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RESEARCH ARTICLE

Bioinformatic analysis of the SPs and NFTs proteomes unravel putative biomarker candidates for Alzheimer's disease

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Abstract

Aging is the main risk factor for the appearance of age-related neurodegenerative diseases, including Alzheimer's disease (AD). AD is the most common form of dementia, characterized by the presence of senile plaques (SPs) and neurofibrillary tangles (NFTs), the main histopathological hallmarks in AD brains. The core of these deposits are predominantly amyloid fibrils in SPs and hyperphosphorylated Tau protein in NFTs, but other molecular components can be found associated with these pathological lesions. Herein, an extensive literature review was carried out to obtain the SPs and NFTs proteomes, followed by a bioinformatic analysis and further putative biomarker validation. For SPs, 857 proteins were recovered, and, for NFTs, 627 proteins of which 375 occur in both groups and represent the common proteome. Gene Ontology (GO) enrichment analysis permitted the identification of biological processes and the molecular functions most associated with these lesions. Analysis of the SPs and NFTs common proteins unraveled pathways and molecular targets linking both histopathological events. Further, validation of a putative phosphotarget arising from the *in silico* analysis was performed in serum-derived extracellular vesicles from AD patients. This bioinformatic approach contributed to the identification of putative molecular targets, valuable for AD diagnostic or therapeutic intervention.

KEYWORDS
biomarkers, kinases, neurofibrillary tangles, phosphatases, senile plaques

Abbreviations: AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid beta; BCA, bicinchoninic acid; CAMK2A, calcium/calmodulin-dependent protein kinase type II subunit alpha (CaMKII-alpha); CAMK2B, calcium/calmodulin-dependent protein kinase type II subunit beta; CDK5, cyclin-dependent kinase 5; CDR, clinical dementia rating; CSF, cerebrospinal fluid; ERK2, extracellular signal-regulated kinase 2; EVs, extracellular vesicles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GRK3, beta-adrenergic receptor kinase 2; GRK5, beta-adrenergic receptor kinase 5; MAPK1, mitogen-activated protein kinase 1; MAPT, synaptosomal-associated protein 25; MMSE, Mini-Mental State Examination; mRNA, messenger ribonucleic acid; MVBs, multivesicular bodies; NFTs, neurofibrillary tangles; NTA, nanoparticle tracking analysis; PET, positron emission tomography; PK, protein kinase; PP, protein phosphatase; PPP1R7, protein phosphatase 1 regulatory subunit 7; PPP2R1A, serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform; PPP3CA, serine/threonine-protein phosphatase 2B catalytic subunit alpha isoform; PPP3CB, microtubule-associated protein Tau; PRKCG, glial fibrillary acidic protein; PTEN, phosphatidylinositol 3,4,5-trisphosphate 3-phosphatase and dual specificity protein phosphatase; PTPRZ1, receptor-type tyrosine-protein phosphatase zeta; RIPA, radioimmunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SNAP25, synaptosomal-associated protein 25; SPs, senile plaques; SYNJ1, synaptojanin-1; TEM, transmission electron microscopy.

1 | INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative, progressive, and thus far incurable disorder affecting cognitive functions, ultimately impacting the individual's capability to live independently. This pathology is the most common type of dementia, accounting for more than 60% of all cases [1, 2]. According to the World Health Organization, nearly 50 million people live with dementia worldwide, with over 10 million new cases diagnosed every year. It is estimated that by 2050, more than 150 million people will be affected by some sort of dementia [3]. Increasing life expectancy and aging as the main risk factor for several diseases, the prevalence of these age-related disorders in older individuals will raise emphasizing the need for better diagnoses and successful treatments.

There are two major forms of AD, the early-onset AD and the late-onset AD, mainly distinguished by the age of symptoms' onset and genetic risk factors [4]. Regardless, the main hallmarks of both forms of disease are extracellular deposition of amyloid beta (Aβ) peptides into senile plaques (SPs) and Tau hyperphosphorylation with neurofibrillary tangles (NFTs) formation [5, 6], leading to synaptic dysfunction and neuron loss. These events occur at specific brain regions like the hippocampus, cerebral cortex, and amygdala [7], impacting brain function and patient capabilities, leading to memory loss, behavior perturbations, and disorientation at speech and visuospatial levels.

Aβ peptides arise from altered processing of the amyloid precursor protein (APP), by β- and γ-secretases, and a shortcoming in clearance pathways [8]. This leads to the production and accumulation of mainly Aβ1-42 and Aβ1-40 in the form of amyloid fibrils, being the former, the major core component of extracellular SPs in AD [2, 9]. Nonetheless, besides Aβ, many other proteins can be found deposited into SPs. These are involved in several processes dysregulated in AD pathology, such as neuroinflammation [10], apoptosis [11], and abnormal APP processing [12, 13].

The molecular nature of intracellular NFTs lies behind its major constituent, the Tau protein [14, 15]. This is a microtubule-associated protein whose function is to maintain the microtubule assembly and stability, where phosphorylation is a key event in these processes. Modifications of this protein can have pathological consequences, provoking tauopathies-related disorders [16]. The progressive hyperphosphorylation and aggregation of Tau in AD brains are linked to neuronal degeneration and synapse loss, which may be, together with Aβ deposition into SPs, the motor behind cognitive impairment [17]. Likewise, many other proteins can also be found associated with NFTs. Even though these two hallmarks can be found in other neurodegenerative/logical diseases, the presence of both SPs and NFTs is typical of AD patients' brains. In this study, the proteomes of SPs and NFTs were collated by an extensive literature search and bioinformatically analyzed. The SPs and NFTs common proteome allowed for the identification of novel pathways and molecular targets linking both hallmarks, which may contribute to better understand the disease's molecular basis or contribute to the development of novel diagnostic or therapeutic approaches.

Statement of significance of the study

Alzheimer's disease (AD) is the most prevalent neurodegenerative disorder worldwide but until now no blood-based molecular tools exist to diagnose AD. The novelty of this study lies on an extensive literature review that was carried out to obtain the proteomes of the senile plaques (SPs) and the neurofibrillary tangles (NFTs), the two main histopathological hallmarks of AD. In addition, a subsequent bioinformatic analysis of the common SPs and NFTs proteome unravel novel putative biomarker candidates for AD. Further, one of the phosphotarget identified was tested in blood-derived extracellular vesicles of Controls and AD cases from distinct cohorts, revealing significant differences. Of note, SPs and NFTs proteomes are available as supplementary tables and may provide a basis for further complementary analysis, either focused on disease molecular mechanisms or novel biomarker candidates' validation.

2 | MATERIALS AND METHODS

2.1 | Senile plaques and neurofibrillary tangles proteomes

An extensive data mining was carried out to collect the SPs and NFTs proteomes, resorting to the Pubmed database (https://pubmed.ncbi.nlm.nih.gov/). Articles published until April 19, 2022 were included. A set of keywords was used for each AD hallmark, as indicated in the flowchart (Figure 1). For abstract reading, only articles written in English or Portuguese, that were not "reviews," were included. These selection criteria were applied, and the remaining article abstracts that had some reference to proteins in the SPs or NFTs, in AD brains, were subjected to a comprehensive analysis. Some of the articles contributed to both SPs and NFTs proteomes. Having filtered the relevant articles, a total of 156, the information of the proteins was organized in tables (Tables S1 and S2) including the following data: "Uniprot ID," "Protein name," "Gene Name," "Method of identification," the "sequence" (when available), "Brain tissue/area" from where the protein was isolated, and if it was "up or downregulated" (when applied). Only proteins isolated from human brains, diagnosed with AD, were included. A total of 90 articles for SPs [18–107] and of 66 [25, 32, 35–37, 41, 46, 47, 50, 51, 63, 66, 67, 78, 79, 83–87, 90, 92, 94, 95, 97, 101, 104–143] articles for NFTs were analyzed.

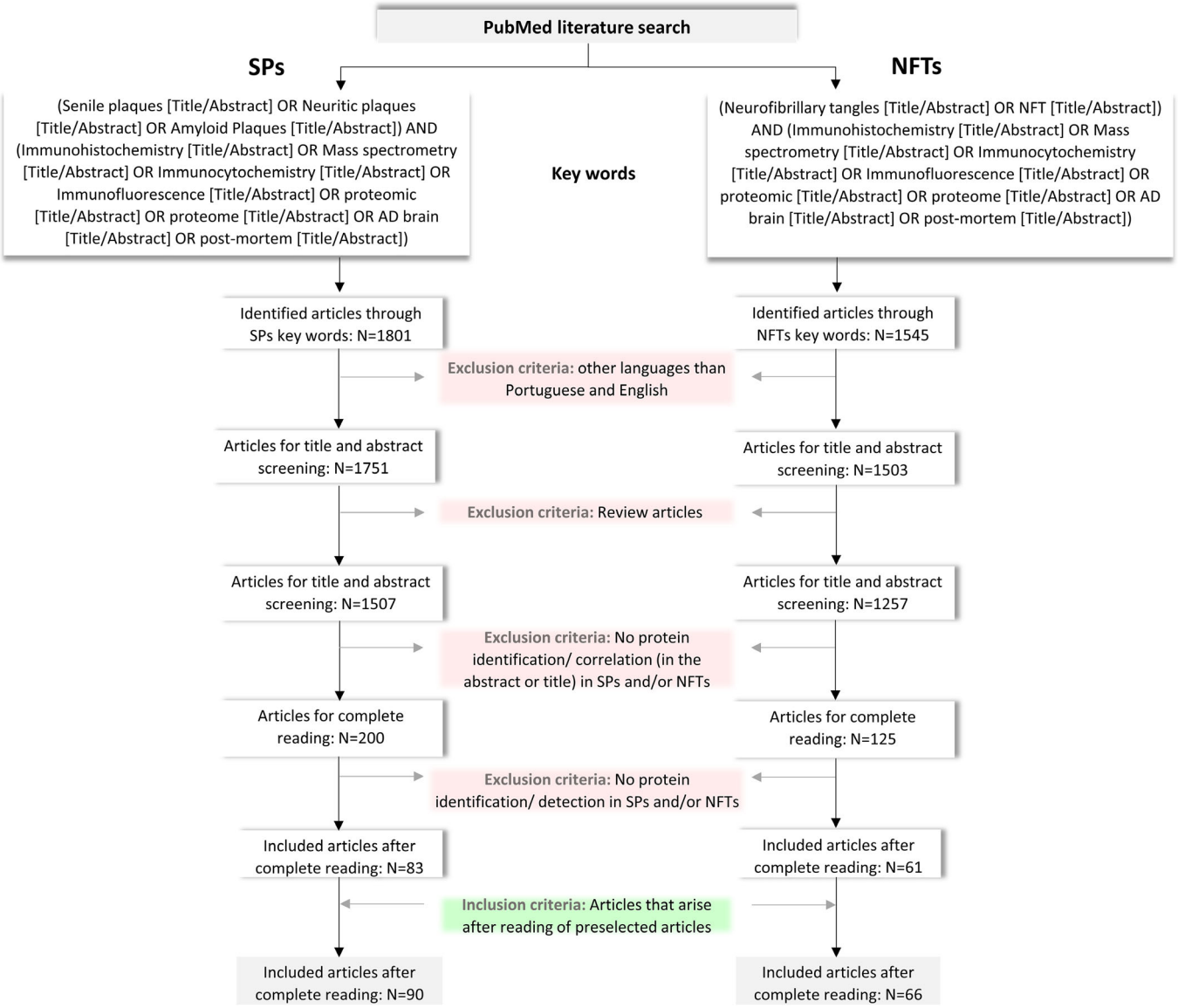


FIGURE 1 Literature review flowchart. Criteria implemented to obtain the final list of proteins present in both senile plaques (SPs) and neurofibrillary tangles (NFTs) are indicated. The literature search was carried on PubMed database (https://pubmed.ncbi.nlm.nih.gov/).

2.2 | Proteomes Gene Ontology analysis

Gene Ontology (GO) enrichment analysis was carried out for each proteome (corresponding gene nomenclature), using ClueGO v2.5.9 + CluePedia v1.5.9 plugins from Cytoscape v3.9.1. (https://cytoscape.org/). On the ClueGO panel, the following variables were defined: 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 *p*-value correction "Bonferroni step down." For the analysis of both SPs and NFTs proteomes, the top 10 most relevant processes (significantly different and most representative) were presented.

2.3 | Networks construction and analysis

The network of the common SPs and NFTs proteome (that included proteins present in both proteomes) was constructed by retrieving the list of gene names corresponding to each proteome and importing them into the Cytoscape software, using the function "Import" → "Network From File."

The protein–protein interaction networks information was retrieved from STRING online database (version 11.5; https://string-db.org/), and the networks were visualized using the Cytoscape software. The networks were analyzed through the "Network Analyzer" using the betweenness centrality option, which permits highlighting central nodes.

To identify the putative biomarker candidates common to both SPs and NFTs, the histopathological hallmarks' proteomes (corresponding gene names) were overlapped with lists of genes obtained from distinct data bases: (i) a list of genes associated with AD imported from DisGenet, https://www.disgenet.org/ on June 20, 2022, using "Alzheimer's Disease" as key words; a list of genes corresponding to the human PKs from http://www.kinhub.org/ on February 2, 2021 and (ii) the lists of genes corresponding to the human PPs downloaded from http://hupho.uniroma2.it/ on November 19, 2020. The lists of genes corresponding to the human PKs and PPs can be found in Table S3.

2.4 | VOSviewer map

Using the bibliometric software VOSviewer (version 1.6.18), a keywords co-occurrence and cluster map was created based on text data collected from the Web of Science database on July 17, 2022. This data search was carried out using the specific keywords: ERK2 OR MAPK1 OR Mitogen-Activated Protein Kinase 1 OR extracellular signal-regulated kinase 2 AND neurodegenerative disease.

2.5 | Human samples

In this work, two distinct study groups were used. One arising from the regional primary health care-based cohort (pcb-cohort), which includes Controls and individuals with dementia, characterized by cognitive testing such as clinical dementia rating (CDR) and Mini-Mental State Examination (MMSE) as previously reported [144, 145]. The pcb-group includes nine individuals, clinically diagnosed as AD cases (mean age 78.67 ± 5.07) and nine age- and sex-matched Controls (mean age 77.56 ± 4.83). 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 UMG-study group includes 12 Control individuals (mean age 67.58 ± 7.74) and 12 AD cases (mean age 73.17 ± 10.66), from the UMG-cohort, established at the University Medical Center of Goettingen, Germany [146]. This cohort is highly characterized by including either cognitive testing, CSF-neurochemical diagnostic biomarkers, PET imaging, or in combination, as described in Shahpasand-Kroner et al. [146]. The collection of these samples and their use was approved by the ethics committee of the University Medical Center of Goettingen (9/2/16). A total of 21 AD cases (mean age 75.52 ± 8.98) and 21 Control (mean age 71.85 ± 8.24) were analyzed. In both cases, all participants gave written informed consent.

2.6 | EVs isolation and characterization

Serum was obtained from participants of the two distinct groups using standard procedures as previously described [144, 146]. Serum-derived EVs, with exosome-like characteristics, were isolated from 250 μL of serum from Controls and individuals with AD, from the two study groups, using the ExoQuick Serum Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, USA). The resulting exosomal pellet was resuspended in 200 μL of RIPA buffer (Sigma-AldrichTM) with protease inhibitors for Western blot; or in PBS for NTA and TEM analyses, as previously described [147, 148]. Following exosome isolation, their nature was assessed by TEM and NTA analysis. NTA analysis was performed using a Nanosight NS300 (Malvern Instruments, Malvern, UK) and NTA 3.2 software (Malvern Instruments, Malvern, UK). NTA analysis was then carried out in duplicate for each sample. Size distribution profiles obtained by NTA analyses confirmed the enriched presence of exosomes within the expected sizes, ranging from 30 to 150 nm (Figure S1). TEM analysis revealed the presence of small extracellular vesicles within the typical exosomal size range and with the expected round shape. A set of EVs markers was also tested.

After protein quantification, using the Thermo Scientific™ Pierce™ BCA Protein Assay Kit, normalized samples (25 μg of protein) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by a wet electrophoretic transfer of the proteins. Nitrocellulose membranes were blocked in nonfat dry milk solution (5%) and incubated overnight with the exosomal marker antibodies, anti-CD63 (1:500) (sc-5275; Santa Cruz Biotechnology), anti-Rab11 (1:500) (610657; BD Transduction Laboratories), or the negative exosomal marker antibody anti-calnexin (1:500) (ADI-SPA-860-J; Enzo Life Sciences). This was followed by incubation with the anti-mouse IgG, HRP-linked antibody (1:2000 or 1:10000) (7076S; Cell Signaling Technology), or anti-rabbit IgG, HRP-linked antibody (1:5000) (7074S; Cell Signaling Technology). Protein bands were detected with the chemiluminescence reagent ECL Select (GE Healthcare Life Sciences™) and images acquired with the Chemidoc gel imaging system (Bio-Rad) with Image Lab Touch Software (Bio-Rad).

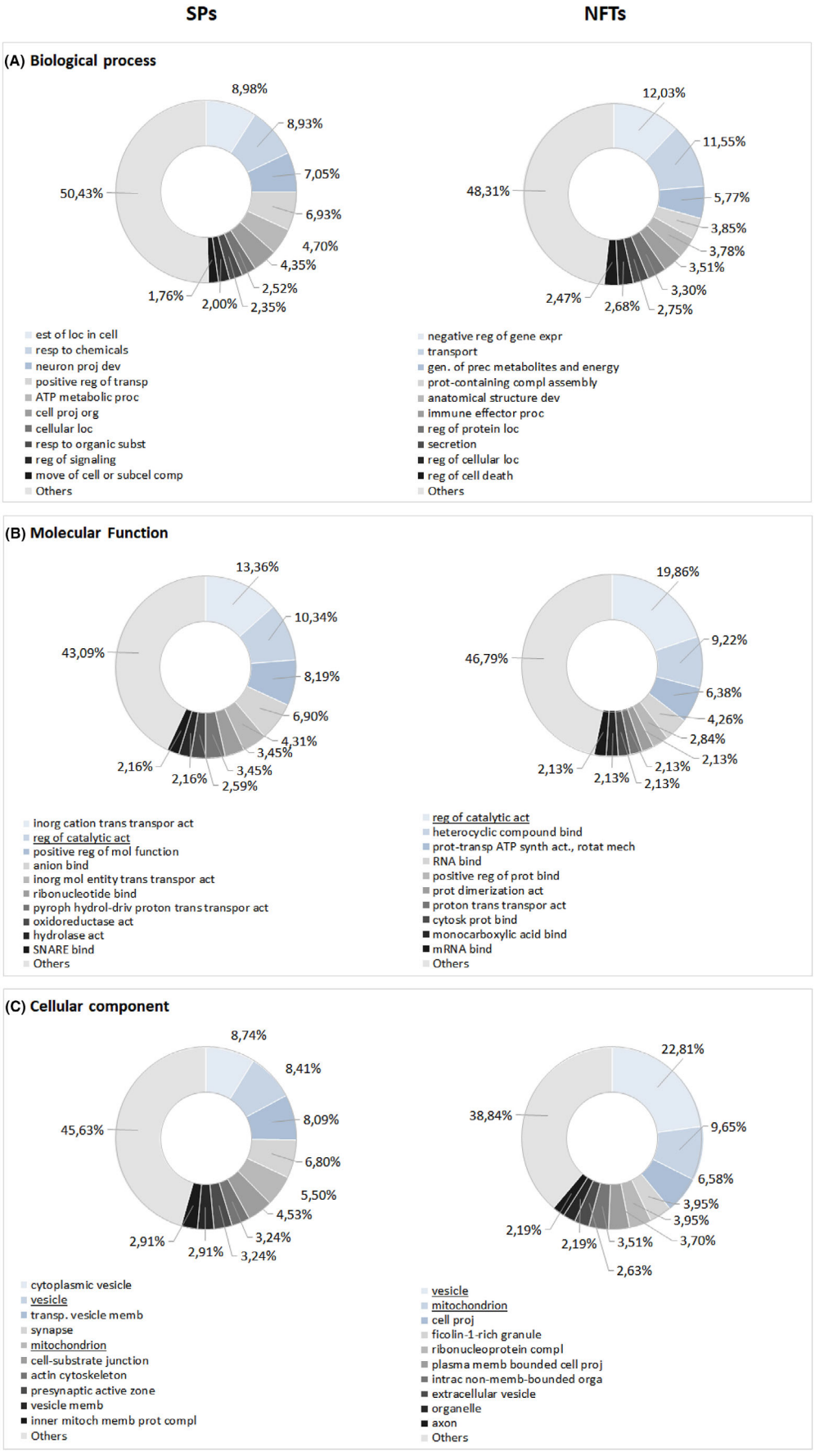
2.7 | Evaluation of MAPK1 levels

From the bioinformatic analysis, various candidates were identified from which MAPK1 was selected for validation in human samples.

Serum-derived EVs samples were normalized for protein content and a total of 25 μg were run by SDS-PAGE for subsequent Western blot analysis of MAPK1. For immunoblotting analysis, the nitrocellulose membranes were blocked, and incubated overnight with primary monoclonal antibody anti-ERK2 (MAPK1) (1:200) (sc-1647; Santa Cruz Biotechnology). The membranes were subsequently incubated with the secondary antibody, anti-mouse IgG, HRP-linked antibody (1:2000) (7076S; Cell Signaling Technology, Danvers, MA, USA). Protein band detection was achieved by chemiluminescence using Crescendo Western HRP Substrate, and images were obtained using Chemidoc gel imaging system (Bio-Rad, Hercules, CA, USA) with Image Lab Touch Software (Bio-Rad, Hercules, CA, USA).

2.8 | Statistical analyses

Statistical analysis was carried out with two-tailed Student's *t*-test, after normal distribution verification; to assess the different levels of



MAPK1 in Controls versus AD samples. A pool of EVs was also loaded for comparative purposes among membranes, and a ratio between the EVs pool/individual EVs sample was performed to normalize data. 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). Receiver operating characteristic (ROC) analysis was performed to assess the discrimination accuracy of MAPK1 differentiating between Controls and AD patients.

3 | RESULTS

3.1 | Gene Ontology analysis of SPs and NFTs proteomes

The literature search was conducted using PubMed database (https://pubmed.ncbi.nlm.nih.gov/) as described in the flowchart (Figure 1). This approach identified 857 proteins (corresponding gene nomenclature was used) associated with the SPs proteome and 627 with the NFTs proteome (Tables S1 and S2).

Characterization of both proteomes was achieved by GO enrichment analysis of the biological process (BP), molecular function (MF), and cellular component (CC) levels (Figure 2). Analyzing the top 10 BPs, no terms were common to both proteomes. For SPs, the top three BPs were "establishment of localization in cell," "response to chemicals," and "neuron projection development," while for NFTs were "negative regulation of gene expression," "transport" and "generation of precursor metabolites and energy." Other processes as "positive regulation of transport" and "regulation of signaling" could also be found in the top 10 BP for SPs, while "secretion" and "regulation of cell death" could be identified for the NFTs proteome.

At the MF level, one term was found common to both proteomes, "regulation of catalytic activity." Additionally, the two proteomes revealed other molecular binding associated functions, like "SNARE binding" for SPs and "cytoskeletal protein binding" for NFTs. For CC, both proteomes presented an association with "vesicle" and "mitochondrion" terms, but others as "synapse" for SPs and "axon" for NFTs were also found. Therefore, although some similarities could be found among the top 10 MFs and CCs differences could be detected characterizing each proteome, supporting that these deposits are indeed very distinct.

3.2 | Characterization of the SPs and NFTs common proteome

From a disease perspective, the analysis of the common SPs and NFTs proteome may unravel putative interesting targets to pursue, more specific to AD.

Overlap of the SPs and NFTs proteomes revealed a total of 375 proteins in common (Figure 3). GO analysis of this common proteome (corresponding gene nomenclature was used) was carried out for the BP (Figure 4A) and for the Reactome pathways associated (Figure 4B). At the BP level, several interesting terms arise related to

FIGURE 2 SPs and NFTs proteome's Gene Ontology analysis. (A) Top 10 biological process, (B) molecular function and (C) cellular component obtained for senile plaques (SPs) proteome and for the neurofibrillary tangles (NFTs) proteome. Underlined terms are common to both proteomes. Analysis carried out on Cytoscape v3.9.1. (https://cytoscape.org/) with ClueGO v2.5.9 + CluePedia v.1.5.9 plugins. act, activity; bind, binding; comp, component; compl, complex; cytosk, cytoskeletal; dev, development; est, establishment expr, expression; gen, generation; hydrol-driv, hydrolysis-driven; inorg, inorganic; intrac, intracellular; loc, localization; mech, mechanism; memb, membrane; mitoch, mitochondrial; mol, molecular; move, movement; org, organization; orga, organelle; prec, precursor; proc, process; proj, projection; prot, protein; prot-transp, proton-transporting; pyroph, pyrophosphate; reg, regulation; resp, response; rotat, rotational; subcel, subcellular; subst, substance; synth, synthase; trans, transmembrane; transp, transport; transport, transporter.

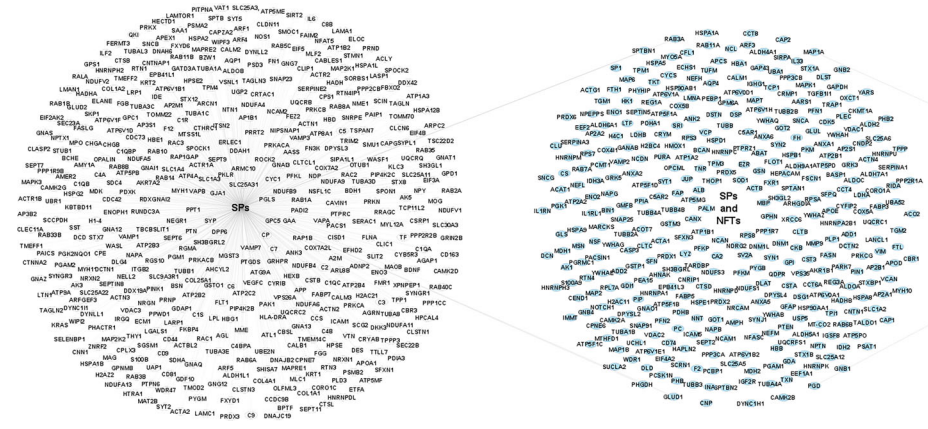


FIGURE 3 SPs and NFTs common proteome. Gene names of the proteins found in the literature review that appeared only associated to senile plaques (SPs) or to neurofibrillary tangles (NFTs). At a blue color are the gene names of the proteins common to both hallmarks, representing a total of 375 proteins. Network created on Cytoscape v3.9.1. (https://cytoscape.org/).

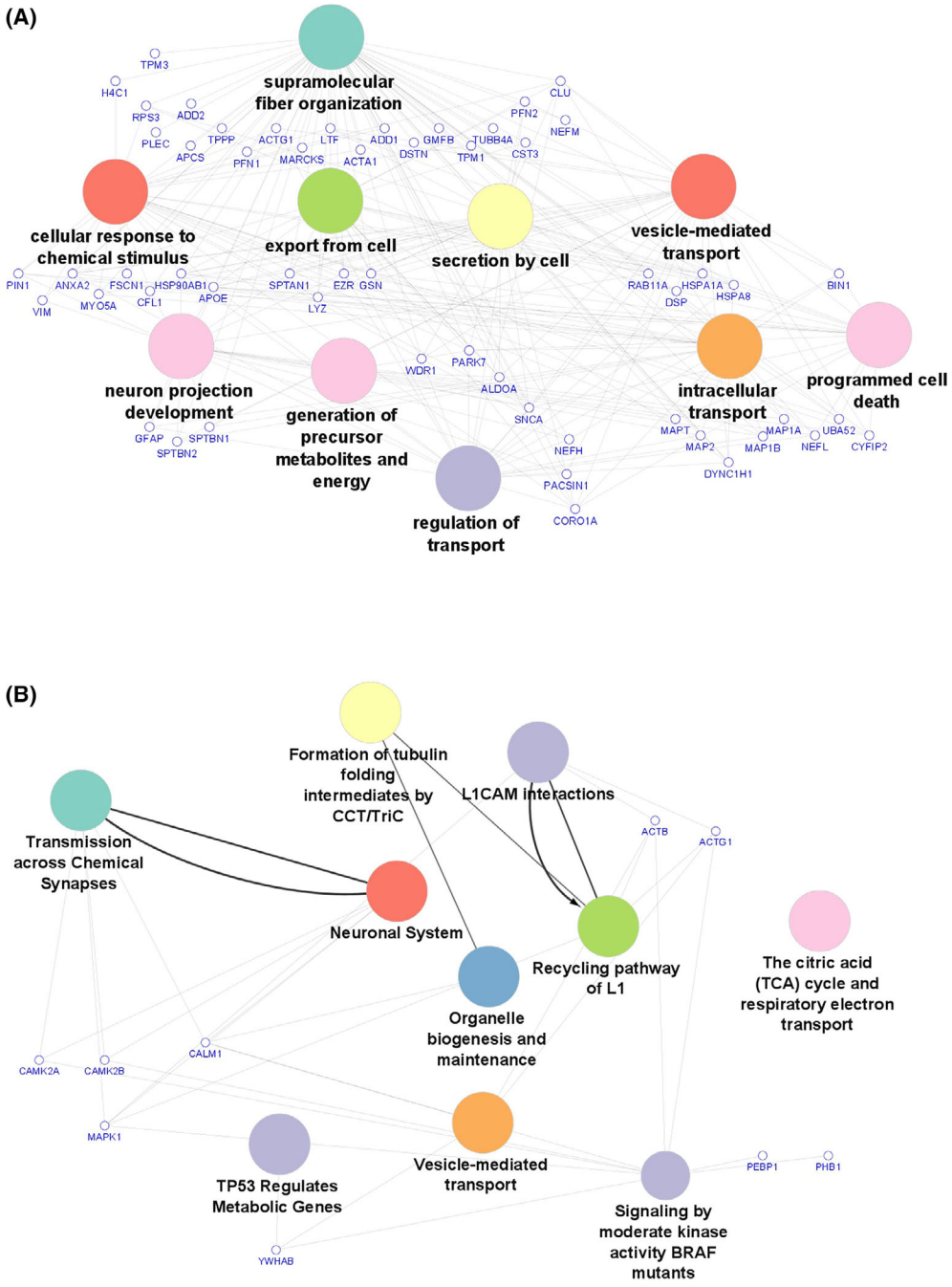


FIGURE 4 GO analysis and Reactome pathways of the SPs and NFTs common proteome. (A) Top 10 biological processes associated to the common senile plaques (SPs) and neurofibrillary tangles (NFTs) proteome. Genes linked to each of the top 10 biological process are indicated in blue. (B) Top 10 Reactome pathways associated to SPs and NFTs common proteome. Genes linked to each of the top 10 Reactome pathways are indicated in blue. Only pathways with p value ≤ 0.05 were considered. Network created on Cytoscape v3.9.1. with ClueGO v2.5.9 + CluePedia v.1.5.9 plugins.

various processes like "vesicle-mediated transport," "neuron projection development," "programmed cell death," and "secretion by cell." Among the main pathways linked to this common proteome were "transmission across chemical synapses," "neuronal system," and also "vesicle-mediated transport." In both cases, these are all processes highly relevant to AD pathogenesis.

3.3 | Identification of putative targets for AD

To identify putative-specific targets relevant to AD two distinct approaches were employed. The first included superimposing the SPs and NFTs proteomes with a list of genes associated with AD available at the DisGenet database, followed by a network construction of these

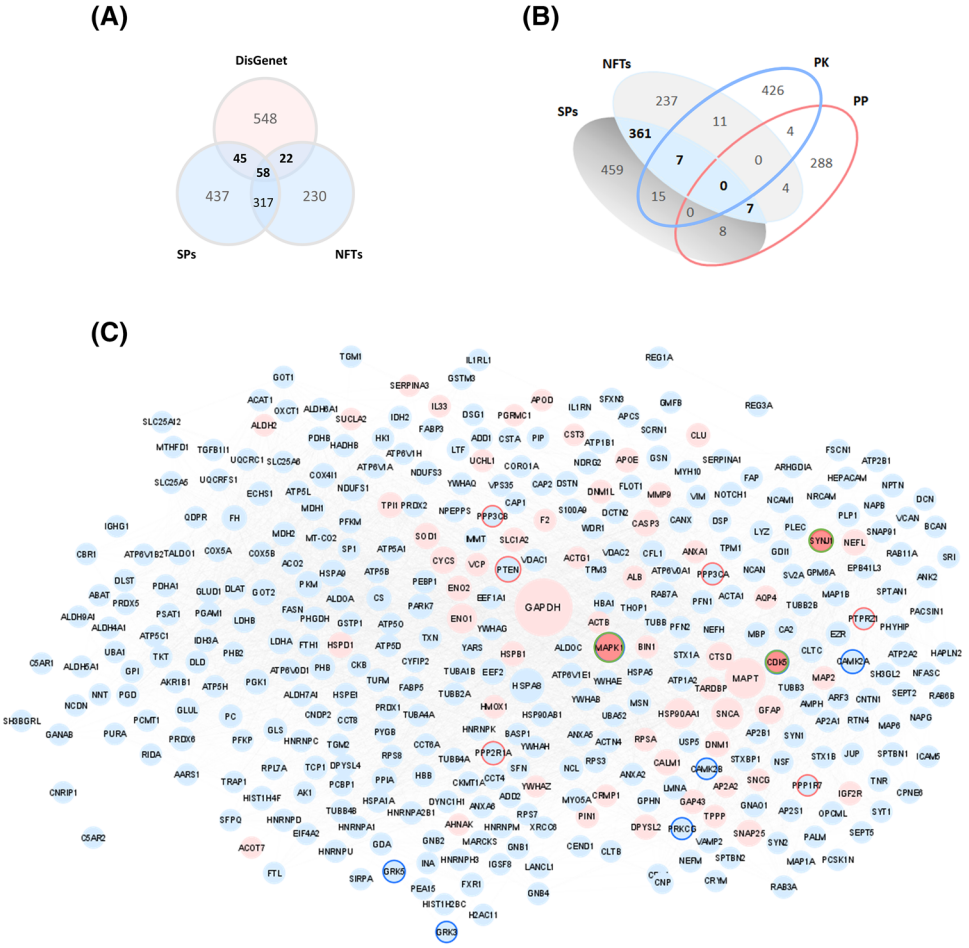


FIGURE 5 Identification of AD putative biomarker candidates common to SPs and NFTs proteomes. Two distinct approaches were carried out to identify putative AD phosphocandidates. (A) Venn diagram representing the overlap of the senile plaques (SPs) and neurofibrillary tangles (NFTs) proteomes with the DisGenet list of genes associated with AD. From the 375 genes that represent the common proteome (in blue), 58 genes were also present in DisGenet list. Only proteins from DisGenet with score ≥0.1 are indicated. (B) Venn diagram representing the overlap of the SPs and NFTs proteomes with the lists of genes corresponding to protein kinases (PKs) and protein phosphatases (PPs). A total of 52 proteins present in SPs and/or NFTs were identified, among which seven kinases and seven phosphatases common to both proteomes. (C) Network obtained for SPs and NFTs common proteome (blue nodes). The 58 genes that overlap with DisGenet list (pink nodes), the kinases (blue contour), and the phosphatases (red contoured) are highlighted. Three common phosphotargets were identified from the two approaches and these are represented in darker pink nodes with green contour: MAPK1, CDK5, and SYNJ1. This interactome was created on STRING and then imported to Cytoscape v3.9.1. AD, Alzheimer's disease.

proteins interaction. This resulted in a set of 58 proteins (corresponding gene names) common to both SPs and NFTs (Figure 5A,C). The central node of this network was GAPDH, a glyceraldehyde-3-phosphate dehydrogenase.

For the second approach, and since phosphorylation is a key event in AD, the SPs and NFTs proteomes were overlapped with the lists of genes corresponding to human PKs and PPs. This allowed for the identification of 33 kinases and 19 phosphatases. Among these, seven kinases (CAMK2A, CAMK2B, CDK5, GRK3, GRK5, MAPK1, PRKCG) and seven phosphatases (including PPP1R7, PPP2R1A, PPP3CA, PPP3CB, PTEN, PTPRZ1, SYNJ1) were common to both proteomes (Figure 5B,C, nodes with contour).

Taken together, both approaches allowed for the identification of a set of proteins, that could constitute possible biomarker candidates for AD diagnosis. Three common phosphotargets appeared, namely CDK5, MAPK1 and SYNJ1 (Figure 5C, darker pink nodes with green contour), from which MAPK1 is the node with more protein interactions. A schematic representation of all the approaches employed to identify and validate biomarker candidates for AD is presented in Figure S2.

In addition, by constructing a keyword co-occurrence and cluster map for MAPK1 based on Web of Science text data collection (Figure S3), it was possible to identify central nodes corresponding to AD. These were also followed by terms related to this disease, such as oxidative stress, neurotoxicity, or apoptosis, supporting the relevance of this kinase in AD pathogenesis. Hence, MAPK1 was selected for follow-up using blood-derived EVs. The EVs constitute an advantage given their capacity to cross the blood–brain barrier, allowing to monitor brain changes in peripheral biofluids. Further, EVs lipid bilayer

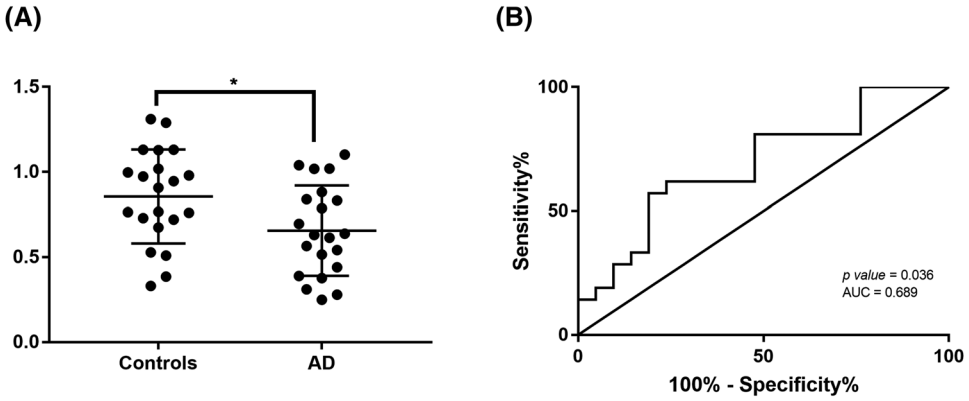


FIGURE 6 MAPK-1 levels in blood-derived extracellular vesicles from AD cases. (A) Quantification of MAPK1 levels in controls and AD cases from pcb- and UMG- groups. Each point represents the densitometry ratio obtained for each individual and the solid horizontal line the median. (B) ROC curve was obtained for controls and AD cases from both cohorts. AD, Alzheimer's disease; AUC, area under the curve; ROC, receiver operating curve. *p value < 0.05.

can protect their content from degradation and EVs cargo is disease-specific. Indeed, AD key molecules such as Aβ, Tau, and phospho-Tau 181 were found significantly different in blood-derived EVs of Controls when compared to AD cases [149–151]. In addition, previous work by the group also supports that blood-derived EVs' proteome is distinct in Controls and AD cases as well as its metabolic profile, assessed by Fourier transform infrared spectroscopy [148, 152].

3.4 | Validation of MAPK1 as a candidate for AD diagnosis

EVs were isolated from the human serum of patients from two distinct cohorts, using the precipitation kit ExoQuick and characterized, as described in the material and methods section (Figure S1). A total of 21 AD cases (mean age 75.52 $\pm$ 8.98) and 21 Control (mean age 71.85 $\pm$ 8.24) were included, composed by nine clinical diagnosed AD cases and the corresponding sex- and age-matched Controls from the pcb-group, and 12 AD patients and corresponding age-matched Controls from the UMG-group. A significant decrease in MAPK1 levels in blood-derived EVs from AD cases was detected by western blot when compared to Controls (Figure 6A). ROC curves were constructed, and the area under the curve (AUC) value obtained was 0.689, when comparing Controls and AD cases from both cohorts (Figure 6B).

4 | DISCUSSION

The increased prevalence of age-related disorders, in particular AD, motivates the search for novel biomarkers useful in early accurate diagnosis and/or therapeutics. In AD, the presence of both SPs and NFTs contributes to generalized neuronal death culminating in mental, physiological, and physical limitations. Unraveling the mechanisms and molecular players underlying the formation of these lesions will be of value to better understand the disease pathogenesis.

The present work, by analyzing both SPs and NFTs proteomes, aimed to provide further insightsinto AD molecular basis and, to identify novel putative biomarker candidates for this disease. The GO enrichment analysis revealed distinct characteristics in both proteomes (corresponding gene names), at the BP, MF, and CC levels. For the SPs, terms related with "neuron projection development" and "regulation of signaling" were found while for the NFTs, terms most prevalent were "regulation of cell death" and "cytoskeletal protein binding" were present. Indeed, abnormal signaling, for example, mediated by phosphorylation, apoptosis, brain inflammation, alterations in cytoskeletal networks, are all key processes in AD pathogenesis [10–13, 153, 154]. The classical link between these two hallmarks is neurodegeneration, and indeed terms, related to neurons, axon, and synapse in the GO enrichment analysis were identified, particularly at the CC level. In GO enrichment analysis, similar terms were found for SPs and NFTs, which is not surprising since 375 proteins were common to both proteomes.

Indeed, the molecular targets and pathways linked to both pathological lesions may reveal novel clues into the events underlying AD pathogenesis. Further, since both SPs and NFTs may be a hallmark present in other neurodegenerative conditions, the common proteome would be more specific for AD and thus it was further analyzed.

The GO enrichment analysis of the genes corresponding to the common proteome also revealed several processes potential linked to AD, as "vesicle-mediated transport," "programmed cell death," "neuron projection development," and Reactome pathways as "transmission across chemical synapses," strengthening the crucial involvement of these events in disease pathogenesis.

To identify phosphotargets relevant to AD in SPs and NFTs common proteome, two additional approaches were implemented, either by overlapping the common proteome (corresponding gene nomenclature) with AD-related genes and the other by overlapping with the lists of genes corresponding to PKs and PPs. These revealed three common targets related with phosphorylation events and AD, namely CDK5, MAPK1, SYNJ1, among which MAPK1 appeared as the node with a higher number of protein–protein interactions.

SYNJ1 is the major phosphoinositide phosphatase, mediating the uncoating of clathrin during clathrin-mediated endocytosis and, it also regulates synaptic vesicle recycling through the interaction with other synaptic activities. This makes SYNJ1 a presynaptic protein essential for synaptic vesicle endocytosis [155–158]. A recent study reported that SYNJ1 accumulates in post-mortem AD brain tissues, particularly in the neuronal soma and in plaque-associated dystrophic neurites, which can be associated to Hirano bodies and occasionally colocalized with hyperphosphorylated Tau in NFTs. Furthermore, the levels of insoluble SYNJ1 as well as its immunoreactivity in neurons were found significantly increased in AD cases [113].

Cyclin-dependent kinase-5 (CDK5), is a proline-directed serine/threonine kinase, abundantly expressed in neural tissues [159, 160]. Different studies revealed that CDK5 is involved in a variety of cellular events, regulating numerous aspects of brain development and function, such as cytoskeletal organization, neuronal differentiation, cell adhesion and membrane trafficking [160, 161]. Different studies have linked CDK5 with AD. For instance, this kinase can influence both Tau and APP phosphorylation [162, 163] or affect the phosphorylation of secretases involved in APP processing [164, 165]. It can also mediate the neurotoxic effect of Aβ1-42 peptide [166]. Further, CDK5 expression levels were found enhanced in frontal cortices of disease patients but decreased in CSF [164, 167].

MAPK1 was the candidate further addressed in this study due to its relevance in AD pathogenesis, the higher number of protein–protein interactions, and since this protein can be found in EVs [168]. MAPK1, also known as ERK2, belongs to the family of mitogen-activated protein kinases (MAPKs), which are serine/threonine protein kinases, that play important roles in cellular signal transduction. Dysregulation of the MAPK signaling pathway has been associated with AD [169–172]. MAPK1 has an important role in regulating Tau functions and Tau phosphorylation, by decreasing its affinity for microtubules and reducing Tau ability to stabilize them [173, 174]. Further, the activation of this kinase in hippocampus is required for contextual, spatial, and long-term memory formation in mammals [175], turning this protein an interesting target for validation as a biomarker for memory decline for diseases such as AD. Also, MAPK1 mRNA levels have been found elevated in different brain regions [169], implicating this kinase in the formation of Tau hyperphosphorylation early in the development of AD.

To address the biomarker potential of MAPK1 in AD, serum-derived EVs with exosomes-like characteristics from AD and Control patients were tested. Exosomes are a subtype of EVs with endocytic origin, formed by inward budding of plasma membrane, which form early endosomes. These can mature in late endosomes or multivesicular bodies (MVBs) that may have two final destinations: exosome secretion or autophagic degradation due to fusion with lysosomes or autophagosomes [176]. Both exosome biogenesis, secretion, and autophagy pathways act as coordinated complex mechanisms for cells to release their content [177]. These pathways are affected in several diseases, thus modulation of exosome biogenesis and secretion or autophagy may also constitute novel therapeutic strategies. In a diagnostic perspective, EVs represent a biomarker resource as these nanovesicles can cross the blood–brain barrier, protecting their cargo from degradation. EVs were shown to transport proteins linked to AD pathology, including Aβ and Tau species, but many other molecules related with events altered in AD, as inflammatory mediators and synaptic proteins, are also present [178, 179]. Hence EVs can carry disease-specific signatures potential useful in AD diagnostics. Herein, MAPK1 levels were found decreased in serum-derived EVs of AD patients, with a discrimination accuracy between Controls and AD cases of approximately 70%. MAPK1 can be found in both SPs deposits and NFTs in AD [20, 21, 79, 108, 111], and this may explain its decreased levels in peripheral biofluids (Figure 7).

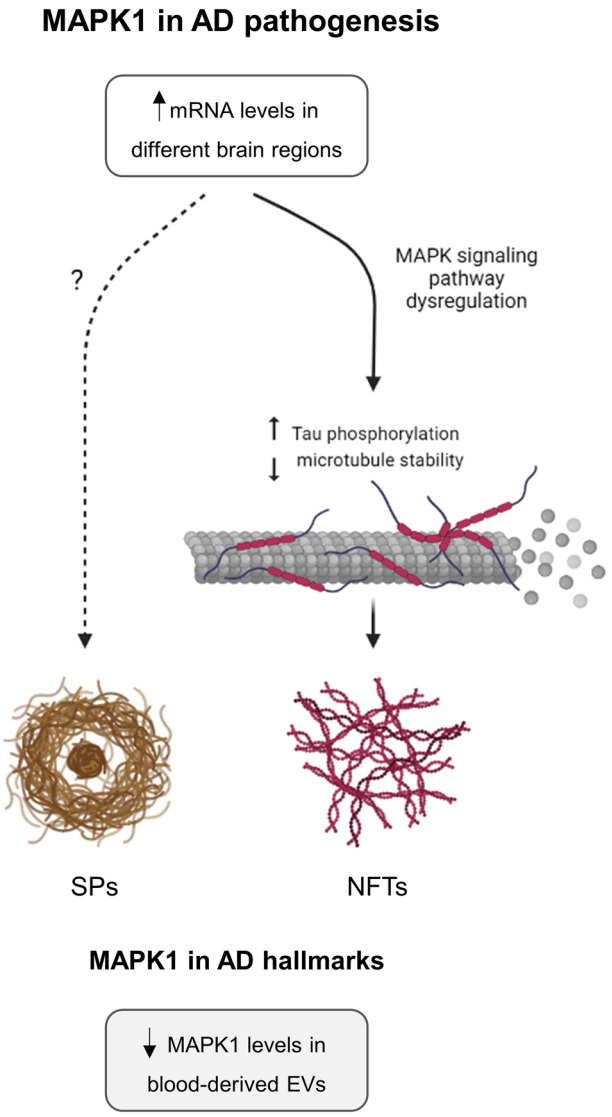


FIGURE 7 Involvement of MAPK1 in AD pathogenesis. Elevated mRNA levels have been found in different AD brain regions, which can be linked to dysregulation of the MAPK signaling pathway, contributing for increased Tau phosphorylation, decreased microtubule stability, and culminating in NFTs formation. MAPK1 contribution to SPs formation is unclear but this protein is found in both AD neuropathological hallmarks. MAPK1 accumulation in AD brain may explain its decreased levels in peripheral biofluids. Created with BioRender.com. AD, Alzheimer's disease; MAPK, mitogen-activated protein kinase; SPs, senile plaques.

5 | CONCLUSION

The work developed identified the common proteome between SPs and NFTs (375 proteins), relevant not only as putative biomarker candidates, but also for the study of molecular mechanisms underlying disease pathogenesis. Further, this study revealed putative phosphorylation-related targets present in both disease histopathological hallmarks that can constitute biomarker candidates for AD diagnosis. Among those is MAPK1, which was tested in human samples revealing the potential of this protein as a putative disease biomarker. Additional complementary studies should be carried out to validate the results obtained by increasing the number of samples and test this protein specificity by addressing other pathologies. Of note, addressing MAPK1 phosphospecific residues could potentially increase diagnostic sensitivity, as the case for P-Tau 181 for Alzheimer's disease [180, 181]. In addition, other analytical approaches, easy to implement in clinical practice, for example, as enzyme-linked immunoassay, should also be tested as the performance characteristics and clinical significance of MAPK-1 in AD diagnosis. Further, due to the complex nature of the disease, it is expected that a panel of biomarker candidates are more useful than a single biomarker, and thus other candidates identified should also be evaluated. A blood-based diagnostics would be cost-effective and easier to implement in clinical settings than the currently available CSF-based molecular diagnosis. Unraveling novel blood-derived biomarker candidates could be of potential value not only for AD early and differential diagnosis but also from a disease therapeutic perspective.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data are available in the article supplementary material.

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