REVIEW

Extracellular vesicles in the study of Alzheimer's and Parkinson's diseases: Methodologies applied from cells to biofluids

Margarida Vaz [ID] | Tânia Soares Martins [ID] | Ana Gabriela Henriques [ID]

Biomarker Discovery Team, Neuroscience and Signalling Group, Institute of Biomedicine (iBiMED), Department of Medical Sciences, University of Aveiro, Aveiro, Portugal

Correspondence
Ana Gabriela Henriques, Biomarker Discovery Team, Neuroscience and Signalling Group, Institute of Biomedicine (iBiMED), Department of Medical Sciences, University of Aveiro, 3810-193 Aveiro, Portugal.
Email: aghenriques@ua.pt

Abstract

Extracellular vesicles (EVs) are gaining increased importance in fundamental research as key players in disease pathogenic mechanisms, but also in translational and clinical research due to their value in biomarker discovery, either for diagnostics and/or therapeutics. In the first research scenario, the study of EVs isolated from neuronal models mimicking neurodegenerative diseases can open new avenues to better understand the pathological mechanisms underlying these conditions or to identify novel molecular targets for diagnosis and/or therapeutics. In the second research scenario, the easy availability of EVs in body fluids and the specificity of their cargo, which can reflect the cell of origin or disease profiles, turn these into attractive diagnostic tools. EVs with exosome-like characteristics, circulating in the bloodstream and other peripheral biofluids, constitute a non-invasive and rapid alternative to study several conditions, including brain-related disorders. In both cases, several EVs isolation methods are already available, but each neuronal model or biofluid presents its own challenges. Herein, a literature overview on EVs isolation methodologies from distinct neuronal models (cellular culture and brain tissue) and body fluids (serum, plasma, cerebrospinal fluid, urine and saliva) was carried out. Focus was given to approaches employed in the context of Alzheimer's and Parkinson's diseases, and the main research findings discussed. The topics here revised will facilitate the choice of

1 | INTRODUCTION

As a worldwide epidemic, neurodegenerative diseases include diverse conditions that primarily cause progressive degeneration and/or neuronal death in specific brain regions, severely impacting patients' quality of life. Amongst the most prevalent neurodegenerative diseases are Alzheimer's disease (AD) and Parkinson's disease (PD). AD is the most prevalent form of dementia worldwide, representing about 60–70% of all cases and is characterized by progressive cognitive impairment and memory loss (Prince et al., 2013). The two main AD hallmarks are the deposition of extracellular amyloid beta (Aβ) peptide into senile plates and the intracellular formation of neurofibrillary tangles as result of Tau hyperphosphorylation. These neurotoxic aggregates, and also Aβ oligomers (Aβo) (Mroczko et al., 2018), cause brain inflammation, mitochondria failure, oxidative damage, synaptic impairment and neurodegeneration (Gao et al., 2018; Reiss et al., 2018). PD is a motor neurodegenerative disorder characterized by the aggregation of α-synuclein into intraneuronal Lewy bodies and Lewy neurites and by the loss of dopaminergic neurons in the substantia nigra pars compacta which lead to muscle rigidity, bradykinesia and resting tremor (Hijaz & Volpicelli-Daley, 2020; Kalia & Lang, 2015).

Extracellular vesicles (EVs) have received increased attention in the field of neurodegenerative diseases, mainly due to its diagnostic and therapeutic potential including in the above-mentioned pathologies. EVs comprise three main subtypes of vesicles, namely exosomes, microvesicles and apoptotic bodies. Exosomes are the smallest type of EVs, with a size range between 30–150 nm. These are formed in the endosomal pathway, by the inward budding of endosomes leading to the formation of multivesicular bodies (MVBs), which later can fuse with the plasma membrane (PM) releasing exosomes. Secreted by most eukaryotic cells, exosomes play an import role in several biological functions including intercellular signaling, cellular homeostasis, immune response, inflammation, angiogenesis, autophagy and apoptosis (Gurunathan et al., 2021). Additionally, since exosomal cargo represent a fingerprint of the cell of origin and these vesicles can be found in diverse biofluids, including blood, plasma, cerebrospinal fluid (CSF), saliva or urine, exosomes represent an important source of valuable diagnostic biomarkers for several diseases. Particularly, in the context of AD and PD, EVs with exosomes-like characteristics have been extensively addressed as potential tools for disease diagnostics and therapeutics (Soares Martins et al., 2021; Zhao & Yang, 2021).

Microvesicles and apoptotic bodies can also be released from cells, however while microvesicles are directly budded from the PM, apoptotic bodies are released as a product of apoptotic cell disassembly. Microvesicles size ranges from 100 nm to 1 μm while apoptotic bodies from 1 to 5 μm (Caruso & Poon, 2018; Elzanowska et al., 2021).

Currently, the main concern is whether EVs isolation procedures can distinguish among different EVs subtypes and whether membrane markers, as proteins or lipids, are specific of a subtype of EVs. Indeed, some proteins of the endosomal sorting complex required for transport (ESCRT), involved in exosomes biogenesis, have been associated with other EVs subtypes (Théry et al., 2018). Hence, the criteria for the classification of EVs into subclass is still matter of debate, in particular, which criteria should be used to distinguish exosomes from microvesicles, since the nanovesicles size seems no longer a good measure. In addition, the International Society for Extracellular Vesicles (ISEV) declared that extensive additional work is needed to identify markers capable of specifically distinguish EVs subtypes, and that can be generally applied to distinct cells (Russell et al., 2019). The recommendations of ISEV are to always indicate EVs morphology/biophysical features and to characterize EVs preparations using at least three positive protein markers of EVs, including at least one transmembrane/lipid-bound protein or a cytosolic protein and one negative marker (Théry et al., 2018).

For the isolation of EVs with exosome-like characteristics several methodologies can be employed, each requiring different biofluid starting volumes and resulting in distinct vesicle yields and purity. All EVs isolation methodologies are subjected to some degree of contamination with larger vesicles or highly abundant proteins present in the biofluids. The gold standard method for EVs isolation is ultracentrifugation (UC) in which samples are first centrifugated at increasing speeds, to remove cells, other vesicles and cellular debris, and then are ultracentrifuged (~100 000 g) pelleting exosomes whereas microvesicles are pelleted by ~10 000 g centrifugation (Kurian et al., 2021; Li et al., 2017). Although this is the most used method, it renders in low exosome yields and EVs preparations may also need additional purifications procedures, as the use of a density gradient (dgUC), to isolate exosomes (Soares Martins, Vaz, & Henriques, 2022). Ultrafiltration (UF) can also be applied to isolate EVs by size, as well as size-exclusion chromatography (SEC) in which samples are passed down a porous resin column. During this process, large vesicles do not enter the pores, smaller particles are not retained and the vesicles with 30–200 nm are trapped and eluted lately. Commercially available kits (e.g. Exospin) already apply this technology. SEC protocols are simple and easy to follow, allowing the isolation of EVs with preserved structure and function. However, EVs preparation may be diluted, since the eluate can be collected in several fractions, and can also suffer

from contamination with other particles (Soares Martins, Vaz, & Henriques, 2022). Other isolation method based on chromatography is membrane affinity (MA)-based spin column. In this method, developed by Qiagen (ExoEasy), EVs bind to the column according to their biochemical properties. Many other commercial kits are also available for the isolation of EVs through precipitation (PP)-based methodology, as ExoQuick (ExoQ), Total Exosome Isolation kit (TEI) or miRCURY. In this method, water-excluding polymers as polyethylene glycol (PEG) are used to change the solubility of EVs in a solution and promote their deposition, only requiring low-speed centrifugations. The main advantages of this method are the easy commercial availability and the high vesicle yields obtained even from small sample volumes. However, this method is prone to co-precipitate protein aggregates and lipoproteins (Soares Martins, Vaz, & Henriques, 2022). EVs isolation can also be carried out based on the immunoaffinity binding to surface EVs proteins, as CD9, CD63 and CD81 coupled on magnetic beads. The interaction between EVs markers and the antibody-coupled to magnetic beads leads to EVs capture that then need to be eluted from the beads, which can be a difficult process and may decrease vesicle yields. This method allows the isolation of EVs subpopulations, but the nature of EVs is highly affected by the binding specificity of the used antibodies (Kurian et al., 2021; Li et al., 2017).

All the above-mentioned techniques result in EVs preparations enriched in exosomes, but still presenting some non-exosome vesicles and other contaminants like high abundant proteins, particularly in biofluids. Indeed, both plasma and serum are highly concentrated biofluids, which presents a challenge when isolating EVs. Typical contaminants of blood-derived EVs are lipoparticles, protein aggregates, albumin and even viruses (Soares Martins, Vaz, & Henriques, 2022). Efforts have been made to reduce these contaminants, including the combination of distinct exosome isolation methodologies or the development of new methods. As an example, the use of a three step protocol, combining an initial EVs enrichment step from cell culture media or plasma using UC or PEG, followed by dgUC and SEC columns could reduce considerably the amounts of apolipoproteins in EVs preparations (Zhang, Borg, et al., 2020). Recent methods, as nanoscale flow cytometry were also developed to decrease viruses contamination when isolating EVs (McNamara & Dittmer, 2020). In contrast with blood, cerebrospinal fluid (CSF), saliva and urine are less concentrated and require higher amounts of the starting biofluid for EVs isolation. CSF is an attractive biofluid for dementia studies since it is in contact with the brain, however its collection involves a lumbar puncture which is highly invasive. Saliva is easy collectable but when isolating EVs it is important to exclude the presence of solid particles and cell contaminants and to remove immunoglobulins or amylase, which are highly abundant. The major contaminants of EVs preparations isolated from urine are Tamm-Horsfall glycoprotein and uromodulin but others may also be relevant to mention as albumin, aquaporin, uroplakin and bacteria (Soares Martins, Vaz, & Henriques, 2022).

After isolation, size and/or concentration of EVs preparations enriched in exosomes can be determined using nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) or tunable resistive pulse sensing (TRPS); EVs morphology can be assessed through cryogenic, scanning or transmission electron microscopy (cryo-EM, SEM, TEM) or atomic force electron microscopy (AFM); and the nature of the preparations can also be tested by Western blot, ELISA or flow cytometry for evaluation of positive and negative EVs markers and of contaminants (Gurunathan et al., 2019; Kurian et al., 2021). Regarding EVs markers, as mentioned, the recommendations of ISEV are to analyze at least one transmembrane/lipid-bound protein (as CD63 and CD81) or a cytosolic protein (as TSG101, ALIX, Flotillins-1 and 2) and at least one negative marker as purity control (like apolipoprotein A1 or 2 (ApoA1/2), albumin and calnexin) (Théry et al., 2018).

In the neurological field, several studies used EVs with exosome-like characteristics isolated from neuronal models or distinct body fluids to better understand the disease related pathways or in biomarker discovery both for AD and PD. However, the methodologies applied are diverse and the results obtained are difficult to compare and relate. In this review, a literature overview was carried out to gather information regarding the methods for EVs isolation and characterization, from neuronal models employed in both neuropathologies, also presenting the main research findings.

2 | METHODOLOGIES EMPLOYED IN EVS ISOLATION FROM AD AND PD MIMICKING MODELS

EVs are key players in several neuronal-related cellular functions, including in neuronal process as the regulation of synapses number (Lee et al., 2018), neurotransmitter receptor levels (Fauré et al., 2006; Smalheiser, 2007) or in the production and turnover of myelin membrane proteins (Tassew et al., 2017). Nonetheless, alterations in EVs production, release, cargo, uptake and degradation can either have a protective or pathogenic role, being involved in neurodegenerative diseases as AD and PD (Mathews & Levy, 2019; Soares Martins et al., 2021). To understand the role of EVs in these pathologies, it is crucial to define the best procedures for the isolation of EVs preparations enriched in exosomes secreted by cells *in vitro*. This will allow to mimic disease conditions and unravel potential molecular mechanisms and novel pathways, linking EVs and disease progression.

To summarize the information on the neuronal cellular models and conditions that have been employed for the study of EVs in AD and PD, a search in PubMed was performed using the following set of keywords: (1)"(Exosomes OR Extracellular Vesicles) AND ((culture and cell) OR neuron OR astrocyte OR microglia OR oligodendrocytes OR brain) and (isolation or extraction) AND (Alzheimer)"; (2) "(Exosomes OR Extracellular Vesicles) AND ((culture and cell) OR neuron OR astrocyte OR microglia OR oligodendrocytes OR brain) and (isolation or extraction) AND (Parkinson)". A total of 205 articles were found. After excluding review articles, articles not written in English or based only in biofluids-derived EVs, a remain of 60 studies were analyzed and the information regarding the experimental conditions for EVs isolation collected (Table 1, Figure 1). Of these

TABLE 1   Cellular and brain-derived EVs as tools in the study of Alzheimer's and Parkinson's diseases

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Neuronal-like cell lines	SH-SY5Y	Alzheimer's disease	Molecular basis - APP-CTFs enrichment in EVs	UC	3×150mm dishes OptiMEM (20–22h)	TEM: cupformed vesicles NTA: mode size of ~100nm	ALIX, Flotilin-2, HSC70, TSG101 and Calnexin*	Lauritzen et al. (2019)
			Molecular basis - Aβ oligomers enrichment in EVs	UC	—	NTA: ~50–200nm	CD9, CD63, CD81 and GM130*	Vrancx et al. (2021)
			Molecular basis - Aβ and Melatonin effect in EVs secretion and cargo	PP (TEI) followed by ALIX IP enrichment	T25 flasks Medium with EVs depleted FBS (for 48h)	—	TSG101	Ozansoy et al. (2020)
			Molecular basis - Tau presence in EVs	UC	4×100mm dishes FBS free medium	TEM: 40–70nm	Flotillin	Santa-Maria et al. (2012)
			Therapeutics - Aβ clearance by EVs upon plant ceramide treatment	UC	FBS free medium (for 24h)	TRPS: 60–160nm (mode size of 120nm)	ALIX and GM1	Yuyama et al. (2019)
		Parkinson's disease	Molecular basis - α-synuclein secretion by EVs	UC	2×140mm dishes Medium with EVs depleted FBS (for 48h)	TEM: uniformly rounded, cup-shaped vesicles; 50–140nm	Annexin II, ALIX, Flotilin, GAPDH and HSC70	Emmanouilidou et al. (2010)
			Molecular basis - α-synuclein transport by EVs after methamphetamine exposure	UC	Complete culture medium (for 24h)	TEM: small vesicles NTA: mode size of 85–104nm	CD63, CD81 and HSC70	Meng et al. (2020)

TABLE 1 (Continued)

Type of cell/tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Neuronal-like cell lines	SH-SY5Y	Parkinson's disease	Molecular basis - α-synuclein transport by EVs	UC	Medium with EVs depleted FBS (for 24h)	TEM NTA: 100–300nm	ALIX, CD63, L1CAM and Flotillin-1	Gustafsson et al. (2018)
			Molecular basis - EVs biogenesis and secretion upon FAF1 transfection	UC and PP (ExoQ)	150mm dishes Medium with EVs depleted FBS (for 48h)	TEM: typical morphology of EVs with exosome-like characteristics NTA: 50–150nm	ALIX, CD63, HSC70 and Hsp90 and Calregulin*	Park et al. (2020)
			Therapeutics - Effect of EVs combined with L-Dopa	UC	Complete culture medium (for 24h)	—	—	Zarrin et al. (2021)
	MC65	Alzheimer's disease	Molecular basis - Aβ identification in EVs	UC	34 mL of CM	TEM: cup-shaped morphology, NTA: mean size of 157 nm; 6.5×10^9 EVs/mL	CD9, CD63, CD81 and MIgG*	Imanbekova et al. (2021)
	N2a	Alzheimer's disease	Therapeutics - EVs as drug delivery vehicles for natural compounds	UC and PP (TEI)	Medium with EVs depleted FBS (for 48h)	NTA: mode sizes of 105nm (by UC) and 135nm (by PP); yield by UC < PP	CD63 and TSG101	Tang et al. (2022)
			Molecular basis - EVs secretion in AD cellular model	UC	Medium with EVs depleted FBS (for 48h)	—	CD63, Flotillin-1 and TSG101	Willén et al. (2017)
			Molecular basis - EVs secretion and Aβ accumulation in EVs	UC	2×10^4 cells/cm^2 (35 mm dishes)	—	Alix, CD63 Flotillin-2, CD40*	Koinuma et al. (2021)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Neuronal-like cell lines	N2a	Alzheimer's disease	Molecular basis - Effect of EVs uptake by recipient cells in APP expression	UC	3.5×10^6 cells/dish (100mm) FBS free medium (for 24h)	TEM NTA: mode size of 136nm for C and 175nm for AD model	ALIX, CD9, Flotillin1, Flotillin2, HSC70, TSG101 and ApoA1*, ApoB* and Calnexin*	Ding et al. (2022)
		Parkinson's disease	Molecular basis - EVs in α-synuclein aggregation	UC	FBS free medium (for 48h)	Cryo-EM: spherical vesicles with 6nm thickness membrane NTA and DLS: mode size of ~100nm	ALIX, Flotillin-1 and Calnexin*	Grey et al. (2015)
	H4 and neurons differentiated from iPSC of AD patient	Alzheimer's disease	Molecular basis - Aβ-associated EVs effect	UC	30 mL of CM (25–30×10^6 cells) Medium with EVs depleted FBS	TEM NTA: mode size at 130nm (H4)	CD9 and Flotillin-1	Eitan et al. (2016)
	SL1SL2	Parkinson's disease	Molecular basis - Internalization of EVs-associated α-synuclein	UC	FBS free medium (for 4 days)	NTA: 90–120nm; 3×10^9 particles/mL	CD9, Flotillin-1, TSG101 and GM130*	Delenclos et al. (2017)
	MN9D	Parkinson's disease	Molecular basis - Manganese in α-synuclein EVs secretion and toxicity	UC	1×10^7 cells per flask/ 2×T175 flasks Medium with EVs depleted FBS (for 24h)	TEM: expected morphology NTA: ~150nm	ALIX, CD9, Flotillin-1, GRP78* and Lamin*	Harischandra et al. (2019)
Neuronal-like cell lines	MN9D	Parkinson's disease	Molecular basis - Manganese in α-synuclein EVs secretion and toxicity	UC	1×10^7 cells per flask/ 2×T175 flasks Medium with EVs depleted FBS (for 24h)	TEM: expected size and morphology NTA: mode size of 129nm	Rab27a	Harischandra et al. (2018)
Neurons	Primary rat neurons	Alzheimer's disease	Molecular basis - Aβ toxicity mediated by EVs from neurotransmitter-stimulated cells	UC	10 mL of CM (1×10^5–1×10^6 cells) per 500μL of EVs Complete culture medium (for 48h)	TEM: typical size and morphology NTA: 50–150nm	CD63, TSG101, COX4*, GM130* and TOM20*	Dou et al. (2021)
			Molecular basis - EVs secretion and Aβ accumulation in EVs	UC	1×10^5 cells/cm^2 (12 well plates)	—	ALIX, CD63 and Flotilin-2	Koinuma et al. (2021)

(Continues)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Astrocytes	Primary mice cortical astrocytes	Alzheimer's disease	Molecular basis - EVs role in apoptosis prompted by Aβ	UC and PP (ExoQ)	FBS free medium	—	ALIX and TSG101	Wang et al. (2012)
	Primary rat cortical astrocytes		Molecular basis - Effect of EVs from astrocytes treated with aFGF	PP (TEI)	Medium with EVs depleted FBS (for 72h)	AFM: spherical in shape NTA: mode size of 115 nm	CD9, CD63 Clathrin and Calnexin*	(Peng et al. (2022)
	Primary rat cortical astrocytes		Molecular basis - Effect of EVs from astrocytes treated with IL-1β	UC	4 × T150 flasks FBS free medium	NTA: ~150nm/ C—1.68×10⁹ particles/ mL; IL-1β—2.52×10⁹ particles/mL	CD63, Flotillin-1, TSG101, α-actinin-4* and Mitofilin*	Li et al. (2020)
Microglia	BV-2 and primary mice microglia	Alzheimer's disease	Molecular basis - Microglia EVs interaction with Aβ	High speed centrifugation (~12000–15 000g)	T75 or T150 flasks	TEM: rougher surface architecture DLS: 160–500nm, mean 274nm	Annexin V	Gouwens et al. (2018)
Microglia	BV-2	Alzheimer's disease	Molecular basis - Effect of EVs from miR-711 transfected BV-2	UC	—	TEM: round or elliptical in shape; mean size 98nm	CD63, Alix and Calnexin*	Zhang, Xu, et al. (2020)
Neurons/ Astrocytes/ Microglia	Primary murine microglia, neurons and astrocytes	Alzheimer's disease	Molecular basis - Microglia depletion on Tau secretion in EVs	UC	2 mL of CM	TEM: 50–100nm	TSG101	Asai et al. (2015)
Neurons and glia co-culture	Primary mice neurons, astrocytes and oligodendrocytes co-cultures	Alzheimer's disease	Molecular basis - EVs cargo after neurons exposure to Aβ protofibrils	UC	32 mL of CM Complete culture medium (for 2 days or 5 days)	TEM: ~50–75nm or >400nm; presence of exosomes and larger microvesicles	CD9, Flotillin-1 and TSG101	Nikitidou et al. (2017)
			Molecular basis - Effect of EVs from neurons exposed to Aβ protofibrils	UC	Complete culture medium	TEM: presence of exosomes and larger microvesicles	—	Beretta et al. (2020)
Neurons/ Microglia	Primary mouse cortical neurons and microglia	Parkinson's disease	Molecular basis - α-synuclein transmission by EVs	PP (ExoQ)	2 × 100mm (20mL of CM) Medium with EVs depleted FBS	TEM and NTA: 50–100nm, mode size of ~80nm (for neurons)	ALIX and TSG101	Guo et al. (2020)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Stem cells	Rat hippocampal NSC (neurospheres) Rat mature hippocampal neurons (derived from NSC)	Alzheimer's disease	Molecular basis - NCS-derived EVs effect in synapses impaired by Aβo	UC	6×10^7 cells (225 mL of CM) Complete culture medium	TEM: rounded and double-membrane structures NTA: mode size 72.3 nm (NSC-EVs) and 69.4 nm (mature neurons-EVs)	CD9, CD63, CD81, HSC70 and GM130*	Micci et al. (2019)
	Differentiated iPSC from AD patient		Molecular basis - EVs effect in Tau phosphorylation	PP (ExoQ)	Complete culture medium (for 2–3 days)	NTA: ~158–268 nm	Flotillin-1	Aulston et al. (2019)
Stem cells	hiPSC from AD patient and hiPSC from other lineages	Alzheimer's disease	Molecular basis - iPSC-derived EVs effect	UC and PP (TEI)	TEI: 8–10 mL of CM; Complete culture medium (for 24–48 h)	TEM: cup-shaped vesicles NTA: ~100 to 120 nm	ALIX, CD63, TSG101 and HSC70	Marzano et al. (2019)
	iPSC differentiated into neurons, microglia, astrocytes and oligodendrocytes		Molecular basis - iPSC-derived EVs characterization	UC followed by SEC (qEV)	7.5×10^6 cells/flask (T75) or 1×10^6 cells/well (6-well) for microglia Medium with EVs depleted FBS	TEM: cup-shaped morphology NTA: most within 50–150 nm (except of astrocytes >150 nm)	ALIX, CD9, CD63, CD81, Flotillin 1, Flotillin 2, GAPDH, HSPA8, Rab7A, Syntenin-1, TSG101 and several negative or contamination EVs markers	You et al. (2022)
	Adult human neural progenitor (AHNP) and patient iPSC	Parkinson's disease	Biomarker discovery—Gene expression from EVs extracted RNA	UC	Medium with EVs depleted FCS (for 48 h)	NTA: mean size of 132.6–234.5 nm; 2–7.08×10^8 particles/mL	—	Candelario et al. (2020)
	F3 cells immortal NSC line (and human fibroblasts)		Therapeutics - Stem cells-derived EVs therapeutic effect	Tangential flow filtration followed by PP (TEI)	500 mL of CM; Medium with EVs depleted FBS (for 48 h)	NTA: 80–150 nm	CD9, CD63, CD81, HSC70, TSG101, Calnexin*	Lee et al. (2022)
Mesenchymal stem cells	Human Wharton's jelly mesenchymal stem cells	Alzheimer's disease	Therapeutics—hMSC-derived EVs therapeutic effect	UC	7×10^3 cells/cm^2 (T75) FBS free medium (for 24 h)	TEM: roughly spherical shapes, 40–100 nm NTA: 30 to 750 nm (mode size 60–70 nm) mixed population of exosomes and microvesicles; 1.7×10^4 particles/cell	CD63, CD9 and CD81	Bodart-Santos et al. (2019)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Mesenchymal stem cells	Human Wharton's jelly mesenchymal stem cells	Alzheimer's disease	Therapeutics—MSC-derived EVs therapeutic effect	UF (Exopure)	FBS free medium (for 48h)	—	CD9, CD63 and CD81	Zhdanova et al. (2021)
	Human umbilical cords mesenchymal stem cell		Therapeutics—MSC-derived EVs therapeutic effect	UC	FBS free medium	TEM: bilayer cup-shaped morphology NTA: homogenous population, ~90nm	CD63, CD81 and Tubulin*	Wei et al. (2020)
			Therapeutics—MSC-derived EVs therapeutic effect	UC	Medium with depleted FBS (for 48h)	TEM: membrane vesicles NTA: 50–150nm	ALIX, CD9, CD63 and TSG101	Yang et al. (2020)
			Therapeutics—MSC-derived EVs therapeutic effect	PP (ExoQ)	FBS free medium (for 48h)	TEM: round shape with a hypodense centre; 30–150nm	CD9 and CD63	Ding et al. (2018)
	Mouse bone marrow mesenchymal stem cells		Therapeutics—MSC-derived EVs therapeutic effect	PP (TEI)	Medium with depleted FBS (for 48h)	TEM: double-layer membrane and cup holder-like structure; ~50nm	CD63, HSC70 and TSG101	Liu et al. (2022)
	Rat bone marrow mesenchymal stem cells		Therapeutics—EVs as vehicles for neprilysin	UC	1×10^7 cells per flask (T75) Medium with depleted FBS (for 24–48h)	SEM: spheroid morphology, 100–300nm DLS: ~223nm	CD9 and HSC70	Izadpanah et al. (2020)
	Human umbilical cords mesenchymal stem cell	Parkinson's disease	Therapeutics—MSC-derived EVs therapeutic effect	UC	FBS free medium (for 48h)	TEM NTA: 30–150nm; mode size ~100nm	CD9, CD63, TSG101 and Calnexin*	Chen et al. (2020)
			Therapeutics—MSC-derived EVs therapeutic effect	UC	6-well FBS free medium (for 48h)	TEM: cup-shaped or spherical morphology NTA: 40–120nm	CD63, CD81, TSG101 and Calnexin*	Bai et al. (2021)
Mesenchymal stem cells	Differentiated into dopaminergic neuron bone marrow mesenchymal stem cells	Parkinson's disease	Therapeutics—MSC-derived EVs therapeutic effect	UC	Complete culture medium	TEM: round vesicles, 60–150nm NTA: 110nm (for control) and 93nm (for differentiated cells)	CD63, CD81 and Cytochrome C*	Li, Li, et al. (2022)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Brain tissue	Human brain tissue	Alzheimer's disease	Biomarker discovery—Proteomics	UC	0.5 g of frozen frontal cortex	TEM: presence of small vesicles	ALIX, CD63 and CD81	Ngolab et al. (2017)
			Biomarker discovery—Multiomics	dgUC	1 g of original brain tissue/150 µl of final EVs solution	TEM: small EVs TRPS: mode size of 89 nm, mean size of 123 nm; 4.16×10^{12} particles/m	CD9, CD81, and Syntenin-1, Calnexin* and GM130*	Cohn et al. (2021)
			Biomarker discovery—miRNAs	dgUC	Between 1.48 g to 1.77 g of original frontal cortex, brain tissue	TEM: 50–200 nm	Syntenin and Calnexin*	Cheng et al. (2020)
			Biomarker discovery—Proteomics	dgUC	0.5 g of unfixed frozen tissue	TEM: cup-shaped morphology	CD9, CD81, Cytochrome C*, histone protein* H2A.Z* and GM130*	You et al. (2022)
	Mice brain tissue		Method—Comparison of different dgUC approaches for EVs isolation and EVs proteomics	dgUC with Top-loaded sedimentation gradient or Bottom-loaded floatation	2 months old-3 mice brains per condition	TEM: highly abundant, clear membrane-bound vesicles between 50 and 200 nm	ALIX, CD63, CD81, HSC70, Rab8a and TSG101 and Calnexin*	Hurwitz et al. (2018a)
			Molecular basis—Effect of EVs reduced secretion in AD mice	UC	—	NTA: 5×10^{11} and 2×10^{11} particles/mL; 100 and 120 nm	ALIX, Flotillin-2 and TSG101	Dinkins et al. (2016)

(Continues)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Brain tissue	Mice brain tissue	Alzheimer's disease	Therapeutics - Aβ clearance by EVs upon plant ceramide treatment	dgUC	Left hemispheres	TRPS: 60–130nm	—	Yuyama et al. (2019)
			Molecular basis—APP proteolytic process in EVs	dgUC	—	—	CD63 and HSC70	Pérez-González et al. (2020)
			Molecular basis - Microglia depletion on Tau secretion in EVs	dgUC	—	TEM: 50–100nm (average ~100nm)	TSG101	Asai et al. (2015)
			Molecular basis - Tau seeding by EVs	dgUC	Half brain per mice	TEM	—	Clayton et al. (2021)
			Biomarker discovery—Proteomics	dgUC	6 mice brains per condition 0.4g per mice	TEM: cup-shaped morphology NTA: 1.40×10^9 particles/µg	CD9, CD63, CD81, TSG101, CYC1*, and GM130*	Muraoka et al. (2021)
	Human and mice brain tissue		Molecular basis - Tau seeding by EVs	dgUC	—	TEM, AFM NTA: mode size of 121nm for C and 110nm for AD (human brain)	CD63	Ruan et al. (2021)
			Molecular basis—APP and APP CTFs presence in EVs	dgUC	Human brain Brodmann area 9 and Murine hemi-brains	TEM: 50–150nm; expected morphology	Flotillin and TSG101	Pérez-González et al. (2012)
	Mice brain extracellular space		Molecular basis - Tau seeding by EVs	dgUC	5 mice brains per condition	—	—	Polanco et al. (2021)
			Molecular basis - APP-CTFs enrichment in EVs	dgUC	—	TEM: appropriate sized and shaped vesicles for EVs with exosome-like characteristics NTA: appropriate size	Flotilin-2 and HSC70	Lauritzen et al. (2019)

TABLE 1 (Continued)

Type of cell/ tissue	Cellular line	Disease	Research topic	EVs isolation methods	Cell number/Culture medium	Size and yield	EVs markers	References
Brain tissue	Mice brain extracellular space	Alzheimer's disease	Molecular basis—Tau seeding by EVs	dgUC	—	TEM: presence of nanovesicles TRPS: mean size 130nm; mode size 74nm	ALIX and Flotillin-1	Polanco et al. (2016)
			Molecular basis - Effect of EVs uptake by recipient cells in APP expression	dgUC	Cortical and hippocampal tissues	TEM: cup-shaped structure NTA: mode size of 148nm for C and 156 for AD	ALIX, CD9, Flotillin1, Flotillin2, HSC70, TSG101, ApoA1*, ApoB* and Calnexin*	Ding et al. (2022)
	Mice brain tissue	Parkinson's disease	Molecular basis - Effect of EVs-containing α-synuclein administration	dgUC	—	TEM	—	Karampetsou et al. (2020)
	Rat midbrain slices		Molecular basis - Effect of EVs derived from activated microglia	UC	Midbrain slices (350-mm thick) cultured in 6-well CM of total 20days in vitro	—	CD63 and Flotillin-2	Tsutsumi et al. (2019)

Abbreviations: AFM, atomic force electron microscopy; APP-CTFs, APP C-terminal fragments; C, control; CM, conditioned media; dg, density gradient; Cryo-EM, cryogenic electron microscopy; DLS, dynamic light scattering; EVs, extracellular vesicles; ExoQ, ExoQuick; FBS, fetal bovine serum; FCS, fetal calf serum; haFGF, human acidic fibroblast growth factor; IP, immunoprecipitation; iPSC, induced pluripotent stem cells; MSC, mesenchymal stem cells; NSC, neural stem cells; NTA, nanoparticle tracking analysis; PP, precipitation; SEC, size exclusion chromatography; SEM, scanning electron microscopy; TEI, total exosome isolation kit; TEM, transmission electron microscopy; TRPS, tunable resistive pulse sensing; UC, ultracentrifugation; UF, ultrafiltration. *Negative or contamination EVs markers.
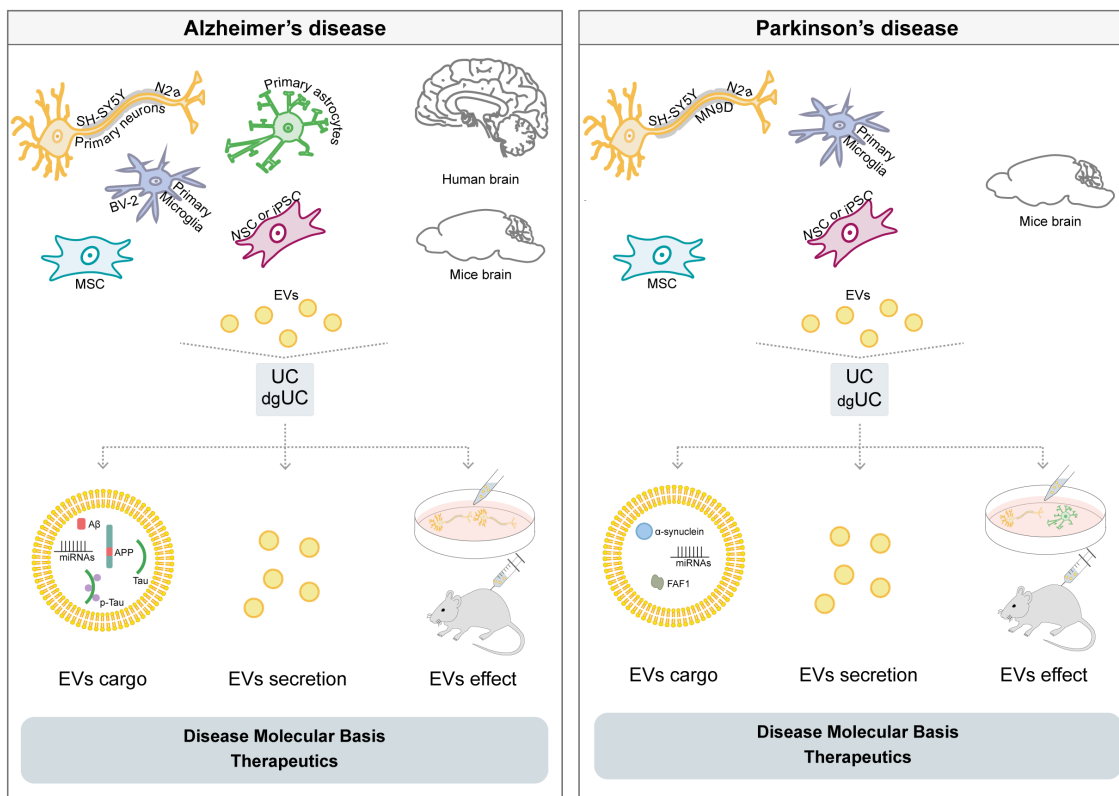
FIGURE 1 EVs isolated from cellular models and brain tissue in the study of Alzheimer's and Parkinson's diseases. N2a, primary neurons, astrocytes and microglia, neuronal and mesenchymal stem cells or even human and mice brain tissue were used to isolate EVs, mostly through ultracentrifugation or density gradient ultracentrifugation. EVs' cargo and secretion were analyzed and/or these vesicles were added to distinct cell models or injected in animals (mice or rats) to evaluate their effect. These approaches greatly contributed to the knowledge of both diseases' molecular basis and to the discovery of new therapeutic tools. Abbreviations: Aβ, amyloid beta; APP, amyloid precursor protein; dgUC, density gradient ultracentrifugation; EVs, extracellular vesicles; FAF1, Fas associated factor 1; iPSC, induced pluripotent stem cells; MSC, mesenchymal stem cells; NSC, neuronal stem cells; p-Tau, phosphorylated Tau; UC, ultracentrifugation.

articles, 41 presented data on EVs derived from cellular cultures, 14 on EVs isolated from brains tissue or brain extracellular space and 5 of both EVs sources.

Regarding the isolation methods, UC is by far the most used in EVs with exosome-like characteristics secreted from cellular models (Asai et al., 2015; Bai et al., 2021; Beretta et al., 2020; Bodart-Santos et al., 2019; Candelario et al., 2020; Chen et al., 2020; Delenclos et al., 2017; Ding et al., 2022; Dou et al., 2021; Eitan et al., 2016; Emmanouilidou et al., 2010; Grey et al., 2015; Gustafsson et al., 2018; Harischandra et al., 2018; Harischandra et al., 2019; Imanbekova et al., 2021; Izadpanah et al., 2020; Koinuma et al., 2021; Lauritzen et al., 2019; Li et al., 2020; Li, Li, et al., 2022; Marzano et al., 2019; Meng et al., 2020; Micci et al., 2019; Nikitidou et al., 2017; Park et al., 2020; Santa-Maria et al., 2012; Tang et al., 2022; Vrancx et al., 2021; Wang et al., 2012; Wei et al., 2020; Willén et al., 2017; Yang et al., 2020; Yuyama et al., 2019; Zarrin et al., 2021; Zhang, Xu, et al., 2020), whereas dgUC was employed in EVs isolation derived from brain tissue in nearly all studies presented (Asai et al., 2015; Cheng et al., 2020; Clayton et al., 2021; Cohn et al., 2021; Ding et al., 2022; Hurwitz et al., 2018a; Karampetsou et al., 2020; Lauritzen et al., 2019; Muraoka et al., 2021; Pérez-González et al., 2012; Pérez-González et al., 2020; Polanco et al., 2016;

Polanco et al., 2021; Ruan et al., 2021; You et al., 2022; Yuyama et al., 2019).

The selection of UC is probably related to the high quantity of conditioned medium necessary to obtain enough EVs yields for the subsequent analysis and also it does not need additional reagents. These quantities varied between 2–30 to 225 ml of conditioned media (Table 1) (Eitan et al., 2016; Guo et al., 2020; Imanbekova et al., 2021; Marzano et al., 2019; Micci et al., 2019; Nikitidou et al., 2017), and therefore, other techniques would require an increasing quantity of reagents and material, depending on the initial volume of conditioned media. Moreover, UC coupled with sucrose or Optiprep density gradient has been applied to improve EVs samples purity and yield. A study that compared UC and Optiprep dgUC, showed that, although dgUC fractions render in low EVs yields, these preparations were more enriched in EVs with exosome-like characteristics, since proteomic analysis identified more proteins present in ExoCarta top100 list (Arab et al., 2019). For the isolation of EVs from brain tissue, the most common employed technique to increase the preparations purity is also dgUC, usually preformed as a top-loaded sedimentation sucrose gradient. However, this type of density gradient usually does not clearly separate the different subtypes of EVs and it was proposed that floatation iodixanol density

gradient separation could improve the purity of EVs-enriched exosomal preparations (Hurwitz et al., 2018a).

Nevertheless, PP-based isolation kits as ExoQ (Aulston et al., 2019; Ding et al., 2018; Guo et al., 2020; Park et al., 2020; Wang et al., 2012) and TEI (Lee et al., 2022; Liu et al., 2022; Marzano et al., 2019; Ozansoy et al., 2020; Peng et al., 2022; Tang et al., 2022) are also being employed in some studies, as well as UF kit Exo:pure (Zhdanova et al., 2021). To note, the employment of different methods for EVs isolation from diverse cell lines render in different results. The use of polymer-based PP kits has been associated with lower exosomal yield when compared with UC, in a study using undifferentiated SH-SY5Y and BE(2)-M17 cells (Seyfizadeh et al., 2019).

Similar profiles of EVs markers were observed in preparations isolated by UC and PP (using ExoQ) from undifferentiated SH-SY5Y or primary mice cortical astrocyte (Park et al., 2020; Wang et al., 2012). On the other hand, comparing EVs preparations from N2a cells, using UC and TEI, revealed that the concentration of EVs with exosome-like characteristics was superior using the PP method. However, comparatively to TEI the size of EVs extracted by UC were closer to the expected exosomal size range (Tang et al., 2022). Furthermore, the enrichment of preparations in exosomes has also been achieved using immuno-affinity with an exosomal marker (e.g. ALIX) after the isolation of EVs by TEI (Ozansoy et al., 2020).

From the studies collected (Table 1), only a few disclose the number of cells, dishes/flasks or quantity of medium used to isolate EVs. This information is very important for comparative proposes and reproducibility since these quantities can diverge even from studies using similar cell types. As an example, for primary neuronal cultures distinct initial conditioned media volumes were used, from 225 ml (Micci et al., 2019), to 32 ml (Nikitidou et al., 2017), 20 ml (Guo et al., 2020), 10 ml (Dou et al., 2021) or even 2 ml (Asai et al., 2015). These experimental differences may relate to the distinct cells' origin (rat and mice) or starting material. Nonetheless, additional studies should be carried out to explore these aspects and to standardize procedures.

The type of cell culture medium used for EVs isolation should also be considered since complete culture medium supplemented with serum (generally fetal bovine serum (FBS)) contain EVs, which can be isolated together with the EVs secreted by cells. These can interfere with downstream analysis, because serum vesicles have a similar morphology to cells-secreted EVs, containing the same markers (e.g. TSG101, CD81 and CD63) and even present similar RNA profiles (Shelke et al., 2014). Most of the studies used culture medium without FBS or with FBS-depleted EVs, generally by long-time UC (18h) (Table 1). However, residual contamination with FBS vesicles is expected and the use of EVs-depleted medium may affect cells growth and viability, as observed in primary astrocytes (Lehrich et al., 2018). Additionally, the use of serum-free medium could impact cells negatively, altering their growth rate and metabolism, or even change EVs production and content. An increased number of particles isolated from undifferentiated N2a and SH-SY5Y cells cultured in serum-free medium (OptiMEM) was observed when compared with EVs depleted medium. Moreover, their content was also significantly different as

EVs from serum-free conditioned medium presented increased levels of G-proteins, small GTPases, kinases and reactive oxygen species, reflecting a situation of cellular stress (Li et al., 2015).

3 | EVS IN THE STUDY OF AD AND PD MOLECULAR BASIS

3.1 | Neuronal-like cell lines and neurons

Concerning the *in vitro* models employed in the context of AD and PD, EVs has been usually isolated from the conditioned medium of diverse neuronal cellular lines as SH-SY5Y (Emmanouilidou et al., 2010; Gustafsson et al., 2018; Lauritzen et al., 2019; Meng et al., 2020; Ozansoy et al., 2020; Park et al., 2020; Santa-Maria et al., 2012; Vrancx et al., 2021; Yuyama et al., 2019; Zarrin et al., 2021), N2a (Ding et al., 2022; Grey et al., 2015; Koinuma et al., 2021; Tang et al., 2022; Willén et al., 2017), MC65 (Imanbekova et al., 2021), H4 (Eitan et al., 2016), SL1SL2 (Delenclos et al., 2017) and MN9D (Harischandra et al., 2018; Harischandra et al., 2019). In all studies undifferentiated cells were used.

SH-SY5Y human neuroblastoma cell line has been widely employed to study both AD and PD. For AD, EVs isolated from SH-SY5Y expressing amyloid precursor protein (APP) Swedish or C99 (which is the β-secretase-derived APP C-terminal fragment (CTF)), were enriched in APP-CTFs and oligomeric CTFs (Lauritzen et al., 2019). Additionally, hexameric Aβo, were only found in EVs derived from SH-SY5Y, while monomeric Aβ was exclusive of the soluble proteins fraction of the culture media (Vrancx et al., 2021). Moreover, Raman spectroscopy analysis of EVs derived from MC65, a human neuroblastoma cell line over-expressing intracellular Aβ, revealed two intense Raman peaks that were attributed to Aβ incorporation in the EVs (Imanbekova et al., 2021). In another study, it has been reported that SH-SY5Y treatment with Aβ$_{1-42}$, melatonin or both reduced EVs released, based on the decrease in TSG101 levels, and Total-Tau content. However, phosphorylated Tau (p-Tau) was not detected in EVs (Ozansoy et al., 2020). Nonetheless, other authors did not detect Tau in EVs secreted by SH-SY5Y (Santa-Maria et al., 2012), which may relate with the different experimental conditions. In a therapeutic perspective, the treatment of SH-SY5Y with a plant ceramide increased the secretion of EVs, which were able to bind to Aβ$_{1-40}$ and Aβ$_{1-42}$. Considering also that the levels of extracellular Aβ species were significantly decreased with treatment, this suggests that the plant ceramide stimulated the clearance of Aβ by EVs (Yuyama et al., 2019).

Regarding PD, α-synuclein was detected in EVs lumen and membrane, being these nanovesicles secreted in a calcium-dependent manner (Emmanouilidou et al., 2010). Another study suggested that the α-synuclein carried in EVs was mainly associated with the vesicles outer membrane. It was also reported that α-synuclein associated to EVs displayed an increased uptake, when compared with the free-floating form (Gustafsson et al., 2018). Additionally, EVs carrying α-synuclein could be transferred from neuronal cultures (SH-SY5Y), after methamphetamine exposure,

to astrocytes (U87MG), inducing an inflammatory response (Meng et al., 2020). Other proteins relevant to PD, as Fas-associated factor 1 (FAF1), were likewise detected in the cargo of EVs with exosome-like characteristics upon SH-SY5Y transfection with FAF1. Under these conditions exosomal yield was also increased when compared to Control cells (Park et al., 2020). Furthermore, SH-SY5Y-derived EVs alone or in combination with L-Dopa were administered to PD rats, resulting in improved movement ability and increased expression of tyrosine hydroxylase and dopamine receptor D2 (Zarrin et al., 2021).

Similar and/or complementary results have been obtained for the N2a mouse neuroblastoma cells. It was reported that EVs secreted by N2a (Control and cytotoxin treated) carried several neural and astroglial proteins, being capable of inducing cytotoxicity in other cells (Kumar et al., 2018). Additionally, Aβ intracellular and extracellular levels were increased and decreased, respectively, upon knockdown of TSG101 to decrease EV secretion, an effect also seen in primary cultured neurons. It was also observed that the induction of autophagosome formation in wild type cells led to a decrease in EVs secretion and the same effect in Aβ intracellular and extracellular levels. Therefore, it seems that EVs are important carriers of Aβ and its accumulation within EVs may relate to down-regulation of autophagy (Koinuma et al., 2021). Interestingly, in cells transfected with Swedish mutation to mimic AD, the secretion of EVs with exosome-like characteristics was increased when ESCRT-dependent intra-luminal vesicles formation was blocked. It was proposed that the secretion of EVs was augmented possibly due to up-regulation of MVB formation in an ESCRT-independent pathway (as CD63 mediator) (Willén et al., 2017). Other study, using N2a cells over-expressing APP, showed that the EVs from these cells were able to promote APP over-expression in recipient cells, in a process possibly involving miR-185-5p (Ding et al., 2022). On the other hand, N2a derived EVs were likewise assessed as drug delivery carriers for AD. In this context, it was reported that the loading method that outperforms the others (endogenous drug loading and the freeze-thaw method) was room temperature incubation of N2a-derived EVs with the natural herbal compounds, which when inside EVs were able to pass blood-brain barrier (BBB) (whereas in the free form the compounds were not permeable to BBB). In addition, exo-herbal compounds were added to PC-12 resulting in a decrease on the levels of proteins related to these neurodegenerative diseases, as Tau or α-synuclein. Further, the administration of the exo-compound neferine to AD mice decreased the deposition of fibrillated Aβ in the brain and improved the mice motor coordination in a higher extent than the compound alone (Tang et al., 2022).

Regarding PD, α-synuclein was also detected in N2a-derived EVs and the presence of these EVs caused an acceleration in exogenous α-synuclein aggregation (Grey et al., 2015).

Other cellular line used to assess the EVs' ability to pack pathogenic proteins was H4. In cells with PS1Δ9 mutation, EVs presented a significantly higher $A\beta_{1-42}/A\beta_{1-40}$ ratio and were toxic to primary rat cortical neurons, possibly by dysregulating calcium homeostasis and impairing mitochondrial function (Eitan et al., 2016).

For PD, it was also observed that SL1SL2-derived EVs associated with α-synuclein were more efficiently internalized by H4 cells than the protein free form (Delenclos et al., 2017). To evaluate the toxic effect of manganese (Mn^{2+}) on EVs secretion and its consequences to PD, the MN9D cellular line was used. It was reported that the Mn^{2+} increased the secretion of EVs, possibly through the up-regulation of Rab27a, and that Mn^{2+} stimulated EVs were able to exert a neurotoxic effect in dopaminergic cellular cultures, promote a brain inflammatory response in primary microglia and induce Parkinsonian-like symptoms in mice (Harischandra et al., 2018; Harischandra et al., 2019).

Besides neuronal-like cellular lines, EVs were also isolated from primary rat neurons stimulated with two neurotransmitters, glutamate or GABA. Opposite effects upon Aβ toxicity were observed since EVs from GABA-treated neurons alleviate the damage caused by Aβ treatments *in vitro* (by increasing cells viability and decreasing apoptosis) and *in vivo* (due to improvements in spatial learning and memory abilities), while EVs derived from glutamate-treated neurons aggravated the damage. These results indicate that the manipulation of EVs with different neurotransmitters may be interesting to unravelling AD pathogenesis pathways (Dou et al., 2021).

3.2 | Astrocytes and microglia

Astrocytes and microglia-derived EVs, commonly isolated from mice primary cultures, were also used as a cellular model to study AD. The incubation of primary mice cortical astrocytes with $A\beta_{25-35}$ triggered the secretion of PARP-4 in EVs, which induced apoptosis in astrocytes that uptake these vesicles (Wang et al., 2012). Also, the EVs isolated from a mixed co-culture of primary mice astrocytes, neurons and oligodendrocytes treated with $A\beta_{1-42}$ protofibrils were enriched in apolipoprotein E (ApoE), suggesting that EVs could have a role in ApoE intercellular transferring (Nikitidou et al., 2017). The exposure of primary mice cortical neurons to these EVs induced apoptosis, mitochondrial stress and synaptic loss (Beretta et al., 2020). In another study, acidic fibroblast growth factor (aFGF) was used to inhibit astrocytes activation. The primary rat astrocytes were also treated with Aβ and EVs were isolated. The intranasal administration of EVs to APP/PS1 mice, lead to ameliorated short-term memory deficits, improved spatial learning, promoted synaptic plasticity, and reduced Aβ deposition in the hippocampus (Peng et al., 2022). On the other hand, EVs from primary rat astrocytes treated with IL-1β increased the APP amyloidogenic processing in EVs-exposed neurons. This process may involve the enrichment of casein kinase 1 in the EVs (Li et al., 2020). Hence, it seems that EVs derived from astrocytes generally have a harmful role in AD progression but if these cells activation is inhibited, the EVs may be beneficial to attenuate disease progression.

It was found that microglia cell line (BV-2) microvesicles interacted strongly with Aβ protofibrils and these vesicles also could inhibit the aggregation of $A\beta_{1-42}$ monomers. Additionally, it was likewise observed that microvesicles derived from primary microglia contained

Aβ protofibrils (Gouwens et al., 2018). Further, EVs isolated from BV-2 transfected with miR-711 were able to reduce inflammation, improve cognitive dysfunction and alleviate neurodegeneration *in vivo* (Zhang, Xu, et al., 2020). Regarding Tau, primary culture murine microglia were found to phagocyte Tau, which were then transmitted, also as p-Tau, inside EVs to neurons, in a process that may be dependent of EVs biogenesis mediated by sphingomyelinase-2 (Asai et al., 2015). Interestingly, sphingomyelinase-2 deficiency in mice led to diminished EVs secretion, as expected, but also reduced Aβ$_{1-42}$ levels, senile plaques loads and p-Tau/Total-Tau ratio, and improved mice cognitive deficits (Dinkins et al., 2016).

In the PD pathogenic pathway, it has been shown that primary mouse microglia can release EVs containing α-synuclein, which can be taken up by primary mouse cortical neurons, triggering α-synuclein aggregation in the recipient neurons *in vitro*, causing dopaminergic neurons degradation and impairing locomotor function *in vivo* (Guo et al., 2020).

3.3 | Stem cells

Stem cells are an innovative type of neuronal cellular model that are beginning to be used. EVs from hippocampal neural stem cells delivered to mice, also injected with Aβo, were reported to eliminate the effect of Aβo in long-term potentiation and memory deficits, in a process that may involve the decrease of Aβo binding to synapses (Micci et al., 2019). Additionally, EVs derived from induced pluripotent stem cells (iPSC) enhanced cell proliferation, axonal growth and protected cells from Aβ$_{1-42}$ toxic effects (Marzano et al., 2019). Nonetheless, EVs derived from AD patient iPSC presented an increase in Aβ$_{1-42}$/Aβ$_{1-40}$ ratio, dysregulated calcium homeostasis and impaired mitochondrial function and were toxic to primary rat cortical neurons (Eitan et al., 2016). It was also reported that the injection of EVs secreted by iPSC of an AD patient led to increased Tau phosphorylation in the injected mice (Aulston et al., 2019). In other study, EVs were isolated from human iPSC differentiated in neurons, microglia, astrocytes and oligodendrocytes. The analysis of EVs proteome revealed novel cell-type specific EVs markers and importantly, it was also found a protein (integrin-β1) enriched in astrocyte-specific EVs, which was also elevated in EVs from AD patients' brain tissue. This protein was also associated with disease progression, since the elevated protein levels correlate with Aβ$_{1-42}$, Total-Tau and p-Tau levels in brain-derived EVs from AD patients (You et al., 2022).

Regarding PD, the analysis of EVs from patients' iPSC revealed their potential as a biomarker source for the disease (Candelario et al., 2020). On the other hand, neuronal stem cells-derived EVs appear to exhibit therapeutic potential since it was observed a reduction in ROS levels, caspase 3/7 activity and inflammation mediators *in vitro* and a decrease in neuronal loss and an anti-inflammatory effect *in vivo* in a PD mouse model (Lee et al., 2022).

The therapeutic potential of EVs has also been addressed using other type of stem cells, mesenchymal stem cells (MSC) which gained increasing relevance in the area, in the late years. MSC-derived EVs

were shown to inhibit apoptosis and promote proliferation and differentiation of PC12 cells (Shariati Najafabadi et al., 2021; Xie et al., 2021). In AD, MSC-derived EVs reduced Aβ$_{1-42}$ accumulation (Yang et al., 2020), prevented synapse damage induced by Aβo, protected hippocampal cells from oxidative stress (through reducing ROS) (Bodart-Santos et al., 2019) and decreased neuronal apoptosis (due to miR-223 delivery) *in vitro* (Wei et al., 2020). In addition, the administration of MSC-derived EVs in an AD mice model reduced Aβ$_{1-42}$ deposition and p-Tau levels in the hippocampus (Liu et al., 2022; Yang et al., 2020), alleviated brain inflammation by modulating microglia and astrocytes activation (Ding et al., 2018; Liu et al., 2022), and improved spatial learning and memory function (Ding et al., 2018; Liu et al., 2022; Zhdanova et al., 2021). MSC-derived EVs were also used as a drug delivery system of neprilysn (an Aβ degrading enzyme), causing improvements spatial memory ability, reducing hippocampus neuronal damage, senile plaques accumulation and alleviating brain inflammation in the AD rat model (Izadpanah et al., 2020).

For PD, the treatment of SH-SY5Y with MCS-derived EVs also promoted proliferation and inhibited apoptosis but, more importantly, these EVs *in vivo* were able to reach the substantia nigra, reducing dopaminergic neurons loss and up-regulating dopamine levels in the striatum (Chen et al., 2020). Similar effects on apoptosis were also observed in other study (in addition to an increase in autophagy) and were associated with miR-106b (Bai et al., 2021). It was also reported that the treatment of neurons (stimulated with a PD neurotoxin) with MSC-derived EVs were able to reverse the effect of the neurotoxin on inflammatory mediators leading to an improvement in brain inflammation, an effect also observed *in vivo* (Li, Li, et al., 2022).

3.4 | Brain tissue

The application of EVs is not only resumed to cellular models (Table 1). Indeed, these vesicles can also be derived from neuronal tissues. A total of 19 articles were found, using the keyword scheme mentioned before. The isolation of EVs from whole brain, in the majority of the articles analyzed, was carried out using a series of low and high speeds centrifugation and a final sucrose gradient ultracentrifugation (Hurwitz et al., 2018b; Pérez-González et al., 2012; Vella et al., 2017).

In the context of AD, it has been found that EVs from human AD brain tissue contained greater amounts of protein (Hurwitz et al., 2018a) and higher levels of Aβ plaques and phosphorylated Tau than EVs from Control brain tissue (Ngolab et al., 2017), among other proteomic, lipidomic and mRNA transcriptomic alterations (Cohn et al., 2021). Also, in EVs derived from AD human and mouse brains, it was observed an increased level of oligomeric Tau and a higher uptake of these EVs by primary cultured murine neurons (Ruan et al., 2021). In an APP-over-expressing mice model, EVs were enriched in full-length APP and APP C-terminal fragments when compared to wild-type animals (Lauritzen et al., 2019; Pérez-González

et al., 2012). Consistently, the mass spectrometry analysis of EVs derived from APP/PS1 mice brains identified more than 3000 unique proteins in AD, including an enrichment in presenilin-1, APP, integrin α-X and annexin V (Muraoka et al., 2021). In other study, EVs with exosome-like characteristics isolated from APP/PS1 mice brain extracellular spaces induced the expression of APP in N2a cells (Ding et al., 2022). Furthermore, APP proteolytic process can occur inside EVs-derived from APP over-expressing mice brain tissue, with generation of α- and β-APP-CTFs and subsequently AICD production, although the levels of exosomal monomeric Aβ decreased dramatically, probably due to peptide dimerization (Pérez-González et al., 2020). Interestingly, it was observed that there was an increase in Aβ clearance by EVs, upon the administration of plant ceramide in APP Swedish mice (Yuyama et al., 2019).

Tau oligomers were likewise found in EVs isolated from the brain tissue of PS19 mice (a specimen that develop Tau aggregation and hippocampal neurodegeneration). These EVs, which seems to be derived from microglia, were uptake by primary cultured neurons (Asai et al., 2015). It was also reported that p-Tau was increased in microglia-derived EVs isolated from brain tissue of App^NL-G-F mice. This AD mice model exhibited robust senile plaque formation suggesting a new potential mechanism that links Aβ deposition and p-Tau spreading (Clayton et al., 2021). It was also found that EVs from rTg4510 mice brain extracellular space were able to seed Tau aggregation by inducing endolysosomal permeabilization (Polanco et al., 2021). This seems to be a slow and inefficient process, which may explain the slowly progresses of Tau pathology in human patients with tauopathy (Polanco et al., 2016).

Regarding PD, α-synuclein was likewise detected in EVs derived from disease mouse brains. Administration of these preparations to wild type mice did not render in increased α-synuclein accumulation, suggesting that EVs can neutralize the toxic effect of α-synuclein (Karampetsou et al., 2020). Other study isolated EVs from the conditioned medium of rat brain slices maintained *in vitro*. Midbrain slice cultures treated with IFN-g/LPS to activate microglia, lead to increased exosomal release and decreased dopaminergic neurons, an event also observed when these EVs were directly added to neuronal cultures. These results suggest that the dopaminergic neurodegeneration that occurs in PD could be triggered by EVs released by activated microglia (Tsutsumi et al., 2019).

3.5 | Disease models treated with biofluids-derived EVs

Additional experiments that address the EVs' role in neurodegenerative disease focus on the use of cells or animals treated with EVs with exosome-like characteristics isolated from human biofluids (Table 2). Serum-derived EVs from AD cases promoted apoptosis and shortened synapses in SH-SY5Y cells, exerting negative effects that could be reversed by the co-treatment with MSC-derived EVs (Wei et al., 2020). In addition, the EVs isolated from CSF and plasma of AD cases could also impair mitochondrial function, promote excitotoxity

and destabilize Ca^{2+} homeostasis in cerebral cortical neurons (Eitan et al., 2016). Further, a recent study reported that plasma astrocyte- and neuronal-derived EVs (NDEVs) from AD cases, but not from non-demented Controls, could exert complement-mediated neurotoxicity when added to rat cortical neurons and human iPSC-derived neurons, once more supporting that EVs may play a role in the disease neurodegenerative process (Nogueras-Ortiz et al., 2020).

Concerning PD, to date, it was shown that mice microglia could uptake and be activated by human plasma-derived EVs from PD patients carrying α-synuclein, and this was easily propagated to neurons via EVs (Xia et al., 2019). In addition, EVs isolated from CSF of PD and dementia with Lewy bodies (DLB) or multiple system atrophy patients were incubated with cortical neurons and H4 cells and induced the aggregation of α-synuclein (Guo et al., 2020; Stuendl et al., 2016). Further, EVs from PD patients injected into A53T mice led to an increase of α-synuclein in several brain regions (Sheng et al., 2020). Interestingly, CSF-derived EVs from DLB and PD patients contained α-synuclein and proteins related to the autophagy-lysosome pathway. It was also shown that the inhibition of autophagy-lysosome pathway could increase the levels of exosomal α-synuclein in human neuroglioma cells and rat primary cortical neurons (Minakaki et al., 2018). On the other hand, serum-derived EVs of PD patients were reported to had a protective effect in rat cortical neurons (Tomlinson et al., 2015). Clearly EVs role either in AD or PD is not completely understood, and additional studies should be carried out to clarify the involvement of these nanovesicles in diseases pathogenesis. Figure 1 summarizes methodologies applied in EVs isolated from cellular models and brains tissue in the study of AD and PD.

4 | EVS AND CLINICAL RESEARCH ON NEURODEGENERATIVE DISORDERS

EVs content can reflect the central nervous system alterations and, thus, these nanovesicles have value not only to unravel disease mechanisms but also as sources of biomarkers either for diagnostic or therapeutic purposes. Compared with biofluids *per se*, EVs cargo is protected from degradation (e.g. by proteases), allowing the exploration of low abundant analytes that otherwise could be masked by more abundant matrix proteins.

To provide an overview on the applications of EVs with exosome-like characteristics from distinct biofluids in the field of AD and PD, an additional literature search on PubMed was carried out using the keywords scheme: (1) "(Exosomes OR Extracellular Vesicles) AND (Serum OR Plasma OR Cerebrospinal fluid OR Urine OR Saliva) AND (Isolation OR Extraction) AND Neurodegenerative Disease"; (2) "(Exosomes OR Extracellular vesicles) AND (serum OR plasma OR cerebrospinal fluid OR saliva or urine) and (isolation or extraction) AND Alzheimer"; (3) "(Exosomes OR Extracellular vesicles) AND (serum OR plasma OR cerebrospinal fluid OR saliva or urine) and (isolation or extraction) AND Parkinson". Studies written in other languages than English and review articles were excluded. Of the 180 articles

TABLE 2 Biofluid-derived EVs isolated from human as tools in the study of Alzheimer's and Parkinson's diseases

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (μL)	Size and yield	EVs Markers	References
CSF	Alzheimer's disease	Biomarker discovery—Proteomics	IA (MagCapture Exosome Isolation Kit PS)	—	TEM: cup-shaped morphology NTA: mode size 110nm and 161nm; 2×10^9 particles in 100μL of isolated EVs	—	Muraoka et al., (2020)
		Biomarker discovery—Tau	dgUC	—	—	ALIX	Saman et al., (2012)
		Biomarker discovery—miRNAs	PP (miRCURY)	1.1mL	—	—	McKeever et al., (2018)
		Biomarker discovery—miRNAs	PP (miRCURY)	300μL	—	—	Riancho et al. (2017)
		Biomarker discovery—circulating small noncoding RNAs	UC	1 mL	NTA: mode size between 84 and 112 nm	CD63, Flotillin-2 and Calnexin*	Jain et al. (2019)
		Biomarker discovery—miRNAs	UF followed by SEC	500μL (miRNA isolation); 2–5 ml (EVs characterization)	TEM: 30–100nm TRPS: mean size of 138nm; 1.4×10^{10} particles/mL	CD9, CD63, CD81, Flotillin, TSG101, Annexin V, Albumin* and ApoA1*	Sandau et al. (2022)
	Alzheimer's disease (and Frontotemporal dementia)#	Biomarker discovery—miRNAs	PP (miRCURY)	500μL	Cryo-EM: double lipid membranes NTA: mean size 111nm; C - 160nm; AD - 176nm, FTD - 168nm; yield trend for FTD > AD > C	—	Tan et al. (2021)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
CSF	Alzheimer' disease (and Frontotemporal dementia and Major depressive episode)#	Biomarker discovery—miRNAs	PP (TEI)	200 (miRNA isolation) or 400 µL (EVs characterization)	—	CD63 and Flotillin-1	Derkow et al. (2018)
	Parkinson's disease	Biomarker discovery—miRNAs	PP (miRCURY)	250 µL	—	—	dos Santos et al. (2018)
		Method—Cryo-EM analysis	UC	5 mL diluted with PBS	Cryo-EM: C – 34 to 435 nm and PD – 26 to 392 nm; intact and round shape NTA: C – 85 nm; 7×10^9 particles/mL; PD – 68 nm; 6×10^9 particles/mL; Pooled: C –77 nm; 2.1×10^{12} particles/mL; PD – 69 nm; 0.6×10^{12} particles/mL	CD9	Emelyanov et al. (2020)
	Parkinson's disease (and Dementia with Lewy bodies)#	Molecular basis—EVs from PD and DLB cases in α-synuclein aggregation	UC	0.5 mL or 2.5 mL (for WB)	TEM: 40-120 nm with cup-shaped morphology NTA: size range of EVs with exosome-like characteristics	Flotillin-2, Calnexin* and IgG*	Stuendl et al. (2016)
		Molecular basis—EVs from PD cases in autophagy-lysosome pathway and EVs from DLB cases in α-synuclein transfer	UC or PP (ExoQ)	1.4–4 mL	—	ALIX, CD63, CD81 and Flotillin-1	Minakaki et al. (2018)
	Parkinson's disease (and Multiple system atrophy) #	Molecular basis—EVs from PD and MSA cases in α-synuclein aggregation	IA with CD11b enrichment	3 mL	—	—	Guo et al. (2020)
CSF	Parkinson's disease, Parkinson's disease dementia (and Dementia with Lewy bodies)#	Biomarker discovery—Proteomics and lipidomics	SEC (qEV)	CSF: 500 µL	Immuno-TEM: presence of EVs markers TRPS: mode size 123 nm	ALIX, CD63, Flotillin 1 and TSG101	Kurzawa-Akanbi et al. (2021)
	Alzheimer's and Parkinson's disease	Biomarker discovery—miRNAs	UC	—	TEM: rounded structures; ~50–80 nm	CD63	Gui et al. (2015)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Plasma	Alzheimer's disease	Diagnostics—Biosensor for Aβ detection	PP (ExoQ) followed by GRIA2 IP enrichment	150µL	—	—	Chae et al. (2017)
		Biomarker discovery—Proteomics	PP (TEI)	200µL	TEM: round morphology and homogeneous size NTA: 40–500nm; mean size C > other groups; particle concentration AD > C	CD63, GAPDH, GFAP, L1CAM TSG101 and Calnexin*	Perrotte et al. (2020)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	250µL	TEM: ~100nm NTA: 89–95nm; 7×10^{11}–10×10^{11} particles/mL	CD81	Winston et al. (2016)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	500µL	TEM and NTA: mode size ~100nm	CD63, L1CAM, TSG101 and GM130*	Zhao et al. (2020)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by NCAM IP enrichment	500µL	TEM: presence of EVs with exosome-like characteristics	ALIX, CD81 and L1CAM	Jia et al. (2019)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	500µL	TEM: size <150nm NTA: high concentration	ALIX, HSP90, CD63, Calnexin*	Gu et al. (2020)
		Biomarker discovery and therapeutics	PP (ExoQ) followed by L1CAM IP enrichment	500µL	NTA: 175–178nm; Similar yield between groups; $\sim3\times10^{11}$ particles/mL	—	Mustapic et al. (2019)

(Continues)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Plasma	Alzheimer's disease	Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	—	—	CD81	Mullins et al. (2017)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM, GLAST or CSPG4 IP enrichment	250 µL	NTA: Similar size; yield NDEVs > ADEVs > CSPG4EVs	CD81	Goetzl et al. (2019)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by CSPG4, L1CAM or GLAST IP enrichment	250 µL	—	CD81	Goetzl, Schwartz, et al. (2018)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by GLAST IP enrichment	250 µL	—	CD81	Winston et al. (2019)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM or GLAST	250 µL	—	CD81	Goetzl, Abner, et al. (2018)
		Biomarker discovery—Proteomics	PP (TEI)	1 mL	TEM: 60–90 nm; round to oval shaped vesicles	CD81	Krishna et al. (2020)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	250 µL	NTA: typical size distribution; C—137×10^9 particles/mL, AD – 132×10^9 particles/mL	ALIX, CD9, CD81, neuron-specific enolase, ApoA1* and GM130*	Yao et al. (2021)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM	500 µL	TEM: typical size and morphology NTA: peak between 50 and 100 nm	CD81 (higher in C vs AD	Zhang, Gu, et al. (2020)
		Biomarker discovery – Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	500 µL	TEM and NTA: round (<100 nm) particles; AD>C; yield C vs AD—similar	ALIX, CD81, CD9 and GM130*	Kapogiannis et al. (2019)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	250 µL	TEM: typical morphology NTA: mode size between 100 and 150 nm; ~2×10^8 particles/mL	CD63, TSG101, Albumin*, and GM130*	Li et al. (2022)
		Biomarker discovery—miRNAs	PP (ExoQ) followed by L1CAM IP enrichment	500 µL	—	—	Cha et al. (2019)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Plasma	Alzheimer's disease	Biomarker discovery—lncRNAs	PP (ExoQ)	300 µL	SEM: spherical nanoparticles; 30–100 nm DLS: 30–150 nm (mean size 39.1 nm)	—	Fotuhi et al. (2019)
		Biomarker discovery—lncRNAs	PP (ExoQ)	500 µL	TEM: spherical vesicles; 30–150 nm NTA: mode size of 99 nm	ALIX and CD63	Wang et al. (2020a)
		Biomarker discovery—miRNAs	UC	4 mL	—	ALIX	Lugli et al. (2015)
		Biomarker discovery—miRNAs	PP (ExoQ) and IA (Exo-Flow) with L1CAM or CD81 IP enrichment	500 µL	TEM: oval shaped, 150 nm NTA: small EVs with peak at ~100 nm; 7×10^{11} particles/mL (total EVs)	CD9, CD81, L1CAM, TSG101, VPS35	Serpente et al. (2020)
		Biomarker discovery—miRNAs	Column (Norgen) followed by L1CAM IP enrichment	1 mL	NTA: peak at ~70 nm; EXOCET: ~ 2–3×10^{10} particles/mL	CD63, β-actin and Calnexin*	Durur et al. (2022)
	Alzheimer's disease (and Frontotemporal dementia)#	Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM or GLAST IP enrichment	250 µL	NTA: 7×10^{10}–3×10^{11} particles/mL	CD81	Goetzl et al. (2016a)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	250 µL	—	CD81, NCAM-1 and neuron-specific enolase	Goetzl et al. (2015a)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	250 µL	—	CD81	Goetzl et al. (2016b)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	500 µL	—	CD81 and HSC70	Goetzl et al. (2015b)
		Biomarker discovery—Proteomics	PP (ExoQ)	250 µL	NTA: ~100 nm of diameter; yield 2.7×10^{11}–3.4×10^{11} particles/mL	CD9, HSC70 and TSG101	Chanteloup et al. (2019)

(Continues)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Plasma	Alzheimer's disease (and Frontotemporal dementia)#	Molecular basis - NDEVs and ADEVs in complement-mediated neurotoxicity	PP (ExoQ) followed by L1CAM, GLAST or CD81 IP enrichment	500µL	TEM and NTA: 50–250nm; mode size of 100nm; 1×10^9–1×10^{10} particles/mL of plasma	ALIX, CD63, CD9, CD81, ApoA1* and GM130*	Nogueras-Ortiz et al. (2020)
	Alzheimer's disease (and Frontotemporal dementia and Type 2 diabetes mellitus)#	Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	500µL	—	CD81	Kapogiannis et al. (2015)
	Alzheimer's disease (and Dementia with Lewy bodies)#	Biomarker discovery—miRNAs	SEC (Sepharose-CL2B)	2 mL	Cryo-EM: vesicles with the expected size and morphology NTA: Similar size or concentration between groups	CD5L, CD9, CD81 and CD63	Gámez-Valero et al. (2019)
	Alzheimer's disease (and vascular dementia)#	Biomarker discovery—Proteomics and miRNA	PP (TEI)	1 mL	TEM and NTA: expected size range	ALIX, CD63, CD81, Calnexin*	Li, Xie, et al. (2020)
	Alzheimer's disease (and small cerebral vascular disease)#	Biomarker discovery—Proteomics	PP (ExoQ) followed by CD31 IP enrichment	250µL	NTA: 96–116nm; 7×10^9–8×10^9 particles/mL	CD81	Abner et al. (2020)
	Parkinson's disease	Biomarker discovery—Proteomics	UC	1 mL	TEM: 40–120nm membrane-bound vesicle NTA: 50–300nm	ALIX, TSG101, Albumin*, HDL* and LDL*	Cerri et al. (2018)
		Biomarker discovery—Proteomics	Immunocapture (beads)	—	—	—	Wang et al. (2018)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP	800µL	TEM: presence of EVs with exosome-like characteristics	—	Zhao et al. (2019)
		Biomarker discovery Proteomics	Immunocapture (beads)	—	TEM: cup-shaped structures; 50–150nm TRPS: 114nm; ~3.5×10^8 particles/mL	CD63 and L1CAM	Niu et al. (2020)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Plasma	Parkinson's disease	Biomarker discovery—Proteomics	Immunocapture (beads)	>300µL	TEM: presence of EVs with exosome-like characteristics	ALIX and L1CAM	Shi et al. (2014)
		Biomarker discovery—Proteomics	PP (TEI) dUC	dUC: 1.5 mL	TEM: cup-shaped vesicles with a diameter of 30–150nm NTA: peak of 137 nm and 113nm; dUC – 5.0×10^9; TEI – 3.8×10^{11} particles/mL	L1CAM	Zheng et al. (2021)
		Biomarker discovery—Proteomics	IA with enrichment for CD235a	300µL	Cryo-TEM: typical morphology NTA: peak <200nm; 2×10^7 particles/mL	CD63, IgG*	Sheng et al. (2020)
		Biomarker discovery—Proteomics	UC	1 mL	TEM: presence of exosomes NTA: ~140nm	ALIX and GM130*	Shim et al. (2021)
		Biomarker discovery—Proteomics	MA (exoEasy Maxi Kit)	1 mL	NTA: peak at 100 nm	CD9, CD63, CD81, TSG101 and Cytochrome C*	Chung et al. (2021)
		Biomarker discovery—Proteomics	MA (exoEasy Maxi Kit)	1 mL	NTA: peak at 100 nm	CD9, CD63, CD81, TSG101, Cytochrome C*	Chan et al. (2021)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP	250µL	TEM and NTA: 30 to 120nm; $\sim5\times10^{12}$ particles/mL	CD63, HSP70, L1CAM, COX4*	Chou et al. (2020)
		Biomarker discovery—Proteomics	Immunocapture (beads)	250µL	TEM: round-shaped intact vesicles NTA: Average of 97 or 115nm	—	Anastasi et al. (2021)
		Biomarker discovery—Proteomics	MA (exoEasy Maxi Kit)	1 mL	NTA: ~100nm	CD9, CD63, CD81, HSC70, TSG101 and Cytochrome C*	Chung, Chan, et al. (2020)

(Continues)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Plasma	Parkinson's disease	Biomarker discovery—Proteomics	MA (exoEasy Maxi Kit)	1 mL	NTA: 35–150 nm	CD9, CD63, HSC70 and TSG101	Chung, Huang, et al. (2020)
		Biomarker discovery—Proteomics	SEC on Drip Column	200 µL	TEM: ~100 nm and binding to CD9 immunogold staining	CD9 and CD81	Kitamura et al. (2018)
		Biomarker discovery—Proteomics	PP (ExoQ)	300 µL	TEM: presence of exosomes	CD63 and HSC70	Leng et al. (2020)
		Biomarker discovery—proteomics and lncRNAs	Immunocapture (beads)	>300 µL	TEM: 40–120 nm membrane-bound vesicles NTA: 50–300 nm	CD9, CD63, L1CAM and TSG101	Zou et al. (2020)
		Biomarker discovery—miRNAs	MA (exoEasy)	—	TEM: round shape NTA: 86 nm; 3.4×10^6 particles/mL	CD9, HSC70 and TSG101	Cai et al. (2021)
		Biomarker discovery—miRNAs	PureExo Exosome Isolation Kit or PP (ExoQ)	500 µL	TEM: 30–100 nm, round shaped or oval-shaped	CD9, CD63 and TSG101	Yao et al. (2018)
		Biomarker discovery—miRNAs	MA (exoEasy)	3 mL	TEM: Typical size and morphology DLS: Peak at ~90 nm	CD63	Xie et al. (2022)
		Biomarker discovery—lncRNAs	UC	—	TEM: small cup-shaped membrane vesicles NTA: small EVs	CD63, Flotillin 1, TSG101 and Calnexin*	Wang et al. (2020b)
		Molecular basis—Effect of EVs from PD patients uptaken by microglia in its' activation	UC	—	TEM: 40–100 nm diameters and cup-shaped morphology	CD63, TSG101 and Calnexin*	Xia et al. (2019)
	Parkinson's disease (and Multiple system atrophy, atypical parkinsonism with tauopathies)#	Biomarker discovery—Proteomics	IA (MACSPlex)	60 µL	NTA: size mainly between 30–150 nm; $>1 \times 10^{12}$ particles/mL (PD > others)	Alix, CD9, CD63, CD81, TSG101, ApoA1* and GRP94*	Vacchi et al. (2020)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (μL)	Size and yield	EVs Markers	References
Plasma	Parkinson's disease (and Multiple system atrophy, atypical parkinsonism with tauopathies)[#]	Biomarker discovery—Proteomics	Anti-CD81-immobilized ELISA plates	40 μL	AFM: round or elliptic protrusions of various sizes NTA: 100–400 nm	CD81	Ohmichi et al. (2019)
	Alzheimer's and Parkinson's disease	Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP enrichment	>300 μL	NTA: Presence of EVs with exosome like-characteristics	—	Shi et al. (2016)
		Biomarker discovery—miRNAs	MA (ExoRNeasy) PP (ExoQ)	ExoRNeasy: 1 mL; ExoQ: 500 μL	—	—	Nie et al. (2020)
	Alzheimer's and Parkinson's disease (and Amyotrophic lateral sclerosis, Frontotemporal dementia)[#]	Biomarker discovery—miRNAs	Small EVs: UC Large EVs: 20000 g centrifugation	—	TEM, NTA: Large EVs > Small EVs (size)	Large EVs: Annexin V Small EVs: ALIX	Sproviero et al. (2021)
		Biomarker discovery—miRNAs	Small EVs: UC Large EVs: 20000 g centrifugation	—	TEM, NTA: Large EVs—diameter ~180 nm to 220 nm; ~1 × 10¹¹ particles/μL); Small EVs – diameter ~140 to 160 nm; > 2 × 10¹¹ particles/μL	Large EVs: Annexin V Small EVs: ALIX	Sproviero et al. (2022)
Serum	Alzheimer's disease	Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP	500 μL	—	ALIX, L1CAM and TSG101	Agliardi et al. (2019)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP	500 μL	TEM: circular structures within 50–150 nm size NTA: 83–159 nm; 1.4 × 10⁹ – 4.3 × 10⁹ particles/mL	CD63 and L1CAM	Nam et al. (2020)
		Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM IP	—	NTA: intact EVs	ALIX	Eren et al. (2020)
		Biomarker discovery—Proteomics	PP (ExoQ)	250 μL	NTA: 125 nm; 3 × 10¹¹–5 × 10¹¹ particles/mL	HSC70, TSG101 and Calnexin*	Soares Martins et al. (2021)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Serum	Alzheimer's disease	Biomarker discovery—Proteomics	PP (ExoQ) or Column (ExoS)	250 µL	TEM: expected size and morphology NTA: expected size, ~120 nm; ExoQ – C −3.6×10^{11} to AD −3.3×10^{11}; ExoS C −6.3×10^{11} to AD −4.4×10^{11} particles/mL	CD63, HSC70, RAB11 and Calnexin*	Soares Martins et al. (2022)
		Biomarker discovery—Proteomics	PP (TEI)	—	TEM: cup-shaped morphology and size within 80–100 nm NTA: 50–150 nm	TSG101, Calnexin*	Haddad et al. (2019)
		Method—Isolation method development and Biomarker discovery—Proteomics	CaTiO$_3$/Al^{3+}/Pr^{3+}/Sm^{3+} nanocomposite	100 µL	TEM and NTA: vesicles within 30 to 200 nm	CD63, CD81, TSG101 and Calnexin*	Wu et al. (2021)
		Biomarker discovery—miRNAs	Plasma/Serum Exosomal RNA Isolation Kit	1 mL	—	—	Cheng et al. (2015)
		Biomarker discovery—miRNAs	PP (Exosome Isolation Q3 kit)	200 µL	TEM: vesicles with expected size and morphology NTA: 120 nm peak	CD9 and CD63	Dong et al. (2021)
		Biomarker discovery—miRNAs	SEC (qEV)	1 mL	TEM, NTA, DLS: round shape, 30–150 nm	ALIX, CD9 and Calnexin*	Song et al. (2022)
		Biomarker discovery—miRNAs	PP (ExoQ)	—	—	—	Ding et al. (2022)
		Biomarker discovery—miRNAs and circular RNAs	MA (exoEasy Maxi Kit)	—	TEM: typical morphology NTA: 30 to 200 nm	CD63, CD81, TSG101	Meng et al. (2022)
		Biomarker discovery—Spectroscopic profile	PP (ExoQ)	150 µL	TEM: typical size range and round shape NTA: 30–150 nm; 3×10^{11}–5×10^{11} particles/mL	—	Soares Martins et al. (2020)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Serum	Alzheimer's disease	Molecular basis—Effect of EVs from AD cases induce apoptosis in AD cellular model	UC	—	—	CD63 and CD81	Wei et al. (2020)
		Biomarker discovery—miRNAs	Spin column chromatography (Norgen kit)	1 mL of serum	—	—	Cheng et al. (2020)
	Alzheimer's disease (and Vascular dementia)#	Biomarker discovery—miRNAs	PP (ExoQ)	—	TEM: presence of exosomes NTA: mode 100nm	CD63 and CD81	Wei et al. (2018)
	Parkinson's disease	Biomarker discovery—Proteomics	UC	—	—	CD9, CD63, CD81, Flotillin and HNRNPA1*	Picca et al. (2020)
		Biomarker discovery—Proteomics	UC	—	TEM: presence of small EVs	CD63, CD9 and CD81	Picca et al. (2019)
		Biomarker discovery—Proteomics	UC	5 mL per group	—	-	Jiang et al. (2019)
		Biomarker discovery—miRNAs	UC	9 mL	NTA: 160–210nm	CD63 and Flotillin	Ruf et al. (2021)
		Biomarker discovery—miRNAs	PP (TEI)	300µL	—	CD63	Cao et al. (2017)
		Therapeutics—EVs as outcome measurement upon exenatide treatment	PP (ExoQ) followed by L1CAM IP enrichment	—	—	—	Athauda et al. (2019)
		Therapeutics—EVs as outcome measurement upon pramipexole treatment	PP (ExoQ)	500µL	TEM: Exosome-like structures of uniform size; ~50nm	CD9 and CD63	Luo et al. (2016)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
Serum	Parkinson's disease	Biomarker discovery—Proteome profile and Molecular basis—Effect of human EVs incubation in rat cortical neurons[#]	UC	7 mL for proteomic profile, or 1 mL for cell-based assays	TEM: Membrane-bound vesicles NTA: mean size of 120 nm; $3–4\times10^{10}$ particles/mL	Flotillin and TSG101	Tomlinson et al. (2015)
	Parkinson's disease and atypical parkinsonism (and Multiple system atrophy, Dementia with Lewy bodies and Frontotemporal dementia)[#]	Biomarker discovery—Proteomics	Immunocapture (beads)	500µL	SEM: presence of exosomes NTA: $\sim4\times10^{11}$ particles/mL	CD81, L1CAM, Syntenin-1 and TSG101	Jiang et al. (2020)
	Parkinson's disease (and Multiple system atrophy, Progressive supranuclear palsy and Corticobasal syndrome)[#]	Biomarker discovery—Proteomics	Immunocapture (beads)	250µL	—	—	Jiang et al. (2021)
	Parkinson's disease (and Progressive supranuclear palsy)[#]	Biomarker discovery—miRNAs	PP (ExoQ)	—	TRPS: <150 nm	—	Manna et al. (2021)
	Alzheimer's and Parkinson's diseases (and Vascular dementia)[#]	Biomarker discovery—miRNAs	PP (TEI)	400µL	NTA: 30–120 nm	CD9 and CD63	Yang, Liu, et al. (2018)
		Biomarker discovery—miRNAs	PP (ExoQ)	400µL	—	—	Barbagallo et al. (2019)
Saliva	Parkinson's disease	Biomarker discovery—Proteomics	XYCQ EV Enrichment KIT	1 mL	TEM: Exosome-like morphology NTA: 30–400 nm; peak 119 nm	ALIX and CD9	Cao et al. (2019)
	Parkinson's disease (and Multiple system atrophy)[#]	Biomarker discovery—Proteomics	XYCQ EV Enrichment KIT	—	—	—	Cao et al. (2020)
Urine	Parkinson's disease	Biomarker discovery—Proteomics	Microfiltration	15 mL of urine	—	TSG101	Ho et al. (2014)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (µL)	Size and yield	EVs Markers	References
CSF and Plasma	Alzheimer's disease	Molecular basis—Toxicity induced by EVs from AD cases	UC	500 µL	NTA: CSF – C, MCI, AD – mean 1×10^8 – 2×10^8; Plasma – AD mean 2×10^{11} – 4×10^{11} particles/mL	ALIX and Flotillin-1	Eitan et al. (2016)
		Biomarker discovery—Proteomics	CSF: UC Plasma: PP (ExoQ) followed by L1CAM IP enrichment	CSF: 1 mL Plasma: 500 µL	NTA: Plasma – 65–305 nm; ~5×10^{10} to 2×10^{11} vesicles/mL	Plasma: CD81 and TSG101	Guix et al. (2018)
CSF and Serum	Alzheimer's disease	Biomarker discovery—miRNAs	PP (TEI)	400 µL	—	CD9	Liu et al. (2021)
CSF, Plasma and Serum	Alzheimer's disease	Biomarker discovery—miRNAs	PP (TEI)	Serum: 400 µL	—	—	Liu et al. (2014)
CSF and Serum	Parkinson's disease	Biomarker discovery—miRNAs	PP (ExoQ)	—	—	CD63	Tong et al. (2022)
CSF and Plasma	Parkinson's disease	Biomarker discovery—Proteomics	CSF: UC Plasma: 20000 g	CSF: 500 µL Plasma: -	NTA: CSF – diameter and concentration in PD > C; Plasma EVs (20.8×10^{11} particles/mL)> CSF EVs (0.07×10^{11} particles/mL); Plasma EVs diameter (171 nm) < CSF EVs (221 nm)	CD9, CD63, CD81	Vacchi et al. (2021)
Plasma and Serum	Parkinson's disease	Biomarker discovery—Proteomics	PP (ExoQ) followed by L1CAM or MOG IP enrichment	250 µL	ELISA: ~$3.1–4.4\times10^{10}$ particles/mL	CD9 and CD81	Dutta et al. (2021)
Urine and CSF	Parkinson's disease	Biomarker discovery—Proteomics	dUC	—	Cryo-EM: Spherical vesicles	ALIX, CD9 and TSG101	Fraser et al. (2013)
Urine, CSF and Serum	Parkinson's disease	Biomarker discovery—Proteomics	dUC	—	Cryo-EM (CSF): typical round morphology; NTA (CSF): ~125 nm	ALIX and Flotillin-1	Wang et al. (2017)

TABLE 2 (Continued)

Biofluids/tissue	Disease	Research topic	EVs isolation methods	Starting volume (μL)	Size and yield	EVs Markers	References
Plasma and Serum	Alzheimer's and Parkinson's diseases (and ischemic cerebrovascular disease)#	Biomarker discovery—Proteomics	PP followed by filtration	200μL	TEM: Typical morphology NTA: Plasma – peak at 138nm; Serum – peak at 127 nm	—	Shan et al. (2022)

Abbreviations: AD, Alzheimer's disease; ADEVs, astrocyte-derived extracellular vesicles; AFM, atomic force microscopy; C, Controls; Cryo-EM, cryogenic electron microscopy; CSF, Cerebrospinal fluid; CSPG4EVs, Chondroitin sulfate proteoglycan 4 type neural precursor cells-derived extracellular vesicles; dg, density gradient; DLS, dynamic light scattering; dUC, differential ultracentrifugation; ELISA, enzyme-linked immunosorbent assay; EV, extracellular vesicle; ExoQ, ExoQuick; ExoS, Exo-spin; FTD, frontotemporal dementia; HDL, high-density lipoproteins; IA, immunoaffinity; IP, immunoprecipitation; LDL, low-density lipoproteins; lncRNA, long non-coding RNA; MA, membrane affinity; MCI, mild cognitive impairment; miRNA, microRNA; NDEVs, neuronal-derived extracellular vesicles; NTA, nanoparticle tracking analysis; PD, Parkinson's disease; PP, precipitation; SEC, size exclusion chromatography; SEM, scanning electron microscopy; TEI, total exosome isolation kit; TEM, transmission electron microscopy; TRPS, tunable resistive pulse sensing; UC, ultracentrifugation; WB, western blot.

*Negative or contamination EVs markers.

In this review, emphasis was given to AD and PD but these articles also present data for other pathologies as indicated.

found, a total of 118 human-derived EVs studies were included. In this review, main focus was given to AD and PD (Table 2, Figure 2).

4.1 | CSF

In particular, the finding of EVs with exosome-like characteristics in CSF (Street et al., 2012) that could carry Tau species (Guix et al., 2018; Saman et al., 2012) encouraged the characterization of EVs proteome for additional biomarker discovery in the field of AD. CSF-derived EVs, isolated through UC, from a moderate AD case were enriched in ALIX and, particularly, in low molecular mass Tau species (Chiasserini et al., 2014). Other disease specific proteins, as the APP involved in AD pathology and the prion protein and DJ-1, involved in PD pathology (Chiasserini et al., 2014) were also found in EVs, supporting the relevance of CSF-derived EVs as an attractive research tool in the neurodegenerative field. Relatively to AD, studies focused on both proteomics approaches and microRNA profiling. CSF-derived EVs from Controls, mild cognitive impairment (MCI) and AD cases were characterized through label-free and tandem mass tag-labelled mass spectrometry. Significantly different levels of three proteins, HSPA1A, NPEPPS and PTGFRN, were reported. These were increased in AD when compared with MCI, possibly reflecting MCI to AD progression (Muraoka et al., 2020). Altered miRNAs levels in EVs isolated from CSF were also found in early and late disease stages when compared with Controls, PD or other dementias (Derkow et al., 2018; Gui et al., 2015; McKeever et al., 2018; Riancho et al., 2017; Sandau et al., 2022; Tan et al., 2021), as well as a combination of circulating small noncoding RNAs (Jain et al., 2019), both holding biomarker interest. Interestingly, changes in miRNA cargo according to gender and ApoE4 genotype were reported for CSF-derived EVs isolated from AD cases (Sandau et al., 2022).

For PD, EVs were applied in the miRNA biomarker discovery area (dos Santos et al., 2018; Gui et al., 2015) (Table 2). Cryo-EM analysis was also employed in Controls and PD cases, revealing the presence of vesicles with distinct sizes and morphology that may represent distinct vesicle subpopulations in both groups (Emelyanov et al., 2020). Moreover, in a clinical perspective, α-synuclein exosomal levels were significantly different between PD and DLB cases (Stuendl et al., 2016) or between PD cases and MSA versus Controls (Guo et al., 2020). Another study identified α-synuclein in brain-derived EVs and increased levels of ceramide in CSF-derived EVs of PD, PD dementia and DLB (Kurzawa-Akanbi et al., 2021).

4.2 | Plasma

Plasma was the most common used biofluid in studies focusing on EVs and biomarker discovery for AD and PD (Table 2). The biomarker potential of several exosome candidates was assessed in total and in neuronal- astrocyte- or endothelial-enriched exosomes, isolated using PP-based methods from small plasma volumes of AD, PD or other dementia types. The targets tested included Aβ, APP, sAPP
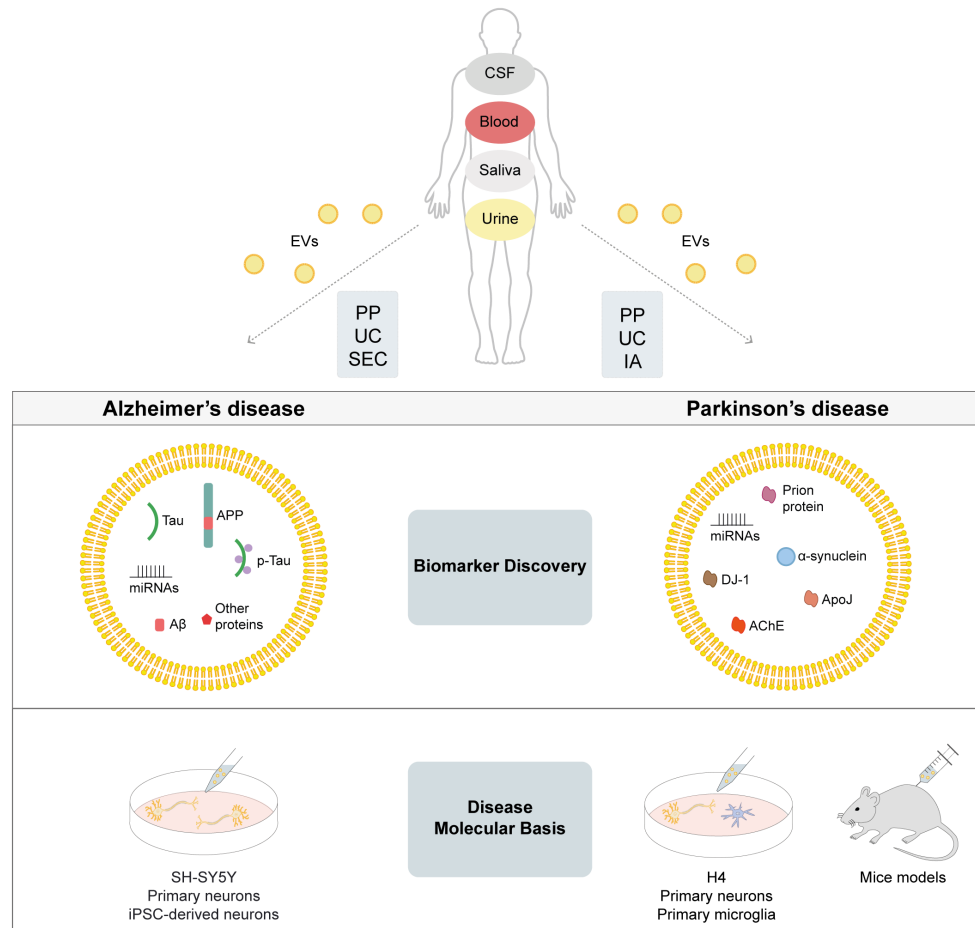
FIGURE 2 Biofluid-derived EVs in the study of Alzheimer's and Parkinson's diseases. EVs were isolated from distinct biofluids (CSF, blood, saliva, urine) of Controls and Alzheimer´s or Parkinson´s disease cases. The most used methodologies were precipitation-based protocols, ultracentrifugation, size exclusion chromatography or immunoaffinity. Biofluid derived-EVs were mainly used for biomarker discovery, in a diagnostic perspective. However, some studies also aimed to unravel molecular mechanisms underlying disease, by biofluids-derived EVs incubation with distinct cellular models or injection into mice. Abbreviations: Aβ, amyloid beta; AChE, acetylcholinesterase; ApoJ, apolipoprotein J; APP, amyloid precursor protein; CSF, cerebrospinal fluid; dgUC, density gradient ultracentrifugation; EVs, extracellular vesicles; IA, immunoaffinity; iPSC, induced pluripotent stem cells; PP, precipitation-based methods; p-Tau, phosphorylated Tau, SEC, size exclusion chromatography; UC, ultracentrifugation.

and Tau species (Total-Tau, p-Tau 181, p-Tau 396, p-Tau 231) (Abner et al., 2020; Chae et al., 2017; Goetzl et al., 2016a; Gu et al., 2020; Guix et al., 2018; Jia et al., 2019; Kapogiannis et al., 2019; Li et al., 2022; Li, Xie, et al., 2020; Perrotte et al., 2020; Shi et al., 2016; Winston et al., 2016; Zhao et al., 2020) to insulin resistance markers (Kapogiannis et al., 2015; Mullins et al., 2017; Mustapic et al., 2019), cell survival and neurotrophic growth factors (Goetzl et al., 2015a; Goetzl et al., 2019), complement effector and regulatory proteins (Goetzl, Schwartz, et al., 2018; Winston et al., 2019), synaptic and lysosomal proteins (Goetzl, Abner, et al., 2018; Goetzl et al., 2015b; Goetzl et al., 2016b; Krishna et al., 2020), cerebrovascular biomarkers (Abner et al., 2020), electron transport chain complexes (Yao et al., 2021), among others (Chanteloup et al., 2019; Zhang, Gu, et al., 2020). In particular, several studies reported increased Aβ levels in NDEVs of AD cases when compared with Controls (Goetzl et al., 2016a; Gu et al., 2020; Jia et al., 2019; Winston et al., 2016; Zhao et al., 2020). Consistently, in a recent study the levels of

$A\beta_{1\text{-}42}$ in NDEVs measured by SIMOA were gradually increased from Control to MCI to AD group (Li et al., 2022). However, distinct results were reported as $A\beta_{1\text{-}42}$ levels in NDEVs evaluated by the same methodology showed no differences between disease and Control groups (Kapogiannis et al., 2019). Differences in AD were also found in NDEVs for T-Tau, p-Tau 396, p-Tau 181 and p-Tau 231 (Goetzl et al., 2016a; Gu et al., 2020; Jia et al., 2019; Kapogiannis et al., 2019; Winston et al., 2016). Nonetheless, the nature of the NDEVs preparations obtained after isolation with ExoQ followed by immunoprecipitation-enrichment using anti-L1CAM antibodies are being focus of debate. This is related with the fact that L1CAM can also be expressed in other tissues beyond brain (e.g. kidneys) and can exist both in transmembrane and soluble forms (Angiolini et al., 2019; Maretzky et al., 2005; Mechtersheimer et al., 2001; Moos et al., 1988). Antibodies used in immunoprecipitation step commonly recognize the L1CAM ectodomain, which exists in both transmembrane-bound EVs and soluble forms. A recent study

showed that most of L1CAM is present in its soluble form in EVs preparations, obtained through SEC and dgUC, from either plasma or CSF (Norman et al., 2021). Considering the biomarker value of these data, it is thus important to characterize the exact nature of these NDEVs preparations immunoprecipitated with LICAM or NCAM and also of astrocyte-enriched EVs (ADEVs). Further studies should focus on the identification of specific brain markers for enrichment in NDEVs preparations which would certainly prompt the EVs research in the neurodegenerative diseases field.

A set of plasma EVs miRNAs and long-non coding RNAs were likewise assessed and hold biomarker potential for AD discrimination from Controls but also from mild cognitive impairment and other dementia types (Cha et al., 2019; Durur et al., 2022; Fotuhi et al., 2019; Gámez-Valero et al., 2019; Li, Xie, et al., 2020; Lugli et al., 2015; Nie et al., 2020; Serpente et al., 2020; Wang et al., 2020a). In these studies, EVs with exosome-like characteristics were isolated from slightly higher samples volumes when compared to the proteomic studies and through PP, MA and SEC methodologies. The reproducibility of small RNA sequencing analysis upon EVs isolation with ExoQ and exoRNeasy was compared, and miRNA expression profiles obtained for ExoQ were more similar to plasma miRNA profiles and presented higher miRNA expression levels, than those obtained for exoRNeasy (Nie et al., 2020). Moreover, in this study, 8 significantly different expressed miRNAs (miR-320e, miR-548k, miR-548ap-3p, let-7e-5p, miR-423-5p, miR378d, miR193b-5p, miR-548ad-5p) were found between AD and PD.

For PD, plasma-derived EVs were isolated through a wider range of methods, as UC, SEC, PP- and immune-based methodologies. Biomarker discovery was again the main purpose of these studies (Table 2). The levels of α-synuclein in plasma-derived EVs were significantly increased in PD cases when compared with controls and could represent a prognostic marker for the disease progression (Cerri et al., 2018; Niu et al., 2020; Sheng et al., 2020; Shi et al., 2014; Wang et al., 2018; Xia et al., 2019; Zhao et al., 2019; Zheng et al., 2021; Zou et al., 2020). However, in other studies a decrease (Chung et al., 2021) or no significant differences (Shim et al., 2021) were found in plasma-derived exosomal α-synuclein levels in controls and PD cases, although lower exosomal acetylcholinesterase (AChE) activity could be detected in PD, possibly reflecting the disease cholinergic system dysfunction (Shim et al., 2021).

The levels of other plasma exosomal proteins, as the prion protein, apolipoprotein J (APOJ), ApoA1, CR1 subcomponent, cytokines, insulin receptor substrate 1 and proteasome complex proteins and several immunologic-associated EVs surface markers, were also capable to distinguish PD cases from controls, while no differences were found for neurofilament light chain or BDNF levels (Anastasi et al., 2021; Chan et al., 2021; Chou et al., 2020; Chung, Chan, et al., 2020; Chung, Huang, et al., 2020; Kitamura et al., 2018; Leng et al., 2020; Vacchi et al., 2020; Vacchi et al., 2021). It was likewise reported that the levels of plasma NDEVs were increased in PD when compared with Controls (Ohmichi et al., 2019).

In addition, several exosome miRNA and lncRNA species were differentially expressed in PD (Cai et al., 2021; Wang et al., 2020b; Xie et al., 2022; Yao et al., 2018). Among these, 35 up-regulated and 72 down-regulated mainly involved in MAPK-PI3K signaling pathway

(Cai et al., 2021) and plasma miRNAs targeting genes, mainly associated with dopaminergic synapse, regulation of neurogenesis or neuron project guidance were also identified as candidate biomarkers for PD. These miRNAs were miR-30c-2-3p (increased in PD), miR-15b-5p, miR-138-5p, miR-106b-3p, miR-338-3p and miR-431-5p (decreased in PD) (Xie et al., 2022).

Moreover, a study compared miRNA profiles in large (130–1000 nm) and small EVs (30–130 nm) isolated from plasma of AD, PD, Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) cases and identified common small RNAs between the two types of vesicles and between neurodegenerative diseases. For example, in AD, 6 common miRNAs were found in both small and large EVs, 33 miRNAs were only found in small EVs and 13 miRNAs in large EVs. Small and large EVs of AD individuals were the less enriched in miRNA species when compared with those secreted in the context of PD, FTD and ALS and, for the four neurodegenerative diseases, miRNA cargo of small EVs presented a higher discriminatory potential than the miRNA cargo of large EVs (Sproviero et al., 2021). Further, comparison of mRNA species found in small and large EVs from AD, PD but also ALS and FTD cases, showed that small EVs were more enriched in mRNA species and that different mRNAs patterns were found between the two vesicles types isolated from all disease cases. No differently expressed mRNA species were found in small EVs from AD however mRNA species were changed in PD, ALS and FTD (Sproviero et al., 2022). In sum, in the same disease, both miRNA and mRNA cargo can differ between small and large EVs and this should also be considered in the search for novel biomarkers.

In the context of AD, plasma-derived EVs were also isolated from disease mice models (Supplementary Table S1). The administration of hAPP-J20 mice plasma derived-EVs to an AD mouse model caused accumulation of EVs around Aβ plaques and activated nearby microglia (Zheng et al., 2017). Using distinct AD mice models, a study observed that plasma-derived NDEVs of 3xTg-AD mice presented higher Total-Tau and P-Tau 181 levels than wild type mice NDEVs, plasma-derived NDEVs of 5xFAD mice had higher levels of $A\beta_{1-42}$ and ADEVs of 3xTg-AD mice had higher levels of C1q than wild-type. The EVs levels of these targets were all moderate to strongly correlated with their values in mice AD brains, reflecting an association between brain pathology and EVs cargo (Delgado-peraza et al., 2021). Others observed increased $A\beta_{1-42/1-40}$ and lower levels of $A\beta_{1-42}$ and $A\beta_{1-40}$ in plasma-derived EVs of two transgenic mice models, APP/PS1 double-mutant and 3xTgAD mice, when compared with wild-type. Of note, higher amounts of $A\beta_{1-42/1-40}$ could be detected in plasma-derived EVs than plasma-depleted EVs isolated from AD transgenic model, which may be an advantage for further biomarker discovery-related studies (Eitan et al., 2016).

4.3 | Serum

Serum-derived EVs begun to be explored more recently as sources of biomarkers for neurodegenerative diseases. Its isolation was in general performed from small sample volumes and, mainly through PP-based methods (Table 2).

In the context of AD, three studies aimed to identify proteomic biomarkers using NDEVs. The synaptic protein SNAP-25 was found decreased in serum-derived neuronal EVs of AD cases when compared with Controls, reflecting the loss of synaptic integrity (Agliardi et al., 2019). Further, exosomal levels of Total-Tau and p-Tau S202 were significantly higher in AD cases when compared to Controls or MCI group (Nam et al., 2020). However, age-related differences for Tau species (Total-Tau, p-Tau 181 and p-Tau 231) in serum-derived EVs were reported; with a tendency for higher values of these species associated with age-associated cognitive decline when compared with cognitively stable individuals (Eren et al., 2020). In addition, novel exosomal biomarker candidates were found through a bioinformatic analysis for AD. Exosomal proteomes from three databases (ExoCarta, Evpedia and Vesiclepedia) were overlapped with a list of exosomal proteins already associated with AD and a total of 42 Aβ-binding proteins were identified. The levels of gelsolin, one of the candidates, decreased in serum-derived EVs with exosome-like characteristics from AD cases of two distinct cohorts when compared with Controls (Soares Martins et al., 2021). In addition, a mass spectrometry analysis of exosome proteomes obtained from Controls and AD cases revealed nine significantly different abundant proteins, among them two other Aβ-binding proteins (AACT and C4BPα). While AACT levels increased, C4BPα levels decreased in EVs of ADs and these protein levels were validated in two distinct cohorts (Soares Martins et al., 2022). Other mass spectrometry-based study revealed two peaks (CXCL12 and CXCL4) that were significantly highly expressed in serum- and plasma-derived EVs isolated from AD and PD patients than Controls (Shan et al., 2022). Another study focused on the development of a nanocomposite that allow highly selective EVs isolation and further identification of proteins with distinct expression patterns in AD, namely serum amyloid protein (Wu et al., 2021).

Several studies have also identified distinct miRNAs altered in serum-derived EVs of AD cases and other dementias (Barbagallo et al., 2019; Cheng et al., 2015; Ding et al., 2022; Dong et al., 2021; Liu et al., 2014; Liu et al., 2021; Meng et al., 2022; Song et al., 2022; Wei et al., 2018; Yang, Liu, et al., 2018). Among these, miR-193b was decreased in serum-, plasma- and CSF-derived EVs of AD cases and it was predicted to target the 3'-untranslated region (UTR) of APP, repressing APP protein expression (Liu et al., 2014; Yang, Liu, et al., 2018). An additional bioinformatic analysis revealed that both miR-193b and miR-125b-5p were putative targets common to both fluids in AD cases (Soares Martins et al., 2021). Interestingly, 45 miRNAs found in serum-derived EVs were also identified in brain-derived EVs (Cheng et al., 2020).

In an innovative approach, Fourier-transformed infrared spectroscopy was applied to serum and serum-derived EVs of Controls and AD cases of two independent cohorts. Spectroscopic profiles were acquired and analyzed through multivariate analysis. Considering the nucleic acids and carbohydrates spectral region, serum-derived EVs preparations enriched in exosomes showed an higher disease discriminatory power than serum, enhancing their value as potential diagnostic tools (Soares Martins et al., 2020).

Serum-derived EVs were likewise used in biomarker identification for PD. A mass spectrometry analysis found 469 exosomal proteins and, among them, 14 proteins with significant different patterns between Controls and PD, as for example apolipoprotein D (ApoD), ApoJ and Complement C1q (Jiang et al., 2019). In addition, exosomal levels of α-synuclein, ApoJ and a panel of mitochondrial and inflammatory molecules were able to distinguish PD from other dementias or synucleinopathies and Controls (Dutta et al., 2021; Jiang et al., 2020; Jiang et al., 2021; Picca et al., 2019; Picca et al., 2020).

Serum-derived EVs miRNAs of Controls and PD were also analysed and miR-19b was found decreased in PD whereas miR-195 and miR-24 levels were increased in PD cases (Cao et al., 2017). Interestingly, miR-24 and other four miRNA species (miR-151a-5p, miR-485-5p, miR-331-5p, and miR-214) were significantly increased in serum- and CSF-derived EVs isolated from PD cases (Tong et al., 2022). In addition, exosomal miR-21-3p, miR-22-3p and miR-223-5p were significantly different between Controls from PD cases and miR-425-5p, miR-21-3p, and miR-199a-5p distinguished between PD cases and progressive supranuclear palsy which are often misdiagnosed (Manna et al., 2021). However, a recent study found altered miRNA patterns between Control and PD cases, with the highest discriminatory value obtained in serum when compared to serum-derived EVs (Ruf et al., 2021).

In a therapeutic perspective, two studies employed human serum-derived EVs to monitor the treatment effects of exenatide, a drug to increase motor function, or pramixole, a dopamine agonist. Increased levels of tyrosine phosphorylated insulin receptor substrate 1 were found in serum-derived EVs of patients after the treatment with exenatide, reflecting increases in brain insulin signaling (Athauda et al., 2019). Insulin resistance plays a role in PD pathogenesis, accelerating the development of the disease (Yang, Wang, et al., 2018). The treatment with pramixole decreased exosomal α-synuclein levels, thus exerting protective effects (Luo et al., 2016) (Table 2).

Like for plasma, EVs were also isolated from serum of AD and PD disease mouse models (Table S1). In a biomarker discovery perspective, various miRNA species have been evaluated in mice biofluids-derived EVs. The miR-185-5p levels were significantly lower expressed in serum-derived EVs from APP/PS1 mice than in Controls. This miRNA pattern is particularly interesting since it is a regulator of APP expression (Ding et al., 2022). In another study, the administration of plant glucosylceramide, a major sphingolipid, to mice lead to increased levels of L1CAM, NCAM and Aβ in serum-derived EVs of the APP mice, suggesting that its administration may increase EVs production to alleviate Aβ pathology (Yuyama et al., 2019). In PD context, the treatment of α-synuclein transgenic rats with manganese, which was a well-known role in protein aggregation, lead to an increase in EVs secretion (Harischandra et al., 2019).

4.4 | Saliva

Interestingly, increased levels of α-synuclein were also found in salivary-derived EVs of PD cases, which would provide a more accessible diagnostics tool (Cao et al., 2019). Moreover, the levels of

total α-synuclein in saliva-derived EVs of PD cases were higher than of multiple system atrophy-parkinsonism cases, showing disease differential diagnosis potential (Cao et al., 2020) (Table 2).

4.5 | Urine

Only three EVs studies were found using urine as biofluid and just for PD (Table 2). LRRK2 and DJ-1 proteins but not α-synuclein were found in urine-derived EVs (Fraser et al., 2013; Ho et al., 2014). In addition, LRRK2 autophosphorylated at serine 1292 was also found in urinary exosomes-enriched preparations and its levels were increased in individuals with LRRK2 mutation when compared to non-carriers (Wang et al., 2017). Significant differences were gender-specific and only detected in LRRK2 and DJ-1 levels between male Controls and male PD cases (Ho et al., 2014). In addition, LRRK2 was also found in CSF-derived EVs (Fraser et al., 2013; Wang et al., 2017). Urine may hold an interesting biomarker resource potential due to its abundance and non-invasive collection and more studies are expected in near future. Figure 2 summarizes the applications of biofluid-derived EVs in the study of AD and PD.

5 | FUTURE PERSPECTIVES

It is undeniable that the use of EVs isolated from human biofluids hold a great potential as biomarker or therapeutic sources for a wide range of diseases, but it is also noticeably that EVs from distinct neuronal cellular models can contribute to unravel important disease mechanisms and hence contribute to the development of new therapeutic approaches. In both research contexts, is fundamental that EVs studies provide higher detail regarding biofluids samples collection, the number of cells and cell culture media volume used, to increase reproducibility of the data. In addition, it would also be relevant to detail exosome isolation and characterization methodologies to reach standardization of procedures and allow validation of the results. This is crucial since differences in samples processing and exosome isolation can impact the purity of EVs preparations or even the downstream applications and consequently the data obtained. The combination of distinct methodologies that can render in high EVs purity is another topic that deserves further attention. Standardize exosome isolation and characterization procedures will certainly boost biomedical and translational research. The promising future of EVs as sources of biomarkers and as therapeutic vehicles in clinic will certainly benefit from additional studies that complement our understanding on EVs role in pathological pathways linked to neurodegenerative diseases.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Literature search and data analysis were performed by MV and TSM. The manuscript was written by MV and TSM and revised by AGH. All authors read and approved the final manuscript.

CONFLICT OF INTEREST

The authors do not have any existing conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

ORCID

Margarida Vaz https://orcid.org/0000-0001-5425-1495
Tânia Soares Martins https://orcid.org/0000-0003-3017-2092
Ana Gabriela Henriques https://orcid.org/0000-0003-0851-6979

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SUPPORTING INFORMATION

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