

There are different methods of exosome isolation from biofluids and cells, such as precipitation, column-based exosome isolation and the standard ultracentrifugation procedure (He *et al.*, 2018; Soares Martins *et al.*, 2021).

In this work, serum-derived EVs, with exosome-like characteristics, were isolated from 200 µL of serum from controls and individuals with AD using the ExoQuick Serum Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, USA), as previously described (Li *et al.*, 2017; Martins *et al.*, 2018). ExoQuick™ is a proprietary polymer that gently precipitates exosomes and microvesicles, from serum for example, with sizes between 30 and 200 nm. The general protocol is illustrated in the Figure 12. The exosomal pellet resulting from the precipitation process was resuspended in RIPA buffer (Sigma-Aldrich) with protease inhibitors for Western blot. The RIPA buffer is a lysis buffer, that minimizes non-specific protein-binding interactions. Besides the extraction of individual samples from each patient, a pool of exosomal sample was run in each blot for data normalization.

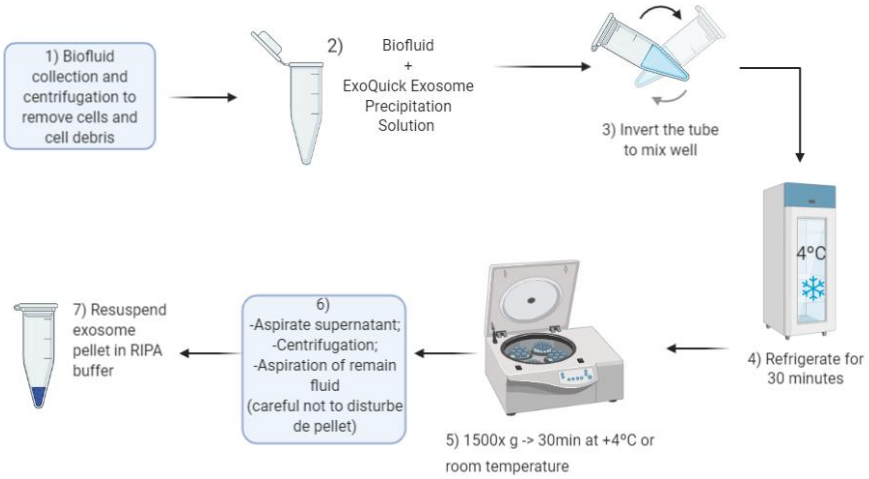



**Figure 12. General protocol of exosome isolation through ExoQuick Serum Exosome Precipitation Solution.** Created with BioRender.com

The samples of serum-derived extracellular vesicles with exosome-like characteristics (just serum-derived exosomes in the rest of the text), are the ones previously characterized by (Martins *et al.*, 2021).

## 1.3. Human cell lysates preparation

SH-SY5Y human cell line were maintained in MEM (Minimum Essential Media)/F12 supplemented with 10% of Fetal Bovine Serum (FBS) and 1% of Antibiotics and Antimycotics (AA). Cells were collected with RIPA buffer, when 80% confluence was reached. Cell lysates were also prepared in RIPA buffer. SH-SY5Y were used for comparative purposes during immunodetection of proteins of interest.

## 1.4. Protein quantification

The serum-derived EVs samples were diluted in a proportion of 1:5, in RIPA buffer (Sigma-Aldrich) with protease inhibitors; SH-SY5Y pool of cells, was not diluted. Protein quantification was performed using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit, which is a two-component, high-precision, detergent-compatible protein assay allowing to determine protein concentration. It is based on a color development reaction fomented by the chelation of copper with the protein in an alkaline environment, known as the biuret reaction, followed by the chelation of two molecules of bicinchoninic acid (BCA) with one cuprous ion, resulting in an intense, purple-colored

reaction product. Depending on the quantity of protein/peptides present in the sample, the final color will be more or less purple and, can be measured at 562 nm.

The standards were prepared as indicated in Table 2, in a 96 well plate. 200 µL of Working Reagent, in a proportion of 50 µL of Reagent A to 1 µL of Reagent B, were added to each well, and was incubated for 30 minutes at 37ºC. After that, the absorbance of BCA assay was measured at 562 nm with the auxiliary of TECAN Infinite M200.

**Table 2. BCA protein assay' standards**

| Standard | Bovine Serum Albumine (BSA) (µL) | Buffer (µL) | Protein Mass (µg) |
|---|---|---|---|
| $P_0$ | - | 25 | 0 |
| $P_1$ | 1 | 24 | 2 |
| $P_2$ | 2 | 23 | 4 |
| $P_3$ | 5 | 20 | 10 |
| $P_4$ | 10 | 15 | 20 |
| $P_5$ | 20 | 5 | 40 |

## 1.5. SDS-PAGE and Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis or simply SDS-PAGE is a method that recurs by electrophoresis allowing protein separation by their molecular weight. In this assay, the proteins are coated with negative charge, masking their intrinsic charge, so they can be separate purely by their weight. The polyacrylamide gel works as a size sorting matrix. The proteins migrate across the gel pores from the positive to the negative anode. A gradient gel 5-20% was used, which allows for the resolution of both high and low molecular weight bands. The protein marker Precision Plus Protein Dual Color Standards (Bio-Rad) was used.

Prior to the run, Loading Buffer (LB) was added to the samples. This buffer contains between other constituents, β-mercaptoetanol, which cleaves disulphide bonds to disrupt tertiary and quaternary protein structure allowing the proteins to become completely unfolded so that they migrate properly.

Serum-derived exosome samples were loaded on the gel, normalized for protein content, 25ug of protein for MAPK-1 assay and 150ug of protein for SYNJ-1 assay. For comparative purposes of protein bands, 50 ug of SHSY5Y were also loaded on the gel. After the addition of the Loading buffer, the samples were boiled at 99◦C for 5 minutes, then loaded in the 5-20% SDS-PAGE gradient gel and separated at constant 90 mV for 2 to 3 hours, in a Hoefer SE 600 vertical electrophoresis system.

Afterwards, proteins were transferred to nitrocellulose membranes in a wet system through the application of an electrical current. Proteins migrate out of the gel to the membranes from the negative to the positive cathode. This method is well known as Western Blotting and permits the detection of proteins from biological samples, with specific antibodies, in a solid membrane. The transference usually occurs for 18h at 200mA. To confirm a successful transfer, the membranes were stained with Ponceau staining solution, which is a red, rapid, and reversible protein stain. Ponceau S binds to the positively charged functional groups of the protein (amino group) and the non-polar regions of the protein. The dye does not interfere with antibody detection. In brief, the membranes were hydrated with TBS 1x (Tris-buffered saline) for 10 min, followed by 5 min submersed in Ponceau staining solution, after which the excess was raised with distilled water, to have a clear background. The Ponceau S was removed out of the membranes with TBS-T 1x (Tris Buffered Saline with Tween 20%).

For immunoblotting with specific antibodies, the membranes were blocked in non-fat dry milk solution (5%) for 4h followed by 3 h at room temperature plus overnight incubation with primary antibody, at 4◦C, Anti-ERK2 (MAPK1) 1:200 (sc-1647; Santa Cruz Biotechnology, Dallas, TX, USA) and Anti-SYNJ1 1:750 (HPA011916; Sigma Prestige Antibodies). After primary antibody incubation, the membranes were washed 3 times with TBS-T1x, followed by the respective secondary antibody incubation, anti-mouse IgG, HRP-linked antibody (1:2000) (7076S; Cell Signaling Technology, Danvers, MA, USA) and anti-rabbit IgG, HRP-linked antibody (1:5000) (7074S; Cell Signaling Technology, Danvers, MA, USA). The incubation occurred at room temperature for 2 h, followed by 3 washes with TBS-T1x. Protein band detection was achieved by chemiluminescence using Immobilon Crescendo Western HRP Substrate and/or ECL Select (GE Healthcare

Life Sciences, Chicago, IL, USA) and images acquired using the Chemidoc gel imaging system (Bio-Rad, Hercules, CA, USA).

## 1.6. Statistical Analyses

Statistical analysis was carried out with two-tailed Student's t-test, when normal distribution was verified and with Mann-Whitney test, when normal distribution was not verified, to assess differences in Controls vs AD samples. Since samples were distributed through different membranes, a ratio between the Pool/individual sample was performed to normalize samples. Only *p*-values equal or less than 0.05 were considered significant. The Analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA).

# 2. Results

## 2.1. MAPK1 levels in serum-derived exosomes of AD cases

Bioinformatic analysis revealed 3 putative phosphotargets interesting to follow: MAPK1, SYNJ-1 and CDK5. Nonetheless, as explained above only two candidates were followed, the MAPK-1 kinase and the SYNJ-1 phosphatase.

Exosomes were isolated from human serum of patients clinical diagnosed as AD cases (putative AD cases) and corresponding sex- and age-matched controls from the pcb-cohort, using a precipitation-based solution commercial kit (ExoQuick). As expected, MAPK-1 appeared around 50 kDa (Figure 13A). For this kinase, a small but significant decrease could be detected between ADs and Controls from the pcb-cohort (Figure 13B and C).

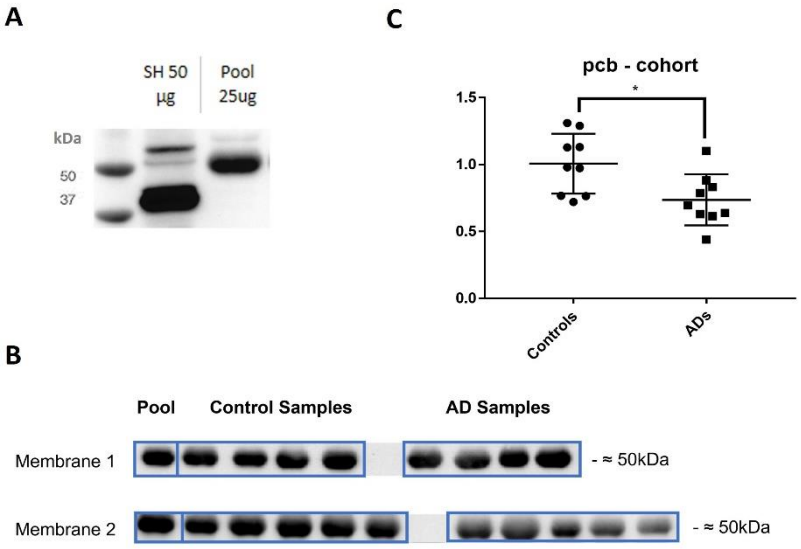



**Figure 13. Exosomal MAPK1 levels in AD.** A & B. Exosomal MAPK1 WB detection. C. Quantification of exosomal MAPK-1 levels. * $p < 0.05$

## 2.2. SYNJ-1 levels in serum-derived exosomes for AD cases

SYNJ-1 antibody was also tested, in SH-SY5Y cell lysates and a pool of exosomes. Several bands at different molecular weights were detected in SH-SY5Y almost immediately, corresponding to the SYNJ-1 isoforms/fragments already described in literature (Ando *et al.*, 2020). For exosomes, and after a longer exposure period, two protein isoform/fragment bands at ≈25kDa and another at ≈70kDa could be detected (Figure 14A). Based on the literature, we speculated that this 70kDa band was a fragment resulting from SYNJ-1 processing (Zou *et al.*, 2021).

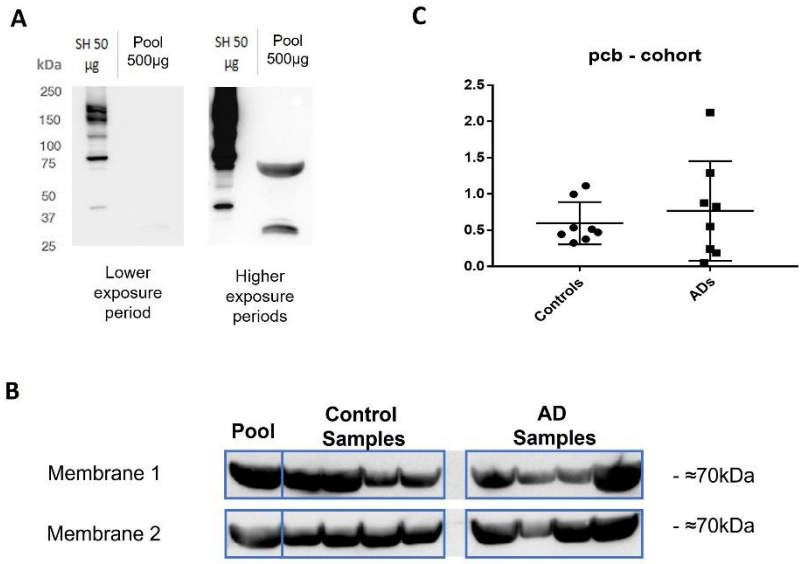



**Figure 14. Exosomal SYNJ-1 fragment levels in AD.** A & B. Exosomal SYNJ-1 WB detection. C. Quantification of exosomal SYNJ-1 levels.

Contrary to MAPK-1, analysis of the SYNJ-1 ≈70 kDa band, that appeared in all exosome samples (Figure 14B), revealed a slight increase of SYNJ-1 levels in serum-derived exosomes from AD patients, compared to the controls (Figure 14C).

# Chapter 4. Discussion

Alzheimer's disease is a devasting, progressive and neurodegenerative disorder, with great impact on patient's quality of life. Intensive research in AD is driven by the need to find an early diagnosis and a cure for the disease. A therapy that targets and influences the underlying disease process of AD was recently approved. "Aduhelm", so called the treatment, is the first to be approved since 2003, showing the lack of therapies available until now (Alexander, Emerson and Kesselheim, 2021; FDA, 2021). This compound indicated to treat Alzheimer's disease is an amyloid beta-directed antibody and was approved under the accelerated approval pathway, which provides patients with a serious disease earlier access to drugs, when there is an expectation of clinical benefit even though FDA have some hesitations about the clinical benefit (FDA, 2021). The availability of this treatment reinforces, more than ever, the need to find a way of diagnosing AD at early stages, since this will allow a more attempt therapeutic intervention and prevent brain functions deterioration and cognitive decline.

Even though it is evident that several processes are dysregulated in AD, and that the presence of both SPs and NFTs can contribute to the general disruption that renders in mental, physiological, and physical constraints in AD, it is still a challenge to completely understand this disease. In this perspective, this work aimed to identify putative biomarker candidates for AD by analyzing both SPS and NFTs proteomes. Although SPs and NFTs, look like two very distinct structures, there are linking points on the formation process of both, that bridges, for instance, the formation of Aβ peptides and Tau hyperphosphorylation. The Gene Ontology analysis herein presented, clearly show distinct characteristics in both proteomes, but with connections in all three levels, biological process, molecular function, and cellular component. Indeed, from the total SPs and NFTs proteomes, 374 proteins where common to both. Further, the classical link between these hallmarks, neurons and synapses is supported by the number of terms, related to those subjects in the top 10, like "synapse", "presynapse" and "presynapse endocytic zone".

There are a lot of hypothesis and processes described to be related with AD. Besides the amyloid cascade hypothesis, that lacks its strength mainly since the therapies directed at Aβ are not completely effective, many other have been proposed, as being involved in AD pathology like, the cholinergic and oxidative stress hypothesis,

the inflammation hypothesis, glucose hypometabolism and of course the Tau hypothesis (Oliveira *et al.*, 2017). It seems however, that there is not one hypothesis that can explain all the abnormal events and be responsible for AD pathology. In fact, like pointed above, AD may be the culmination of different processes, and that would explain the huge diversity of terms/pathways in which the proteins found in each lesion's proteome are involved. For instance, decrease metabolism in AD brains and the damage stimulated by reactive oxygen species (ROS), can arise from mitochondrial dysfunction, proving its involvement in the pathology (Oliveira *et al.*, 2017; Arora *et al.*, 2021). Further, the changes in the metabolism lead to glucose hypometabolism with studies suggesting that it precedes clinical symptoms (Jagust *et al.*, 2006; Mosconi *et al.*, 2009; Kyrtata *et al.*, 2021). Curiously, a great percentage of proteins, by the GO analysis, are involved in metabolism related process, oxidative stress, and electron transfer activity. Different dysfunctions can be explained if there is an impairment in the conformation or function of proteins involved in the pathways, but the main question is "which ones are the most relevant targets?" (Oliveira *et al.*, 2017; Arora *et al.*, 2021).

The GO analysis also revealed several processes related to vesicles, its secretion, endocytosis, and exocytosis, which gives more strength to the possible involvement of vesicle-mediated transport in AD. In this matter, it is probable that exosomes, may play a relevant role, since these nanovesicles have been discovered to transport key proteins or fragments, and other metabolites, linked to disease development. These conjectures, come to support the multifactorial and diverse elements involved in the pathology.

Noticeably, phosphorylation, a fundamental regulatory process, has been shown to be dysregulated in AD (Gong *et al.*, 1993; Liu *et al.*, 2005). In the bioinformatic analysis performed, the protein phosphatases and kinases present in both lesions may constitute putative phosphotargets candidates for AD. MAPK-1, SYNJ1 and CDK5 were the three targets uncovered, that arise from the overlap with the list of AD associated genes from Disgenet. All these protein candidates have been previously found altered in AD pathology (Martin *et al.*, 2014; Hugon *et al.*, 2018). In subsequent studies the biomarker potential of MAPK-1, a kinase, and SYNJ-1, a phosphatase, was tested in AD samples.

Recent focus has been given to exosomes as biomarkers resources for different pathologies, in a minimal invasive way. Exosome's relevance is also related to the fact that these type of EVs are produced in a spectrum of different cell types and can be found in every peripheric biofluids. In AD, since there is evidence that these nanovesicles can surpass the blood brain barrier, and contain many proteins linked to AD pathology, it is possible that exosomes may carry other undiscovered disease relevant biomarkers (Haney *et al.*, 2015; Yang *et al.*, 2015; Soares Martins *et al.*, 2021). Therefore, the levels of MAPK-1 and SYNJ-1 were accessed in serum-derived exosomes from AD patients, since these two proteins have already been discovered in exosomes of peripheric fluids (Table 1).

For MAPK-1, a significant decrease could be detected in serum-derived exosomes from AD patients comparatively to Controls, for the pcb-cohort. Mitogen-activated protein kinases (MAPKs), which are serine/threonine protein kinases, play important roles in cellular signal transduction and in AD. Dysregulation of the MAPK signaling pathway has been associated with the disease (Gerschütz *et al.*, 2014; Qi *et al.*, 2016; Hugon *et al.*, 2018; Li *et al.*, 2019). MAPK-1, also known as ERK2, has an important role in regulating tau functions and tau phosphorylation, by decreasing its affinity for microtubules, reducing the ability of tau to stabilize them. Phosphorylated MAPK-1 has been linked to the early stages of neurofilament formation and tau phosphorylation in neurons and glial cells, both in AD and other tauopathies (Ferrer *et al.*, 2001; Mazanetz and Fischer, 2007). MAPK-1 seems to be activated in all AD neurons that display tau and neurofilament hyperphosphorylation, suggesting an absolute requirement for its deregulation in neurofibrillary degeneration (Mazanetz and Fischer, 2007). Also, MAPK1 mRNA levels have been found elevated in different brain regions, implicating this kinase in the formation of Tau hyperphosphorylation early in the development of AD (Gerschütz *et al.*, 2014; Qi *et al.*, 2016; Hugon *et al.*, 2018; Li *et al.*, 2019). The fact that MAPK-1 may be found in SPs deposits and NFTs in AD, may be the cause of its diminished levels in serum-derived exosomes from AD patients, compared to the controls.

Relative to SYNJ-1 we hypothesized that a fragment of ≈75KDa was found in exosomes, being that the full length of SYNJ-1 form is not detected. The fragment levels were slightly elevated in exosomes from AD cases compared to the controls. SYNJ-1 is a

presynaptic protein essential for Synaptic Vesicle Endocytosis (SVE) (Zou *et al.*, 2021). It is the major phosphoinositide phosphatase which mediates the uncoating of clathrin during clathrin-mediated endocytosis and, regulates synaptic vesicle recycling by interacting with other synaptic activities (Farsad *et al.*, 2001; Geng *et al.*, 2016; Nguyen *et al.*, 2019). It is a protein highly concentrated in synapses, and besides that it was recently found that SYNJ-1 was a substrate of a cysteine proteinase, asparagine endopeptidase (AEP) Li Zou (Zou *et al.*, 2021). Neuronal AEP is activated during ischemia and apoptosis, and in the substantia nigra (SN), in an age dependent manner (Liu *et al.*, 2008; Zhang *et al.*, 2017). SYNJ-1 can be cleaved by AEP at N599, mediating synaptic dysfunction and dopaminergic neuronal degeneration in Parkinson's Disease (PD). One of the resulting fragments, which had around 72kDA, with a flag, interrupted clathrin-mediated endocytosis and induced presynaptic dysfunction (Zou *et al.*, 2021). Bringing all this information together, there is a possibility that the fragment found in the exosomes in our experimental conditions could be the same fragment resulting from the cleavage with AEP, overexpressed in PD brains. This needs to be addressed in future experiments.

In sum, this study identified putative phosphotargets that can constitute biomarkers candidates for AD diagnosis. MAPK-1 and SYNJ-1 were tested in human samples and additional complementary studies should be carried out to validate the results obtained. Other antibody-based methodologies can be employed, like ELISA (enzyme-linked immunosorbent assay), which is a more sensitive and quantitative assay which rely on highly specific antibody-antigen interactions. For SYNJ-1, to confirm the nature of the fragment that appears in the exosomes, is the same has the one resulting from the cleavage by AEP, mass spectrometry analysis can be employed. In addition, the number of samples used in the study could be increased and samples from other cohort, could be tested for comparison. The identification of novel biomarker candidates could be of potential value not only for AD early and differential diagnosis but also from a therapeutic perspective.

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# Annexes (Chapters 2 & 3)

**Supplementary Material 1:** List of Gene Names from SPs Proteome

| | | | | | | |
|---|---|---|---|---|---|---|
| A2M | APOD | C4B | COL1A2 | EEF1A1 | GNA13 | HSP90AA1 |
| AARS1 | APOE | C4BPA | COL25A1 | EEF2 | GNAI1 | HSP90AB1 |
| AASS | APP | C5 | COL4A1 | EFHD2 | GNAI2 | HSPA12B |
| ABAT | AQP1 | C5AR1 | CORO1A | EIF2AK2 | GNAI3 | HSPA1A |
| ACAT1 | AQP4 | C5AR2 | CORO1C | EIF3A | GNAO1 | HSPA1B |
| ACLY | ARCN1 | C6 | COX4I1 | EIF4A2 | GNAQ | HSPA1L |
| ACO2 | ARF1 | C7 | COX5A | EIF4B | GNAS | HSPA2 |
| ACOT7 | ARF3 | C8B | COX5B | EIF5 | GNAT1 | HSPA5 |
| ACTA1 | ARF4 | C9 | COX7A2 | ELANE | GNAZ | HSPA8 |
| ACTA2 | ARF5 | CA2 | COX7A2L | ELOC | GNB1 | HSPA9 |
| ACTB | ARHGDIA | CABLES1 | CP | ENO1 | GNB2 | HSPB1 |
| ACTBL2 | ARL8B | CALB1 | CPLX3 | ENO2 | GNB4 | HSPD1 |
| ACTG1 | ARMC10 | CALM1 | CPNE6 | ENO3 | GNG12 | HSPE1 |
| ACTN1 | ARPC2 | CALM2 | CPNE7 | ENOPH1 | GNG7 | HSPG2 |
| ACTN2 | ATG9A | CALM3 | CPS1 | EPB41L1 | GOT1 | HTRA1 |
| ACTN3 | ATL1 | CAMK2A | CRMP1 | EPB41L3 | GOT2 | ICAM1 |
| ACTN4 | ATP1A2 | CAMK2B | CRYAB | ERLEC1 | GPC1 | ICAM5 |
| ACTR1A | ATP1A3 | CAMK2D | CRYM | ETFA | GPC5 | IDE |
| ACTR1B | ATP1B1 | CAMK2G | CS | EZR | GPD1 | IDH2 |
| ACTR2 | ATP1B2 | CANX | CSRP1 | F12 | GPHN | IDH3A |
| ADD1 | ATP2A2 | CAP1 | CST3 | F2 | GPI | IGF2R |
| ADD2 | ATP2B1 | CAP2 | CSTA | FABP3 | GPM6A | IGHG1 |
| ADNP2 | ATP2B2 | CAPZA2 | CSTB | FABP5 | GPNMB | IGSF8 |
| AGAP1 | ATP2B3 | CASP3 | CTHRC1 | FABP7 | GPS1 | IL1RL1 |
| AGL | ATP2B4 | CAVIN1 | CTNNA2 | FAIM2 | GRHPR | IL1RN |
| AGRN | ATP2C2 | CBR1 | CTSB | FAP | GRIN2B | IL33 |
| AHCYL2 | ATP4A | CBR3 | CTSD | FASLG | GSN | IL6 |
| AHNAK | ATP5F1A | CBSL | CTSL | FASN | GSTM3 | ILF2 |
| AK1 | ATP5F1B | CCDC9B | CYB5R3 | FBXO2 | GSTO1 | IMMT |
| AK3 | ATP5F1C | CCS | CYC1 | FERMT3 | GSTP1 | INA |
| AK5 | ATP5F1D | CCT4 | CYCS | FEZ2 | H1-4 | IRGQ |
| AKR1B1 | ATP5ME | CCT6A | CYFIP2 | FGB | H2AC11 | ITGB2 |
| AKR7A2 | ATP5MF | CCT8 | CYRIB | FGG | H2AC21 | ITSN2 |
| ALB | ATP5MG | CD163 | DCD | FH | H2AZ2 | JUP |
| ALDH1L1 | ATP5PB | CD44 | DCN | FKBP4 | H2BC4 | KBTBD11 |
| ALDH2 | ATP5PD | CD74 | DCTN1 | FLNA | H4C1 | KLC3 |
| ALDH4A1 | ATP5PO | CD81 | DCTN2 | FLOT1 | HADH | KRAS |
| ALDH5A1 | ATP6V0A1 | CD9 | DDAH1 | FLT1 | HADHA | KRT1 |
| ALDH6A1 | ATP6V0D1 | CDC42 | DDX19A | FMR1 | HADHB | KRT2 |
| ALDH7A1 | ATP6V1A | CDC73 | DDX42 | FN1 | HAPLN2 | LAMA1 |
| ALDH9A1 | ATP6V1B1 | CDK5 | DES | FN3K | HBA1 | LAMC1 |
| ALDOA | ATP6V1B2 | CEND1 | DKK3 | FSCN1 | HBB | LANCL1 |
| ALDOB | ATP6V1D | CFL1 | DLAT | FTH1 | HBD | LARP1 |
| ALDOC | ATP6V1E1 | CHGA | DLD | FTL | HBE1 | LASP1 |
| AMER2 | ATP6V1F | CHGB | DLG4 | FXR1 | HBG1 | LDHA |
| AMPH | ATP6V1H | CISD1 | DLST | FXYD1 | HECTD1 | LDHB |
| AMY1A | ATP8A1 | CKB | DNAH6 | FXYD6 | HEPACAM | LGALS1 |
| ANK2 | BASP1 | CKMT1A | DNAJB2 | GAA | HEXB | LMAN1 |
| ANK3 | BCAN | CLASP2 | DNAJC19 | GANAB | HK1 | LMNA |
| ANXA1 | BCHE | CLDN11 | DNM1 | GAP43 | HLA-DRA | LPL |
| ANXA2 | BDH1 | CLEC11A | DNM1L | GAPDH | HMOX1 | LRP1 |
| ANXA5 | BDNF | CLIP1 | DPP6 | GATD3A | HNRNPA1 | LTF |
| ANXA6 | BIN1 | CLSTN3 | DPYSL2 | GDA | HNRNPA2B1 | LTN1 |
| AP1B1 | BPTF | CLTB | DPYSL3 | GDAP1 | HNRNPC | LYZ |
| AP2A1 | BSN | CLTC | DPYSL4 | GDF10 | HNRNPD | MAG |
| AP2A2 | BZW1 | CLTCL1 | DSG1 | GDI1 | HNRNPDL | MAOB |
| AP2B1 | C1QA | CLU | DSP | GFAP | HNRNPH2 | MAP1A |
| AP2M1 | C1QB | CNDP2 | DSTN | GJA1 | HNRNPH3 | MAP1B |
| AP2S1 | C1QBP | CNN1 | DYNC1H1 | GLS | HNRNPK | MAP2 |
| AP3B2 | C1R | CNP | DYNC1I1 | GLUD1 | HNRNPM | MAP2K1 |
| AP3S1 | C1S | CNRIP1 | DYNLL1 | GLUD2 | HNRNPU | MAP2K2 |
| APCS | C2 | CNTN1 | DYNLL2 | GLUL | HPCAL4 | MAP6 |
| APEX1 | C3 | CNTNAP1 | ECHS1 | GMFB | HPSE | MAPK1 |
| APOA1 | C4A | COL1A1 | ECM1 | GNA12 | HPSE2 | MAPK3 |

MAPRE1
MAPRE2
MAPT
MARCKS
MAT2B
MBP
MDH1
MDH2
MDK
MGST3
MLC1
MLF2
MME
MMP9
MOG
MPO
MSN
MT-CO2
MTHFD1
MTSS1L
MYH1
MYH10
MYH11
MYL12A
MYO5A
NAPA
NAPB
NAPG
NCAM1
NCAM2
NCAN
NCDN
NCL
NDRG2
NDUFA11
NDUFA13
NDUFA4
NDUFA5
NDUFA6
NDUFA9
NDUFB4
NDUFB9
NDUFS1
NDUFS3
NDUFV1
NDUFV2
NEFH
NEFL
NEFM
NEGR1
NFASC
NFAT5
NIPSNAP1
NME1
NNT
NOS1
NOTCH1
NPEPPS
NPTN
NPY
NQO1
NRCAM
NRGN
NRXN1
NSF
NSFL1C
NTN1
OLFML3
OPALIN
OPCML
OTUB1
OXCT1
PACS1
PACSIN1
PADI2
PAICS
PAIP1
PAK1
PALM
PARK7
PC
PCBP1
PCMT1
PCSK1N
PDHA1
PDHB
PDIA3
PDXK
PEA15
PEBP1
PFKL
PFKM
PFKP
PFN1
PFN2
PGAM1
PGAM2
PGD
PGK1
PGK2
PGLS
PGM1
PGRMC1
PHACTR1
PHB
PHB2
PHGDH
PHYHIP
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PIP4K2C
PITPNA
PKLR
PKM
PLD3
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PLP1
PPIA
PPP1CC
PPP1R7
PPP1R9B
PPP2CB
PPP2R1A
PPP2R2B
PPP3CA
PPP3CB
PPT1
PPWD1
PRDX1
PRDX2
PRDX3
PRDX5
PRDX6
PRKACA
PRKACB
PRKCA
PRKCB
PRKCG
PRKN
PRKX
PRND
PRNP
PRRT2
PSAT1
PSD3
PSMA2
PSMB2
PTEN
PTGDS
PTN
PTPN6
PTPRC
PTPRZ1
PURA
PYGB
PYGM
QDPR
QKI
RAB10
RAB11A
RAB11B
RAB14
RAB1A
RAB1B
RAB2A
RAB33B
RAB35
RAB3A
RAB3B
RAB40C
RAB5C
RAB6A
RAB6B
RAB7A
RAB8A
RAB8B
RAC1
RAC2
RAC3
RALA
RAP1B
RAP1GAP
RDX
REG1A
REG3A
RGMA
RGS10
RIDA
ROCK2
RPL7A
RPS3
RPS7
RPS8
RPSA
RTN1
RTN3
RTN4
RTN4IP1
S100A9
S100B
SAA1
SCCPDH
SCG2
SCIN
SCRN1
SDC4
SDHA
SEC22B
SEC23A
SELENBP1
SEPT11
SEPT2
SEPT6
SEPT7
SEPT9
SEPTIN5
SEPTIN8
SERAC1
SERPINA1
SERPINA3
SERPINE2
SFN
SFPQ
SFXN1
SFXN3
SGSM1
SH3BGRL
SH3BGRL2
SH3GL1
SH3GL2
SHISA7
SIPA1L1
SIRPA
SIRT2
SKP1
SLC1A2
SLC1A3
SLC1A4
SLC25A11
SLC25A12
SLC25A22
SLC25A3
SLC25A31
SLC25A5
SLC25A6
SLC30A3
SLC9A3R1
SLIT1
SLIT2
SMOC1
SMU1
SNAP23
SNAP25
SNAP91
SNCA
SNCB
SNCG
SNRPE
SOD1
SORBS1
SP1
SPOCK1
SPOCK2
SPON1
SPTAN1
SPTB
SPTBN1
SPTBN2
SRI
SST
STMN1
STUB1
STX1A
STX1B
STX7
STXBP1
SUCLA2
SV2A
SYN1
SYN2
SYNGR1
SYNGR3
SYNJ1
SYP
SYPL1
SYT1
SYT2
SYT5
TAGLN
TAGLN2
TAGLN3
TALDO1
TARDBP
TBCB
TCP1
TCP11L2
TF
TGFB1I1
TGM1
TGM2
THOP1
THY1
TKT
TMEFF1
TMEFF2
TMEM14C
TMOD2
TNR
TOMM22
TOMM70
TPI1
TPM1
TPM3
TPM4
TPP1
TPPP
TPPP3
TRAP1
TRIM2
TSC22D2
TSPAN7
TTLL7
TUBA1A
TUBA1B
TUBA1C
TUBA3C
TUBA3D
TUBA3E
TUBA4A
TUBA8
TUBAL3
TUBB
TUBB1
TUBB2A
TUBB2B
TUBB3
TUBB4A
TUBB4B
TUFM
TXN
UAP1
UBA1
UBA52
UBE2N
UBR1
UCHL1
UGP2
UQCRB
UQCRC1
UQCRC2
UQCRFS1
UQCRQ
USP5
VAMP1
VAMP2
VAMP3
VAPA
VAPB
VAT1
VCAN
VCP
VDAC1
VDAC2
VDAC3
VEGFC
VIM
VPS26A
VPS35
VSNL1
VTN
WASF1
WASL
WDR1
WDR47
WIPF3
WIPI2
XPNPEP1
XRCC6
YARS
YWHAB
YWHAE
YWHAG
YWHAH
YWHAQ
YWHAZ
ZNRF2

**Supplementary Material 2:** List of Gene Names from NFTs Proteome

| | | | | | | |
|---|---|---|---|---|---|---|
| | ATP6V0A1 | COX5A | FLOT1 | HNRNPD | MAP6 | PDHB |
| AARS1 | ATP6V0D1 | COX5B | FSCN1 | HNRNPH1 | MAPK1 | PDHX |
| ABAT | ATP6V1A | COX6C | FTH1 | HNRNPH3 | MAPRE3 | PDIA6 |
| ABI1 | ATP6V1B2 | CPNE6 | FTL | HNRNPK | MAPT | PDXP |
| ACAT1 | ATP6V1E1 | CRMP1 | FUBP1 | HNRNPM | MARCKS | PEA15 |
| ACHE | ATP6V1H | CRYM | FUS | HNRNPR | MATR3 | PEBP1 |
| ACO2 | AZGP1 | CRYZ | FXR1 | HNRNPU | MBP | PFKM |
| ACOT7 | BASP1 | CS | GANAB | HP1BP3 | MC1R | PFKP |
| ACTA1 | BCAN | CSNK1D | GAP43 | HRNR | MCCC1 | PFN1 |
| ACTB | BECN1 | CST3 | GAPDH | HSD17B10 | MDH1 | PFN2 |
| ACTG1 | BIN1 | CSTA | GDA | HSP90AA1 | MDH2 | PGAM1 |
| ACTN4 | BRCA1 | CTSD | GDI1 | HSP90AB1 | MIF | PGD |
| ADD1 | C5AR1 | CYCS | GDI2 | HSP90B1 | MMP9 | PGK1 |
| ADD2 | C5AR2 | CYFIP2 | GFAP | HSPA12A | MSN | PGM2L1 |
| AHCYL1 | CA2 | DBN1 | GGCT | HSPA1A | MT-CO2 | PGRMC1 |
| AHNAK | CACNA2D1 | DCLK1 | GLO1 | HSPA4 | MTHFD1 | PHB |
| AIFM1 | CALM1 | DCN | GLS | HSPA4L | MUCL1 | PHB2 |
| AK1 | CALML3 | DCTN2 | GLUD1 | HSPA5 | MYEF2 | PHGDH |
| AKR1B1 | CALML5 | DDB1 | GLUL | HSPA6 | MYH10 | PHYHIP |
| ALB | CAMK2A | DDX17 | GMFB | HSPA8 | MYH9 | PICALM |
| ALDH2 | CAMK2B | DDX39B | GNAO1 | HSPA9 | MYL6 | PIN1 |
| ALDH4A1 | CAND1 | DDX5 | GNB1 | HSPB1 | MYO5A | PIP |
| ALDH5A1 | CANX | DECR1 | GNB2 | HSPD1 | NACA | PKM |
| ALDH6A1 | CAP1 | DEFA3 | GNB4 | HSPE1 | NAPB | PKP1 |
| ALDH7A1 | CAP2 | DHX9 | GOT1 | HSPH1 | NAPG | PLCB1 |
| ALDH9A1 | CAPZB | DLAT | GOT2 | HYOU1 | NASP | PLEC |
| ALDOA | CASP14 | DLD | GPD2 | ICAM5 | NCAM1 | PLP1 |
| ALDOC | CASP3 | DLST | GPHN | IDH2 | NCAN | POF1B |
| AMPH | CASP7 | DMTN | GPI | IDH3A | NCCRP1 | PPIA |
| ANK2 | CASP8 | DNM1 | GPM6A | IDH3B | NCDN | PPP1R7 |
| ANXA1 | CAT | DNM1L | GRB2 | IGF2R | NCL | PPP2R1A |
| ANXA2 | CBR1 | DPYSL2 | GRIA2 | IGHA1 | NDRG2 | PPP3CA |
| ANXA5 | CCAR2 | DPYSL4 | GRN | IGHG1 | NDUFA2 | PPP3CB |
| ANXA6 | CCT2 | DSC1 | GSK3A | IGLC3 | NDUFS1 | PRDX1 |
| AP2A1 | CCT3 | DSG1 | GSN | IGSF8 | NDUFS3 | PRDX2 |
| AP2A2 | CCT4 | DSP | GSTM3 | IL1RL1 | NEFH | PRDX5 |
| AP2B1 | CCT5 | DSTN | GSTP1 | IL1RN | NEFL | PRDX6 |
| AP2S1 | CCT6A | DTD1 | H1-2 | IL33 | NEFM | PRKAR1A |
| APCS | CCT7 | DYNC1H1 | H2AC1 | ILF3 | NFASC | PRKAR2A |
| APMAP | CCT8 | DYRK1A | H2AC11 | IMMT | NME2 | PRKCG |
| APOD | CD47 | ECHS1 | H2AC12 | IMPA1 | NNT | PRKCSH |
| APOE | CD74 | EEF1A1 | H2AC14 | INA | NONO | PRKDC |
| AQP4 | CDC37 | EEF1B2 | H2AC18 | JCHAIN | NOTCH1 | PSAP |
| ARF3 | CDK1 | EEF1D | H2AC4 | JUP | NPEPPS | PSAT1 |
| ARG1 | CDK5 | EEF1G | H2AC6 | KHSRP | NPM1 | PSEN1 |
| ARHGDIA | CDS2 | EEF2 | H2AC7 | KPNB1 | NPTN | PSEN2 |
| ARL8A | CDSN | EIF4A2 | H2AFX | LANCL1 | NRAS | PSMA3 |
| ARPC4 | CEND1 | ELAVL4 | H2AJ | LCN1 | NRCAM | PSMA5 |
| ASAH1 | CFL1 | ENO1 | H2AW | LDHA | NSF | PSMA6 |
| ATG12 | CFL2 | ENO2 | H2BC11 | LDHB | NUCB1 | PSMA7 |
| ATG5 | CHCHD3 | EPB41 | H2BC4 | LEPR | OGG1 | PSMB5 |
| ATP1A1 | CHMP4B | EPB41L3 | H3-2 | LGALS7 | OPCML | PSMC2 |
| ATP1A2 | CKAP4 | EPRS1 | H3C15 | LICAM | OXCT1 | PSMD14 |
| ATP1B1 | CKB | ERP44 | H4C1 | LMNA | P4HB | PSMD2 |
| ATP1B3 | CKMT1A | ESR1 | HADHB | LMNB1 | PA2G4 | PSPC1 |
| ATP2A2 | CLTA | ETFB | HAPLN2 | LMNB2 | PABPC1 | PTEN |
| ATP2B1 | CLTB | EZR | HBA1 | LORICRIN | PACSIN1 | PTPRZ1 |
| ATP5F1A | CLTC | F2 | HBB | LRPPRC | PALM | PURA |
| ATP5F1B | CLU | FABP3 | HEPACAM | LRRC47 | PARK7 | PXN |
| ATP5F1C | CMAS | FABP5 | HK1 | LTF | PARP1 | PYGB |
| ATP5F1D | CNDP2 | FAP | HMOX1 | LYZ | PC | QDPR |
| ATP5F1EP2 | CNP | FARSA | HNRNPA1 | MAP1A | PCBP1 | RAB11A |
| ATP5MG | CNRIP1 | FARSB | HNRNPA2B1 | MAP1B | PCCA | RAB3A |
| ATP5PD | CNTN1 | FASN | HNRNPA3 | MAP1LC3B | PCMT1 | RAB6B |
| ATP5PF | CORO1A | FH | HNRNPAB | MAP2 | PCSK1N | RAB7A |
| ATP5PO | COX4I1 | FKBP1A | HNRNPC | MAP4 | PDHA1 | RACK1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| RAN | RPS14 | SEPTIN7 | SND1 | SV2A | TRAP1 | VCAN |
| RBM14 | RPS18 | SERPINA1 | SOD1 | SYN1 | TRIM28 | VCL |
| RBM39 | RPS19 | SERPINA3 | SOD2 | SYN2 | TUBA1B | VCP |
| RBMXL1 | RPS2 | SERPINB12 | SP1 | SYNGAP1 | TUBA4A | VDAC1 |
| REG1A | RPS20 | SERPINB3 | SP3 | SYNJ1 | TUBB | VDAC2 |
| REG3A | RPS27A | SET | SP4 | SYT1 | TUBB2A | VIM |
| RIDA | RPS28 | SF3B3 | SPRR2B | TALDO1 | TUBB2B | VPS35 |
| RNH1 | RPS3 | SFN | SPTAN1 | TARDBP | TUBB3 | WASF2 |
| ROCK1 | RPS3A | SFPQ | SPTBN1 | TCP1 | TUBB4A | WDR1 |
| RPL10A | RPS6 | SFXN3 | SPTBN2 | TGFB1I1 | TUBB4B | XRCC5 |
| RPL11 | RPS6KB1 | SH3BGRL | SQSTM1 | TGM1 | TUBB8 | XRCC6 |
| RPL12 | RPS7 | SH3GL2 | SRI | TGM2 | TUFM | YARS |
| RPL13 | RPS8 | SIRPA | SRPK2 | TGM3 | TXN | YWHAB |
| RPL13A | RPSA | SLC12A5 | SRSF1 | THOP1 | UBA1 | YWHAE |
| RPL15 | RTN4 | SLC1A2 | SRSF2 | TKT | UBA2 | YWHAG |
| RPL22 | RUFY3 | SLC25A12 | SRSF6 | TMPO | UBA52 | YWHAH |
| RPL27 | RUVBL2 | SLC25A3 | SRSF7 | TMSB4X | UBC | YWHAQ |
| RPL31 | S100A8 | SLC25A4 | SSBP1 | TNR | UBQLN1 | YWHAZ |
| RPL4 | S100A9 | SLC25A5 | ST13 | TOM1 | UCHL1 | |
| RPL6 | SBSN | SLC25A6 | STIP1 | TPI1 | UQCRC1 | |
| RPL7 | SCRN1 | SNAP25 | STX1A | TPM1 | UQCRFS1 | |
| RPL7A | SDHB | SNAP91 | STX1B | TPM3 | USP14 | |
| RPL8 | SEPT2 | SNCA | STXBP1 | TPPP | USP5 | |
| RPLP2 | SEPTIN5 | SNCG | SUCLA2 | TRA2B | VAMP2 | |

**Supplementary Material 3**

## Exosome isolation

RIPA buffer (Sigma-aldrich)

Add 20 µl protease cocktail inhibitors to 980 µl of RIPA buffer. Store at 4◦C.

## Protein quantification

Working reagent:

Working reagent preparation considering the proportion 50 reagent A: 1 reagent B.

## SDS-PAGE

Lower gel buffer (LGB) (4x):

To prepare 1L, add 181.65 g of Tris to 900 mL of distilled $H_2O$ and mix until complete dissolution. Adjust pH to 8.9. After adjust volume to 1L with distilled water and store at 4◦C.

Upper gel buffer (UGB):

To prepare 1L, add 75.69 g of Tris to 900 mL of distilled $H_2O$ and mix until complete dissolution. Adjust to pH 6.8, adjust the volume to 1L with distilled water and store at 4◦C.

Ammonium Persulfate (APS) 10x:

Dissolve 0.1g of APS in 1mL of distilled $H_2O$. Prepare it fresh before use.

10% Sodium dodecyl sulfate (SDS) (100 ml):

Dissolve 10g of SDS in 100 mL of distilled H2O.

Resolving gel 3.5%:

| Reagents | ½ system |
|---|---|
| dH2O | 6.92mL |
| Acrylamide | 0.88mL |
| UGB (4x) | 2mL |
| 10% SDS | 100µL |
| 10% APS | 100µL |
| TEMED | 10µL |

Resolving gel (5% and 20%):

| Reagents (½ system) | Resolving gel 5% | Resolving gel 20% |
|---|---|---|
| dH2O | 9,29mL | 3,67mL |
| LGB (4x) | 3,75mL | 3,75mL |
| Acrylamide | 1,875mL | 7,5mL |
| 10% APS | 75µL | 75µL |
| TEMED | 7,5µL | 7,5µL |

Loading gel buffer (4x) (10 ml):

| Reagents | V= 10mL |
|---|---|
| Tris 1M | 2.5 mL |
| SDS | 0.8 g |
| Glicerol | 4 mL |
| β- Mercaptoetanol | 2 mL |
| Azul bromofenol | 1mg |

Tris 1M (250 ml):

Dissolve 30.3g of Tris in 250 mL of distilled $H_2O$. Adjust pH to 6.8.

Running buffer (10x):

To prepare 1L, dissolve 30.3g of Tris (250 mM), 144.2g of glycine (2.5 M) and 10g of SDS (1%) in ≈800mL of distilled $H_2O$. Adjust pH to 8.3 and adjust the volume to 1L.

## Western blot

Transfer buffer (1x):

To prepare 1L, dissolve 3.03g of Tris (25mM) and 14.41g of Glycine (192mM), to 700 mL of distilled $H_2O$. Adjust the pH to 8.3. Adjust the volume to 800 mL of distilled $H_2O$. Before use, add 200 mL of methanol (20%).

Tris buffered saline (TBS) (10x):

To prepare 1L, add 12.11g of Tris (10 mM) and 87.66g of NaCl (150 mM), to 700 mL of distilled $H_2O$. Adjust the pH to 8.0 and the volume to 1L with distilled $H_2O$.

Tris buffered saline + Tween (TBS-T) (10x) (1L):

Add 12.11g of Tris (10 mM), 87.66g of NaCl (150 Mm) and 5 mL of Tween 20 (0.05%) of distilled H2O. Adjust the pH to 8.0 with HCl and adjust the volume to 1L with distilled H2O.

Blocking solution (5%):

To prepare 50mL, dissolve 2.5g of non-fat dry milk in 50 mL of 1x TBS-T.

Antibody solution (3%):

To prepare 15mL, dissolve 0.45g of non-fat dry milk in 15 mL of 1x TBS-T. Add antibody according to pretended dilution, mix gently (no vortex). Store at -20◦C.

Ponceau S Staining solution (0.1%) (50 ml):

Dissolve 0.05g of Ponceau S in 2.5mL of acetic acid and adjust the volume to 50mL with distilled $H_2O$. Store at 4◦C, protected from light.