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Emergence of Extracellular Vesicles as “Liquid Biopsy” for Neurological Disorders: Boom or Bust

Ashish Kumar, Michael A. Nader, and Gagan Deep

Departments of Cancer Biology (A.K., G.D.), Physiology and Pharmacology (M.A.N.), Radiology (M.A.N.), and Center for Addiction Research (M.A.N., G.D.), Wake Forest University School of Medicine, Winston-Salem, North Carolina; Atrium Health Wake Forest Baptist Comprehensive Cancer Center, Winston-Salem, North Carolina (G.D.); and Sticht Center for Healthy Aging and Alzheimer’s Prevention, Wake Forest School of Medicine, Winston-Salem, North Carolina (G.D.)

Abstract	200
Significance Statement	200
I. Introduction	200
II. Current Approaches for the Isolation of Brain Cell–Derived Extracellular Vesicles from Biofluids	201
III. Characterization of Brain Cell–Derived Extracellular Vesicles for Purity and Cargo	204
IV. Brain Cell–Derived Extracellular Vesicles’ Usefulness in Characterizing Molecular Biomarkers of Various Neurological Disorders	206
A. Alzheimer’s Disease	206
1. Protein Cargo of Extracellular Vesicles as an Important Source of Biomarkers for Alzheimer’s Disease	206
2. microRNAs in Extracellular Vesicles as Potential Biomarkers for Alzheimer’s Disease	209
3. Lipid Cargo of Extracellular Vesicles as Novel Biomarkers for Alzheimer’s Disease	210
B. Parkinson’s Disease	211
1. Protein Cargo of Extracellular Vesicles as Promising Biomarkers for Parkinson’s Disease	211
2. microRNAs in Extracellular Vesicles as Potential Biomarkers for Parkinson’s Disease	212
3. Lipid and Carbohydrate Cargo of Extracellular Vesicles as a Novel Source for Biomarkers for Alzheimer’s Disease	213
C. Schizophrenia	213
1. Protein Cargo of Extracellular Vesicles as Potential Biomarkers for Schizophrenia	213
2. Extracellular Vesicle–Derived microRNAs as Potential Biomarkers for Schizophrenia	214
3. Metabolites in Extracellular Vesicles as Novel Biomarker for Schizophrenia	214
D. Bipolar Disorder	214
1. Protein Cargo of Extracellular Vesicles as Potential Biomarkers for Bipolar Disorder	215
2. Extracellular Vesicle–Derived microRNAs as Potential Biomarkers for Bipolar Disorder	215
3. Extracellular Vesicle Metabolites as Biomarkers for Bipolar Disorder	215
E. Major Depressive Disorder	215
1. Protein Cargo as Potential Biomarkers for Major Depressive Disorder	215
2. Extracellular Vesicle–Derived microRNAs as Biomarkers for Major Depressive Disorder	216
F. Substance Use Disorders	216
G. Human Immunodeficiency Virus–Associated Neurocognitive Disorder	217
H. Cancer-Related Neurodegeneration	218

Address correspondence to: Gagan Deep, Wake Forest University School of Medicine, Medical Center Boulevard, Hanes 5048, Winston-Salem, North Carolina 27157. E-mail: gdeep@wakehealth.edu

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V. Brain Cell–Derived Extracellular Vesicles’ Usefulness in Predicting Treatment Response in Neurological Disorders	219
VI. Conclusion: “Boom or Bust”	220
Acknowledgments	221
References	221

Abstract—Extracellular vesicles (EVs) have emerged as an attractive liquid biopsy approach in the diagnosis and prognosis of multiple diseases and disorders. The feasibility of enriching specific subpopulations of EVs from biofluids based on their unique surface markers has opened novel opportunities to gain molecular insight from various tissues and organs, including the brain. Over the past decade, EVs in bodily fluids have been extensively studied for biomarkers associated with various neurological disorders, such as Alzheimer’s disease, Parkinson’s disease, schizophrenia, bipolar disorder, major depressive disorders, substance use disorders, human immunodeficiency virus–associated neurocognitive disorder, and cancer/treatment-induced neurodegeneration. These studies have focused on the isolation and cargo characterization of either total EVs or brain cells, such as neuron-, astrocyte-, microglia-, oligodendrocyte-, pericyte-, and endothelial-derived EVs from biofluids to achieve early diagnosis and molecular characterization and to predict the treatment and intervention outcomes. The findings of these studies have demonstrated that EVs could serve as a repetitive and less invasive source of valuable molecular information for these neurological disorders, supplementing existing costly neuroimaging techniques and relatively invasive measures, like lumbar

puncture. However, the initial excitement surrounding blood-based biomarkers for brain-related diseases has been tempered by challenges, such as lack of central nervous system specificity in EV markers, lengthy protocols, and the absence of standardized procedures for biological sample collection, EV isolation, and characterization. Nevertheless, with rapid advancements in the EV field, supported by improved isolation methods and sensitive assays for cargo characterization, brain cell–derived EVs continue to offer unparalleled opportunities with significant translational implications for various neurological disorders.

Significance Statement—Extracellular vesicles present a less invasive liquid biopsy approach in the diagnosis and prognosis of various neurological disorders. Characterizing these vesicles in biofluids holds the potential to yield valuable molecular information, thereby significantly impacting the development of novel biomarkers for various neurological disorders. This paper has reviewed the methodology employed to isolate extracellular vesicles derived from various brain cells in biofluids, their utility in enhancing the molecular understanding of neurodegeneration, and the potential challenges in this research field.

I. Introduction

The term extracellular vesicle (EV) refers to a variety of heterogeneous vesicles released by all cell types. EVs are delimited by a lipid bilayer and cannot replicate (Théry et al., 2018). EVs have been broadly categorized into subtypes based on their size and origin, such as exosomes, microvesicles, and apoptotic bodies (Rani et al., 2015) (Fig. 1). Exosomes, a type of small-sized EV with diameters ranging from approximately 30 to 150 nm, originate from the endocytic pathway within the cell. These small vesicles are formed through the inward budding of the endosomes, leading to the formation of a multivesicular body (MVB) in the lumen of endosomes (Hessvik and Llorente, 2018). MVBs are then either degraded by fusion with lysosomes or fuse with the cell’s plasma

membrane, releasing the exosomes into the extracellular space (Colombo et al., 2014). In contrast, microvesicles originate by outward budding of the plasma membrane into the extracellular milieu and have diameters ranging from approximately 100 to ≥ 1000 nm. Apoptotic bodies originate from the blebbing of the plasma membrane in cells, undergoing apoptotic stress, and have a variable size range of ~ 50 to 4000 nm in diameter (Battistelli and Falcieri, 2020; Kakarla et al., 2020; Jeppesen et al., 2023). Due to the difficulty in fully separating these subtypes of vesicles from biofluids, the broader term EV has been recommended, although various names have been used in the literature to describe these vesicles. Despite the lack of consensus on the nomenclature of these vesicles, there is a wealth of literature highlighting the biological importance of EVs in maintaining

ABBREVIATIONS: ABCA1, ABC transporter A1; AD, Alzheimer disease; ADE, astrocyte-derived extracellular vesicle; ADRD, Alzheimer disease and related dementia; ADT, antidepressant therapy; APP, amyloid precursor protein; ATN, amyloidosis, tauopathy, and neurodegeneration; $A\beta$, amyloid β ; BD, bipolar disorder; BDE, brain cell–derived extracellular vesicle; BDNF, brain-derived neurotrophic factor; circRNA, circular RNA; CNS, central nervous system; CSF, cerebrospinal fluid; EDE, endothelial-derived extracellular vesicle; EV, extracellular vesicle; FGF, fibroblast growth factor; GAP43, growth associated protein 43; GLAST, glutamine aspartate transporter; HAND, human immunodeficiency virus–associated neurocognitive disorder; HIV, human immunodeficiency virus; IL, interleukin; IRS-1, insulin receptor substrate-1; L1CAM, L1 cell adhesion molecule; lncRNA, long noncoding RNA; MCI, mild cognitive impairment; MDD, major depressive disorder; MDE, microglia-derived extracellular vesicle; miRNA, microRNA; MMKD, modified Mediterranean-ketogenic diet; MRI, magnetic resonance imaging; MSA, multiple system atrophy; MVB, multivesicular body; NDE, neuron-derived extracellular vesicle; NFL, neurofilament light; NLGN3, Neuroligin-3; NMDA, N-methyl-D-aspartate; ODE, oligodendrocyte-derived extracellular vesicle; p, phosphorylated; PCR, polymerase chain reaction; PD, Parkinson disease; PDD, PD dementia; PDE, pericyte-derived extracellular vesicle; PET, positron emission tomography; qPCR, quantitative PCR; sAPP, soluble amyloid precursor protein; SCZ, schizophrenia; SNAP-25, synaptosome-associated protein-25; TEM, transmission electron microscopy; TMEM119, transmembrane protein 119; TNF- α , tumor necrosis factor- α .

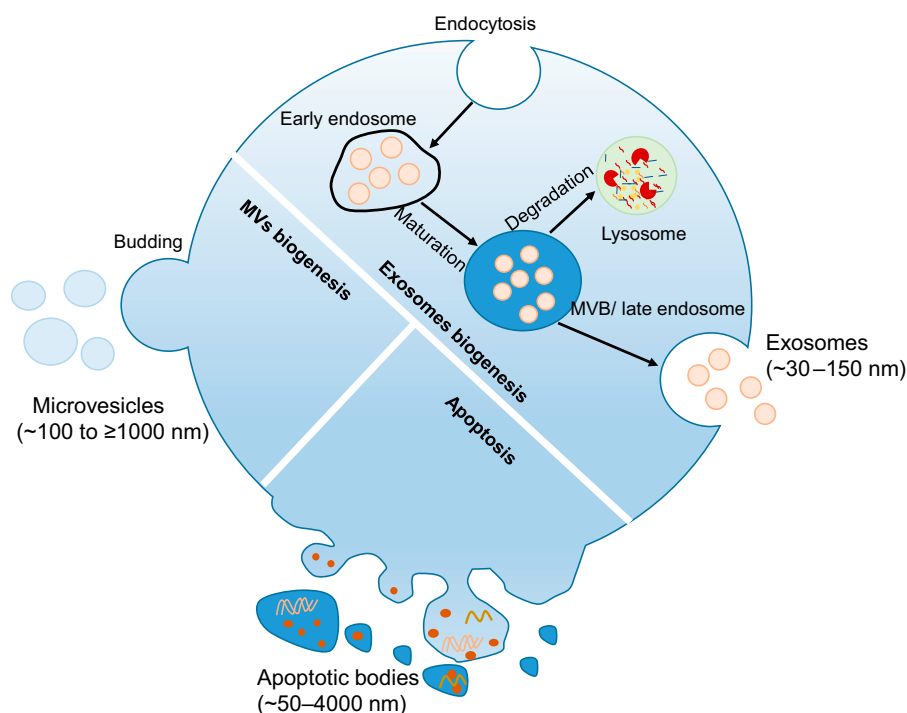


Fig. 1. Biogenesis of various subtypes of EVs: exosomes, microvesicles, and apoptotic bodies.

cellular homeostasis and facilitating intercellular communication.

The role of EVs in intercellular communication, both local and distant, is well recognized. EVs, through their cargo (proteins, lipids, metabolites, and nucleic acids), stimulate widespread effects on systemic processes, including immune function, inflammation, and a host of disease- and organ-specific processes. Consequently, there is a growing interest in the potential of EVs to serve as noninvasive biomarkers for the diagnosis and prognosis of various diseases, such as cancer, metabolic disorders, cardiovascular diseases, neurological disorders, infectious diseases, and others (Panigrahi and Deep, 2017; Shah et al., 2018; Kumar and Deep, 2020; Kumar, 2021). EVs are released by every cell type into circulation, and their easy accessibility and isolation from almost any biofluid make them an attractive and feasible candidate for biomarker discovery. Furthermore, to a great extent, the cargo of EVs reflects the pathophysiological state of the parent cell; their analysis could provide treasured information about the parent cells in a given condition (Hedlund et al., 2011; de Jong et al., 2012; Sánchez-Melgar et al., 2020; Berumen Sánchez et al., 2021; Kumar, 2021; Phan et al., 2022). For these reasons, EVs have been suggested as a key component of liquid biopsy for various diseases, particularly those affecting difficult-to-access organs, like the brain. In this review, we highlight studies related to the isolation and characterization of brain cell-derived extracellular vesicles (BDEs) as a less-invasive approach for better understanding the molecular

aspects of various neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia (SCZ), bipolar disorder (BD), major depressive disorder (MDD), substance use disorders (SUD), human immunodeficiency virus (HIV)-associated neurocognitive disorder (HAND), and cancer/treatment-induced neurodegeneration. We also discuss the major challenges in this field and potential opportunities that lie ahead.

II. Current Approaches for the Isolation of Brain Cell-Derived Extracellular Vesicles from Biofluids

Like other cell types in the body, different brain cells also secrete EVs, and there is ample evidence that these EVs can find their way into the peripheral circulation. The mechanism underlying EVs crossing the blood-brain barrier is actively being researched and includes transcytosis or disrupted blood-brain barrier. Advances in the EV field have made it possible to isolate cell type-specific EVs, including BDEs, from the biofluids.

The isolation of cell type-specific EVs from biofluids is a multistep process (Fig. 2). The first step involves isolating total EVs from the biological sample, such as blood plasma or serum. This is achieved through methods such as ultracentrifugation, polymer-based precipitation, density gradient centrifugation, size exclusion chromatography, filtration, immuno-affinity capture, and microfluidic chip (Contreras-Naranjo et al., 2017;

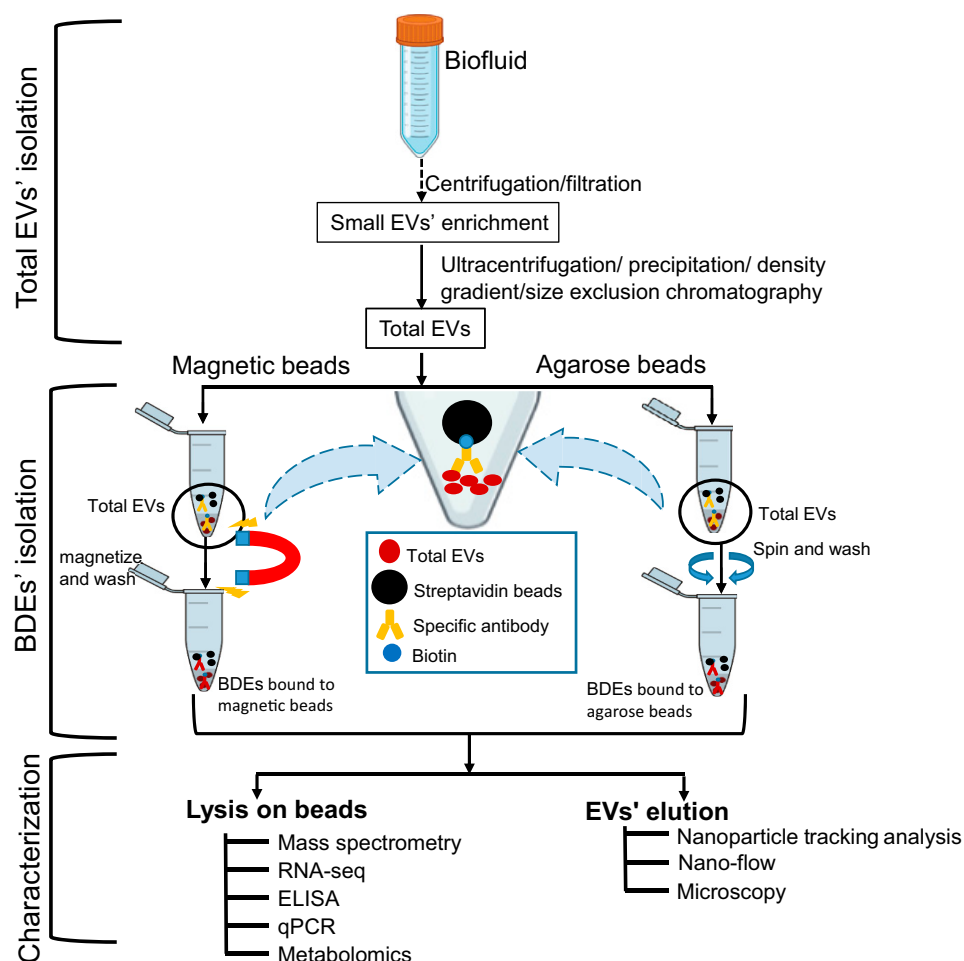


Fig. 2. An overview of approaches to isolate BDEs from biofluids. The initial step involves enriching small extracellular vesicles (sEVs) from biofluids through low-speed sequential centrifugation steps (up to 10,000g) and/or filtration (0.22 μ m, filters). The subsequent isolation of total sEVs (TEs) can be achieved through various methods, including ultracentrifugation, precipitation, density gradient centrifugation, or size exclusion chromatography. Further, immunocapture is the most preferred method for isolating different BDEs using cell type-specific biotin-labeled antibodies with streptavidin-coated magnetic/agarose beads. Following incubation of TEs with specific antibodies and streptavidin-coated beads, unbound nonspecific sEVs can be removed by washing after magnetization (for magnetic beads) or centrifugation (for agarose beads). Specific BDEs bound to the beads can either be eluted for characterization of their biophysical properties or can be lysed directly on the beads for cargo analysis. In the latter scenario, the beads can be removed after magnetization or centrifugation.

Li et al., 2017, 2019; Doyle and Wang, 2019; Zhang et al., 2019a). The choice of method for isolating total EVs depends largely on the amount of starting biological sample, and the resultant yield depends on the method used, with the aim of maximizing the concentration of total EVs for subsequent isolation of specific EVs. The most implemented methods for isolating total EVs are ultracentrifugation and polymer-based precipitation. However, the consensus on the choice of method for the clinical use of EVs as a biomarker for various neurological diseases is yet to be made, which will be driven by the feasibility, sensitivity, and specificity of the method.

For isolating BDEs from plasma/serum, immunocapture is the preferred method, which involves a biotin-tagged antibody specific to the marker (s) present on the surface of EVs related to the cell of origin. This is combined with streptavidin-coated magnetic/agarose/protein A/G bead/epoxy beads. The first demonstration of neuron-derived EV (NDE) isolation was provided by

Zhang and colleagues, who directly isolated them from plasma diluted 1:3 (after centrifugation at 2000 and 12,000g) in phosphate-buffered saline without prior isolation of total EVs. The diluted plasma samples were incubated with anti-L1 cell adhesion molecule (L1CAM) antibody-coated on M-270 Epoxy beads with gentle rotation before proceeding to exosome release or lysis (Shi et al., 2014). However, subsequent studies have preferred a two-step methodology, where total EVs are first isolated from plasma or serum, followed by immunocapture of BDE using specific markers (Goetzl et al., 2016b; Mateescu et al., 2017; Mustapic et al., 2017; Kapogiannis et al., 2019; Kumar et al., 2021a, 2022; Pounders et al., 2022; Roseborough et al., 2023). The two-step method can circumvent the interference of free proteins present in plasma for the isolation of BDEs. In recent years, several companies, such as NeuroDex, Novus Biologicals, Miltenyi Biotec, and FUJIFILM Wako Pure Chemical Corporation, have offered

commercial kits based on the immunocapture principle for the isolation of total EVs or NDE from biofluids. A few studies have also presented methods to isolate BDEs from the brain tissues (Gomes et al., 2023), which have been discussed in detail by Brenna et al. (2021). However, for this review, we will primarily focus on the methods of BDE isolation from the biofluids. The most common choice of biofluid for BDE isolation is mainly plasma (Mustapic et al., 2017; Pulliam et al., 2019; Kumar et al., 2021a, 2022, 2023) or serum (Dagur et al., 2019; Edwards et al., 2021; Qin et al., 2022); however, other biofluids, such as cerebrospinal fluid (CSF), urine, saliva, and ascites (Sun et al., 2019b; Gomes and Witwer, 2022), have also been used for their potential as biomarkers for neurodegenerative diseases.

The isolation and characterization protocols for BDEs have primarily been targeted toward NDEs (Kumar et al., 2022), with limited reports on astrocyte-derived EVs (ADEs), microglia-derived EVs (MDEs), oligodendrocyte-derived EVs (ODEs), pericyte-derived EVs (PDEs), or endothelial-derived EVs (EDEs) (Table 1). Several markers have been used to isolate NDEs from plasma or serum, with L1CAM (or CD171) being the most commonly used for biomarkers discovery and validation (Pulliam et al., 2019; Gomes and Witwer,

2022). L1CAM is a member of the L1 family of adhesion proteins. It is typically expressed as a single-pass transmembrane protein, with its extracellular N-terminal containing immunoglobulin domains and fibronectin-like repeat domains, whereas its cytosolic C-terminal region is involved in various signaling pathways (Samatov et al., 2016; Gomes and Witwer, 2022). However, the utility of L1CAM in isolating NDEs has been challenged for several reasons. First, L1CAM specificity to neurons or even the central nervous system (CNS) has been questioned as this protein is expressed by other brain cell types, like oligodendrocytes, as well as cells outside the CNS (such as kidney and urinary bladder) and cancer cells (Gutwein et al., 2005; Colombo and Meldolesi, 2015; Altevogt et al., 2020). Additionally, the transient expression of L1CAM due to the susceptibility of proteolytic cleavage of its ectodomain results in the majority of protein being in soluble form (Linneberg et al., 2019; Norman et al., 2021). Nevertheless, there is sufficient evidence confirming the presence of L1CAM on NDEs' surface established by immunogold labeling coupled with transmission electron microscopy, confocal microscopy, and flow cytometry (Winston et al., 2019; Kumar et al., 2021a, 2022, 2023; Lee et al., 2021). Alternatively, neural cell adhesion molecule (NCAM), GluR2/3 subunits of

TABLE 1
List of potential markers used to isolate different BDEs

BDE Subtype	Surface Markers Used for Isolation	BDE Characterization Methods	References
NDE	L1CAM (CD171), NCAM, SNAP-25, Synaptophysin, GluR2/3, ATP1A3, GAP43, NLGN 3	NTA, TRPS, dynamic light scattering, western blot, TEM, immuno-gold labeling, flow cytometry	Shi et al., 2014; Goetzl et al., 2015a,b, 2018a, 2021; Sun et al., 2017, 2019a; Kuwano et al., 2018; Agliardi et al., 2019; Dagur et al., 2019; Kapogiannis et al., 2019; Pulliam et al., 2019; Wijtenburg et al., 2019; Winston et al., 2019, 2022; Zhao et al., 2019; Gu et al., 2020; Hiramoto et al., 2020; Hornung et al., 2020; Niu et al., 2020; Nogueras-Ortiz et al., 2020; Song et al., 2020; Arioiz et al., 2021; Delgado-Peraza et al., 2021; Kumar et al., 2021a, 2022, 2023; Lee et al., 2021; Nasca et al., 2021; Saeedi et al., 2021; Yao et al., 2021; Alvarez et al., 2022; Du et al., 2022a; Durur et al., 2022; Gomes and Witwer, 2022; Kluge et al., 2022; Perluigi et al., 2022; Pounders et al., 2022; Tasdelen et al., 2022; Yousif et al., 2022; Eitan et al., 2023; Meloni et al., 2023; Visconte et al., 2023; You et al., 2023
ADE	GLAST (ACSA-1)	NTA, western blotting, TEM, immuno-gold labeling, flow cytometry	Goetzl et al., 2016b, 2018b; Winston et al., 2019, 2022; Nogueras-Ortiz et al., 2020; Delgado-Peraza et al., 2021; Kumar et al., 2021a, 2023; Lee et al., 2021; Valle-Tamayo et al., 2022; Xie et al., 2023b
MDE	TMEM119, CD11b	NTA, western blotting, TEM, immuno-gold labeling, flow cytometry	Cohn et al., 2021; Kumar et al., 2021a, 2023; Winston et al., 2022; Roseborough et al., 2023; Visconte et al., 2023
ODE	MOG, PDGFR α , CNPase	NTA, western blotting, TEM, immuno-gold labeling, flow cytometry	Yu et al., 2020; Dutta et al., 2021; Kumar et al., 2023
PDE	PDGFR β	NTA, TEM, immuno-gold labeling, flow cytometry	Kumar et al., 2023
EDE	CD31, CD146	NTA, TEM, immuno-gold labeling, flow cytometry	Elahi et al., 2018; Abner et al., 2020; Kumar et al., 2023

ACSA-1, astrocyte cell surface antigen-1; ATP1A3, ATPase Na⁺/K⁺ transporting subunit α 3; CNPase, 2,3-cyclic nucleotide-3-phosphodiesterase; GAP43, growth associated protein 43; GluR2/3, GluR2/3 subunits of α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; MOG, myelin oligodendrocyte glycoprotein; NLGN3, Neuroligin-3; NTA, nanoparticle tracking analysis; PDGFR α , platelet-derived growth factor α ; TRPS, tunable resistive pulse sensing.

α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (GluR2/3), synaptosome-associated protein 25 (SNAP-25), synaptophysin, and ATPase Na⁺/K⁺ transporting subunit α 3 (ATP1A3) have also been used for NDE isolation (Hornung et al., 2020; Song et al., 2020; Saeedi et al., 2021; Kumar et al., 2022; Yousif et al., 2022; You et al., 2023) (Table 1). The isolation of plasma NDEs using two separate markers, L1CAM and SNAP-25, from the independent cohort showed high concordance in microRNA (miRNA) cargo analysis, demonstrating the utility of these markers in isolating similar NDE populations (Kumar, 2021; Saeedi et al., 2021). However, the decreased expression of SNAP-25 in serum NDEs from patients with AD compared with healthy controls has also been reported (Agliardi et al., 2019), which may impact the efficiency of NDE isolation from individuals with AD if SNAP-25 is used.

In recent years, there has been increasing attention on non-neuronal cells and their EVs to gain a more comprehensive understanding of the pathophysiological characteristics of various neuropsychiatric disorders. Astrocytes, the most prevalent type of glial cells in the CNS, and their EVs (ADEs) have been shown to play a crucial role in several neuropathological conditions (You et al., 2019; Rouillard et al., 2021; Zhao et al., 2021). ADEs have been reported to be associated with the pathogenesis of diverse neuropathological diseases by horizontally transferring their cargos to neighboring brain cells as well as peripheral immune cells. Furthermore, ADEs have also been implicated in neuroprotection, neuro-regeneration, repair, and maintenance of normal neuronal function, making their isolation from patients' biofluids and cargo analysis vital for the diagnosis, treatment, and prevention of neurological diseases (Zhao et al., 2021). The isolation of ADEs from the peripheral system has been demonstrated using a biotinylated antibody against glutamine aspartate transporter [GLAST; also known as astrocyte cell surface antigen-1 (ACSA-1)] (Goetzl et al., 2016b, 2018b; Delgado-Peraza et al., 2021; Kumar et al., 2021a, 2023; Valle-Tamayo et al., 2022) (Table 1). Similarly, microglia, the resident macrophagic cells of the brain, play a crucial role in maintaining the integrity of the CNS by regulating neuronal mapping and activities, CNS development, and tissue homeostasis through the maintenance of a basal inflammatory state (Ransohoff and El Khoury, 2015; Thion et al., 2018). Recently, we demonstrated the utility of microglia-specific transmembrane protein 119 (TMEM119) biotin-tagged antibody in combination with streptavidin-coated magnetic beads to isolate MDEs from plasma (Kumar et al., 2021a) (Table 1). This method has been further validated by us and others (Kumar et al., 2023; Roseborough et al., 2023; Visconte et al., 2023). Recently, the isolation of NDEs, ADEs, and MDEs using biotin-tagged L1CAM, GLAST, and TMEM119 antibodies, respectively, tagged on streptavidin-coated magnetic beads was demonstrated

following sorting of beads-Ab-EV complex on a flow cytometer before their elution (Lee et al., 2021; Winston et al., 2022). Additionally, the utility of CD11b to isolate MDEs from cryopreserved brain tissue (Cohn et al., 2021) and myelin oligodendrocyte glycoprotein (MOG), CD140a/platelet-derived growth factor α (PDGFR α), and 2,3-cyclic nucleotide-3-phosphodiesterase (CNPase) for ODEs has also been demonstrated (Yu et al., 2020; Dutta et al., 2021; Kumar et al., 2023) (Table 1).

Similar to glial cells, brain pericytes and endothelial cells have traditionally been described as bystanders in the development of various neurological disorders. Yet, these vascular cells may play an active role in the pathogenesis of various diseases, where neuron-glial-endothelial cells work as a cohesive unit. Recent studies have reported the usefulness of EDEs to better understand cerebral vascular disease and cognitive function (Goetzl et al., 2017; Elahi et al., 2018; Abner et al., 2020). For example, Abner et al. (2020) reported the isolation of EDEs from plasma using surface markers CD31 and CD146 to assess the role of small cerebral vascular disease in the development of dementia in AD. Recently, we also reported the isolation of EDEs and PDEs, along with other BDEs, and showed the usefulness of their cargo (specific miRNAs) as a potential biomarker for AD (Kumar et al., 2023). Table 1 lists various surface biomarkers that are being used to isolate BDEs from blood, with references of a few of these studies.

III. Characterization of Brain Cell-Derived Extracellular Vesicles for Purity and Cargo

BDEs have been extensively characterized for their concentration (particle number per ml), size distribution, expression of EV biomarkers, and cargos (protein, metabolites, and nucleic acids) to identify disease-specific biomarkers (Goetzl et al., 2015b, 2016b; Doyle and Wang, 2019; Kumar et al., 2021a, 2022, 2023; Eitan et al., 2023) (Fig. 3). Size distribution analysis not only helps determine the prevalence of specific types of EVs, such as small extracellular vesicles, but also identifies differences due to pathology or any therapeutic intervention. For instance, nanoparticle tracking analysis showed a decreased concentration of NDE in AD, indicating higher diagnostic performance for discriminating AD from control individuals (Visconte et al., 2023). On the other hand, the concentration of MDEs was significantly higher in the mild cognitive impairment (MCI) group compared with the control group, with no change in size reported for both NDEs and MDEs (Visconte et al., 2023). Similarly, another study using nanoparticle tracking analysis showed a significantly reduced concentration of NDEs in patients with amnesic MCI or AD dementia compared with amyloid β ($A\beta$)⁻ or $A\beta$ ⁺ normal controls (Li et al., 2022). However, contrasting results were reported by Noguerras-Ortiz et al. (2020), who found a higher particle concentration of NDEs and ADEs isolated

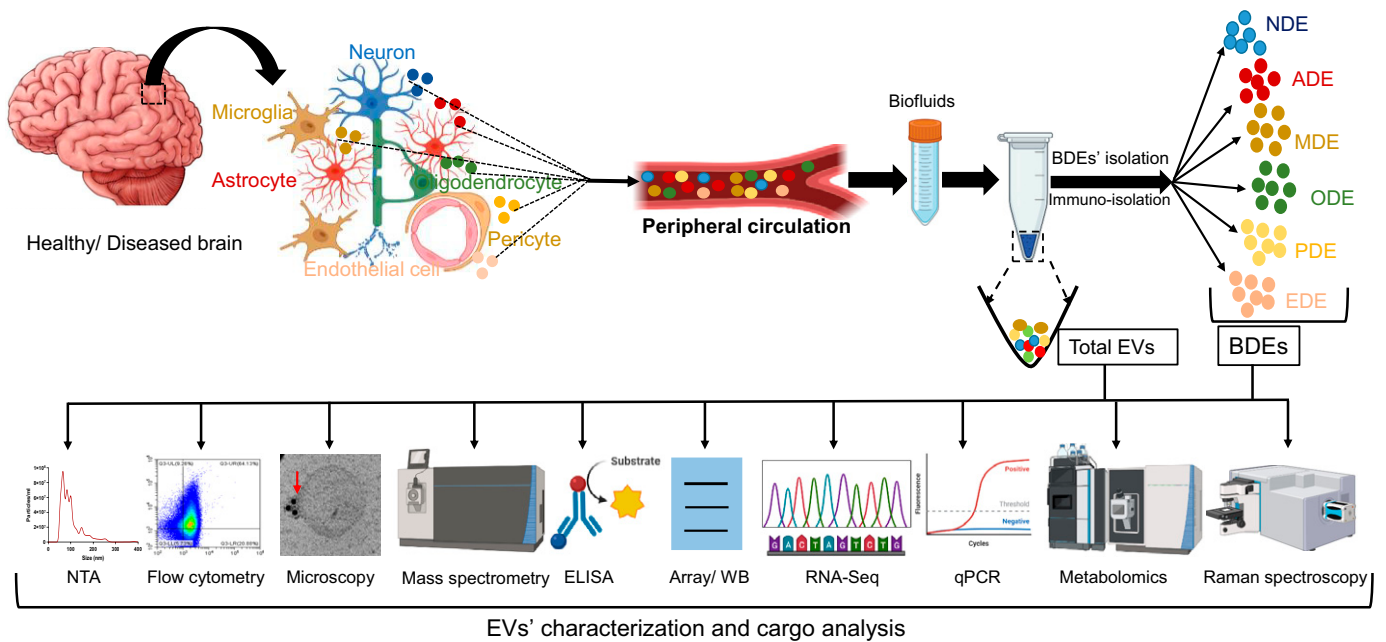


Fig. 3. BDEs in biofluids could serve as liquid biopsy for various neurological disorders. EVs secreted by various brain cells find their way into peripheral circulation. Based upon their specific surface markers, various BDEs can be isolated by immunocapture method. BDEs are characterized for their biophysical properties, purity, and/or expression of specific biomarkers by multiple techniques, such as nanoparticle tracking analysis (NTA), nano-flow cytometry, and electron microscopy. Furthermore, the cargo of BDEs can be characterized by multiple techniques. For proteins, mass spectrometry, ELISA, arrays, and western blotting (WB) can be used. Nucleic acids can be analyzed through RNA sequencing (RNA-seq) and qPCR, and metabolites and lipids can be studied using targeted or untargeted mass spectrometry and Raman spectroscopy.

from plasma using L1CAM and GLAST markers, respectively, in AD individuals compared with controls. Further, Saeedi et al. (2021) reported a smaller size of NDEs and total EVs, measured using tunable resistive pulse sensing technology, in MDD subjects compared with controls, which increased after 8 weeks of antidepressant therapy (ADT) and showed associations with better therapeutic response.

In addition to characterizing EVs for size and concentration, the proteins present on their surface are routinely characterized using immunogold labeling coupled with transmission electron microscopy (TEM) or cryo-TEM to confirm the purity, specificity, and size of BDEs (Dickens et al., 2017; Men et al., 2019; Willis et al., 2020; Kumar et al., 2022, 2023; Roseborough et al., 2023). We recently reported the extensive characterization of BDEs using immunogold labeling (Kumar et al., 2022, 2023) and also detailed the method of immunogold labeling for EV characterization (Su et al., 2022). For BDEs, imaging of typical EV markers (i.e., CD63 or CD9) along with specific EV markers [i.e., L1CAM (for NDEs), GLAST (for ADEs), TMEM119 (for MDEs), PDGFR α (for ODE), PDGFR β (for PDEs), and CD31 (for EDEs)], using gold-labeled antibodies, confirmed their purity and specificity (Kumar et al., 2022, 2023). We also reported the coexpression of CD63 and L1CAM on the surface of NDEs by this approach using two different-sized “gold particles (10 and 20 nm)-tagged antibodies” (Kumar et al., 2022). We further confirmed immuno-gold labeling–TEM results by confocal microscopy as well as ELISA assays (Kumar et al., 2022).

Recent advancements in flow cytometry instrumentation and protocols, including the integration of a high-power 488-nm laser and modifications of the optical detection systems, have made it possible to quantify EVs’ surface proteins, size, and even proteins loaded in EVs using fluorescent antibodies targeting EV-associated membrane/cargo proteins (Stoner et al., 2016). Flow cytometry has been used in many recent studies to detect and quantify specific subpopulations of BDEs. We have extensively characterized NDEs using a fluorescent antibody (L1CAM) to isolate these EVs, which was further validated by other NDE-specific markers, such as enolase 2 (ENO2) (a neuron-specific enolase) and synaptophysin (Kumar et al., 2022). Furthermore, we used flow cytometry to analyze the purity of six different BDEs (neurons, astrocytes, microglia, oligodendrocytes, pericytes, and endothelial) and determine the relative percentage of these brain cell-specific EVs in the total EV pool isolated from the plasma of control and individuals with dementia (Kumar et al., 2023). Similarly, other studies have also characterized different BDEs using flow cytometry (Mustapic et al., 2017; Silverman et al., 2019; Ali Moussa et al., 2022; Huang et al., 2022). Recently, Hong et al. (2021) demonstrated an assay based on the novel Apogee nanoscale flow cytometry technology for the detection of α -synuclein or aggregated α -synuclein using specific antibodies with high specificity and sensitivity in controls and Parkinson’s disease patients. Due to the small size of EVs and the challenges in their detection with most conventional flow cytometers, EVs

can be tagged to beads and labeled with specific antibody/antibodies that detect EV surface markers, and conventional flow cytometry instruments can be used for analysis (Campos-Silva et al., 2019).

Although western blotting of typical EV markers has been extensively used to characterize EVs isolated from different biofluids and cell-cultured media, the low recovery of specific cell-derived EVs, including BDEs, limits its utility, especially when isolated from human biofluids. Still, the expression of neuron-specific markers, such as L1CAM, SNAP25, NeuN, and GluR2, as well as general EV markers, like CD9, Flot-1, CD9, and TSG101, has been confirmed by western blotting in many studies (Dagur et al., 2019; Serpente et al., 2020; Saeedi et al., 2021; Eitan et al., 2023). Similarly, the expression of typical EV markers, like Alix, flotillin-1, Annexin-A2, and CD81, has been reported in ADEs isolated from cultured astrocytes media (Pascua-Maestro et al., 2019; D'Arrigo et al., 2021). Moreover, commercial EV protein arrays are available and being used to characterize typical markers for total EVs as well as NDEs (Joerger-Messerli et al., 2018; Kumar et al., 2022). In addition to the analysis of typical EV markers, the western blotting approach has also been used to analyze the expression of markers related to neuron/astrocyte-derived EVs (Mustapic et al., 2017; Patel and Weaver, 2021).

The analysis of BDEs' cargo as a biomarker has primarily been focused on proteins and miRNAs/mRNA, with some sparse studies on lipids. The methods of characterization of BDEs, along with the analyzed cargo proteins and miRNAs in different neurological disease conditions, are listed in Table 2. The preferred method for these analyses includes mass spectrometry and ELISA-based assays for proteins and real-time polymeric chain reaction (PCR) and RNA sequencing for miRNA/RNA quantification (Fig. 3). The studies adopting these methods for the analysis of BDEs in different disease contexts are discussed below.

IV. Brain Cell-Derived Extracellular Vesicles' Usefulness in Characterizing Molecular Biomarkers of Various Neurological Disorders

A. Alzheimer's Disease

AD, the most common form of dementia, is currently a public health crisis. AD becomes more prevalent with age and is considered one of the most expensive diseases to manage in the United States (Hurd et al., 2013; Alzheimer's Association, 2016). Histopathologically, AD is characterized by extracellular amyloid plaques, intracellular neurofibrillary tangles, synaptic degeneration, and hippocampal neuronal loss (Swerdlow, 2007). The principal explanation of AD pathogenesis is the "amyloid hypothesis," which posits that A β peptides accumulate in the extracellular space, leading to a series of downstream

events, such as synaptic dysfunction, inflammation, tau pathology, neuronal cell death, and, ultimately, dementia (Hardy and Selkoe, 2002; Palop and Mucke, 2010; Bloom, 2014; Tu et al., 2014; Marsh and Alifragis, 2018; Chen et al., 2020). A β peptide is formed through the sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretase enzymes to generate the amyloidogenic A β peptide, whereas cleavage of APP with α -secretase followed by γ -secretase precludes A β formation (Chow et al., 2010; Zhang et al., 2011).

Despite well-defined histopathological hallmarks and the increasing prevalence of the disease, the clinical diagnosis of AD is complex and relies primarily on extensive cognitive measures. Neuroimaging, including positron emission tomography (PET) and magnetic resonance imaging (MRI), and CSF biomarkers provide objective evidence of the underlying neuropathology of cognitive impairment, although these techniques have not been routinely implemented for early diagnosis. Moreover, using these standard methods, clinical diagnosis is mostly possible only when significant and irreversible neuronal damage has already occurred. Although the assessment of pathologic proteins like A β , total- and phosphorylated (p)-tau, and synaptic proteins are ideal for AD diagnosis, their concentrations in different biofluids differ significantly, and their detection by standard assays is only possible in the advanced disease condition (Candelise et al., 2020). Furthermore, the research framework for AD diagnosis, developed under the National Institute on Aging and Alzheimer's Association, proposed a classification scheme known as the ATN classification system based on amyloidosis (A), tauopathy (T), and neurodegeneration (N). This system divides seven major AD biomarkers into three binary categories: β -amyloid (amyloid PET and CSF A β 1-42), tau (CSF p-tau, or tau PET), and biomarkers of neurodegeneration or neuronal injury ([¹⁸F]-fluorodeoxyglucose-PET, structural MRI, or CSF total tau) (Jack et al., 2016; Ebenau et al., 2020; Baldeiras et al., 2022). This framework provides a reference for AD diagnosis. However, evidence suggests that the ATN classification does not always reflect the phenotypic and pathogenic complexity of AD. Therefore, ATN biomarkers are not routinely used in clinical practice for late-onset dementia or to diagnose non-AD dementia and are mostly used for identifying pre-clinical AD in research settings (Niemantsverdriet et al., 2017). Moreover, the collection of CSF for routine and repeated assessment over time is invasive, and the cost associated with neuroimaging measures is high. This has promoted researchers to seek a more feasible, affordable, and reliable blood-based biomarker for AD diagnosis.

1. *Protein Cargo of Extracellular Vesicles as an Important Source of Biomarkers for Alzheimer's Disease.* As highlighted above, the possibility of isolation of cell type-specific EVs from the peripheral biofluid has provided an

TABLE 2
BDE isolation markers and analyzed cargos for different neurodegenerative diseases

BDE	Cargo Analysis		References	
	Markers Used for BDE Isolation for Cargo Analysis	Protein		
Alzheimer's disease				
NDE	L1CAM (CD171), NCAM, ATP1A3, GAP43, NGLN 3	Total tau, p-T181-tau, p-S396-tau, A β 1-42, NRG1, REST, GAP43, cathepsin D, LAMP1, ubiquitin, synaptotagmin 1, pSer312-IRS-1, p γ -IRS-1, A β 1-42, SNAP-25, PSD-95, proBDNF, GluR2, Syntaxin-1, NPTX2, AMPA4, NLGN1, NRXN2 α , SOD1, electron transport chain complexes, α -, β -, Δ -globin, hemoglobin, IL-6, MMP-9,	miR-132, miR-212, miR-23a-3p, miR-223-3p, miR-190a-5p, miR-100-3p, miR-9-5p, miR-29a-5p, miR-106b-5p, miR-125b-5p, miR-132-5p, miR-135b-5p, miR-210-3p, miR-373, miR-204, Let-7e	Fiandaca et al., 2015; Goetzl et al., 2015a, b, 2016a, 2018a; Winston et al., 2016; Agliardi et al., 2019; Cha et al., 2019; Kapogiannis et al., 2019; Gu et al., 2020; Noguera-Ortiz et al., 2020; Serpente et al., 2020; Arioiz et al., 2021; Delgado-Peraza et al., 2021; Jia et al., 2021; Yao et al., 2021; Alvarez et al., 2022; Durur et al., 2022; Liu et al., 2022; Tasdelen et al., 2022; Ertan et al., 2023; Kumar et al., 2023; You et al., 2023
ADE	GLAST (ACSA-1, EAAT-1), LRP-1	BACE-1, γ -secretase, sAPP α , sAPP β , Septin-8, p-T181-tau, P-S396-tau, A β 42, GDNF, Integrin β 1, LRPL, C1q	miR-29a-5p, miR-107, miR-125b-5p, miR-132-5p, miR-210-3p	Goetzl et al., 2016b, 2018b; Noguera-Ortiz et al., 2020; Delgado-Peraza et al., 2021; Liu et al., 2022; Kumar et al., 2023
MDE	CD11b, TMEM119	FTH1, TREM2, tau	miR-28-5p, miR-381-3p, miR-651-5p, and miR-188-5p, miR-29a-5p, miR-106b-5p, miR-125b-5p, miR-132-5p, miR-210-3p, miR-29a-5p, miR-107, miR-125b-5p, miR-135b-5p, miR-210-3p	Kumar et al., 2023
ODE	PDGFR α		miR-9-5p, miR-125b-5p, miR-132-5p, miR-135b-5p, miR-210-3p	Kumar et al., 2023
PDE	PDGFR β		miR-29a-5p, miR-125b-5p, miR-132-5p, miR-135b-5p, miR-210-3p	Kumar et al., 2023
EDE	CD31, CD146	LAT-1, Glut-1, p-gp, A β 40, A β 42, PrPc, p-181T-tau	miR-29a-5p, miR-125b-5p, miR-132-5p, miR-135b-5p, miR-210-3p	Abner et al., 2020; Kumar et al., 2020
Parkinson's disease				
NDE	L1CAM (CD171), NCAM	α -Synuclein, tau aggregates, syntaxin-1A, VAMP-2, SNAP-25, Dj-1		Shi et al., 2014; Si et al., 2019; Zhao et al., 2019; Jiang et al., 2020; Niu et al., 2020; Agliardi et al., 2021; Dutta et al., 2021; Kluge et al., 2022; Meloni et al., 2023; Dutta et al., 2021
ODE	MOG	α -Synuclein		
Schizophrenia				
NDE	L1CAM	Insulin resistance markers (Akt and signaling effectors), A β 42, A β 40, P-T181-tau		Wijtenburg et al., 2019; Lee et al., 2021
ADE	GLAST	A β 42, A β 40, P-T181-tau		Lee et al., 2021
Bipolar disorder				
NDE	L1CAM	IRS-1, pS812-IRS-1, Akt, GSK-3 β , p70S6K, ERK1/2, JNK, p70S6K, p38-MAPK		Mansur et al., 2021
Major depressive disorder				
NDE	L1CAM, SNAP-25	IRS-1, mitochondrial proteins, IL-34, Synaptophysin	miR-423-3p, miR-191-5p, miR-486-5p, miR-30d-5p, miR-425-5p, miR-25-3p, miR-21-5p, miR-335-5p, and miR-126-5p	Kuwano et al., 2018; Goetzl et al., 2021; Nasca et al., 2021; Saeedi et al., 2021
ADE	ACSA-1	Interferon- γ , IL-12p70, IL-1 β , IL-2, IL-4, IL-6, TNF- α , and IL-17A		Xie et al., 2023b

(continued)

TABLE 2—Continued

BDE	Markers Used for BDE Isolation for Cargo Analysis	Cargo Analysis		References
		Protein	miRNA	
Substance use disorders				
NDE	L1CAM,	NFL, α -synuclein, A β 1–42	Let7a-5p, miR16-5p, miR-18-5p, miR107, miR-125-5p, miR-144-3p, miR-210-3p, miR-339-3p, and miR-361-5p	Kumar et al., 2021b
ADE	GLAST	NFL, α -synuclein, A β 1–42	Let7a-5p, miR16-5p, miR-18-5p, miR107, miR-125-5p, miR-144-3p, miR-210-3p, miR-339-3p, and miR-361-5p	Kumar et al., 2021b
MDE	TMEM119	NFL, α -synuclein, A β 1–42	Let7a-5p, miR16-5p, miR-18-5p, miR107, miR-125-5p, miR-144-3p, miR-210-3p, miR-339-3p, and miR-361-5p	Kumar et al., 2021b
HIV-associated neurocognitive disorder				
NDE	L1CAM	NFL, synaptophysin, HMGB1, NFL, A β , CD63, CD81, High mobility group box 1, p-T181-tau, cathepsin S, total tau, NCAM, contactin 5 and other 184 proteins by proximity extension assays.		Sun et al., 2017, 2019a

ACSA-1, astrocyte cell surface antigen-1; AMPA4, glutamate ionotropic receptor 4; ATP1A3, ATPase Na+K+ transporting subunit 3; BACE-1, β secretase-1; C1q, complement component 1q; FTH1, ferritin heavy chain 1; GAP43, growth-associated protein 43; GDNF, glial-derived neurotrophic factor; GSK-3 β , glycogen synthase kinase 3 β ; HMGB1, high-mobility group box 1; LAMP1, lysosome-associated membrane protein 1; LRP1, lipoprotein receptor-related protein 1; MOG, myelin oligodendrocyte glycoprotein; NCAM, neural cell adhesion molecule; NLGN1, neuroligin 1; NLGN3, Neuroligin-3; NPTX2, neuron pentraxin 2; NRGN, neurogranin; NRXN2x, α -neurexin 2; p70S6K, p70 ribosomal S6 kinase; PDGFR α , platelet-derived growth factor α ; PSD-95, postsynaptic density protein-45; REST, repressor element 1-silencing transcription factor; TREM2, triggering receptor expressed on myeloid cells 2; VAMP-2, vesicle-associated membrane protein-2.

opportunity to study the complex molecular changes in the brain. Profiling the levels of p-T181-tau, p-S396-tau, A β 1–42, neurogranin (NRGN), glutamate ionotropic receptor AMPA type subunit 4 (AMPA4), repressor element 1-silencing transcription factor (REST), growth-associated protein 43 (GAP43), cathepsin D, lysosome-associated membrane protein (LAMP)-1, ubiquitin, and synaptotagmin 1 in NDEs has shown differential levels in control, MCI, and AD patients. These levels were reported to predict the development of AD 5–10 years prior to clinical onset (Fiandaca et al., 2015; Goetzl et al., 2015a,b, 2016a, 2018a; Winston et al., 2016; Jia et al., 2021). Studies consistently showed higher levels of total/p-tau and A β 1-42 in NDEs isolated from the plasma of MCI and AD individuals as well as in EVs isolated from CSF and brain tissues (Saman et al., 2012; Fiandaca et al., 2015; Polanco et al., 2016; Guix et al., 2018; Liu et al., 2022; Ruan, 2022). The higher levels of A β in NDEs can be justified by other evidence, which showed the cleavage of APP by β -secretase, mainly in the late Golgi/trans-Golgi network (TGN) and endosomes. The release of A β peptide utilizes the endocytic pathway and is secreted into the extracellular space in EVs (or exosomes) after the fusion of the MVB with the plasma membrane (Koo and Squazzo, 1994; Rajendran et al., 2006). Moreover, the expression of different APP cleavage forms [soluble amyloid precursor protein (sAPP)- β , sAPP α , and soluble A β 1-42], and the enzymes responsible for its cleavage (β - and γ -secretase) were detected in both NDEs and ADEs, with comparatively higher levels reported in ADEs (Goetzl et al., 2016b). Significantly higher levels of β secretase-1 (BACE-1) and sAPP β and a lower level of glial-derived neurotrophic factor (GDNF) were detected in ADEs from AD patients compared with controls (Goetzl et al., 2016b).

Analysis of EVs' cargo in a mixed co-culture of astrocytes, neurons, and oligodendrocytes treated with synthetic A β 42 protofibrils showed significantly higher levels of apolipoprotein E (apoE) in the isolated EVs. Interestingly, the apoE-containing EVs were primarily derived from the astrocytes in the co-culture system (Nikitidou et al., 2017). Additionally, exposure of astrocytes to synthetic A β peptide increased the secretion and diameter of ADEs, with higher levels of ceramide and GM1 ganglioside (involved in A β aggregation, seeding, and sequestration) loaded as cargo (Dinkins et al., 2016). Furthermore, microglia in the brain have been shown to be positively correlated with tau pathology, and their involvement in tau propagation was suggested to be mediated by their EVs (Asai et al., 2015). MDEs isolated from AD brains were reported to be loaded with higher levels of disease-associated microglia markers, such as ferritin heavy chain 1 (FTH1) and triggering receptor expressed on myeloid cells 2 (TREM2), along with higher levels of tau and proinflammatory lipids (Cohn et al., 2021). EVs secreted from affected brain cells transmit pathologic cargo to other

brain cells, contributing to disease propagation. For example, EVs isolated from frozen brain tissues of human AD, prodromal AD, and nondementia control individuals showed higher levels of tau in AD brain tissue derived EVs. Injecting these tau-containing EVs into the hippocampus of 18-month-old female wild-type mice induced the accumulation of oligomeric and fibrillar tau in mouse brain compared with recipients of prodromal or control EVs, demonstrating the potential for tau propagation loaded in EVs (Ruan et al., 2021). Further, You et al. (2022) identified brain cell type-specific markers from human-induced pluripotent stem cell-derived neural cell types, including excitatory neurons (ATP1A3, NCAM1), astrocytes [lipoprotein receptor-related protein 1 (LRP1), integrin subunit α 6 (ITGA6)], microglia-like cells [integrin subunit α M (ITGAM), lymphocyte cytosolic protein 1 (LCP1)], and oligodendrocyte-like cells (LAMP2, FTH1). Using label-free quantitative liquid chromatography with tandem mass spectrometry proteomics analysis, they demonstrated that proteins enriched in ADEs were most significantly associated with AD pathology and cognitive impairment. Moreover, a significantly elevated level of integrin β 1 (ITGB1) was also reported in ADEs enriched from total brain tissue EVs of AD subjects, which was further associated with brain A β and tau load in an independent cohort.

Synaptic dysfunction and loss are common early features and contributing factors in AD pathogenesis and positively relate to the severity of dementia (Masliah et al., 2001; Selkoe, 2002; Reddy et al., 2005; Morrison and Baxter, 2012). Increased levels of both pre- and postsynaptic proteins, such as SNAP-25, GAP-43, postsynaptic density protein-95 (PSD-95), synaptotagmin-1, and neurogranin, have been reported in CSF at early-stage AD due to their release from degrading synapses (McGrowder et al., 2021; Kivisakk et al., 2022). However, contrary to their CSF levels, decreased levels of synaptic proteins, such as SNAP-25, neurogranin, PSD-95, neuron pentraxin 2 (NPTX2), AMPA4, neuroligin 1 (NLGN1), and α -neurexin 2 (NRXN2 α), have been reported in plasma NDEs of AD dementia patients compared with preclinical AD individuals with normal cognition (Goetzl et al., 2018a). Additionally, other risk factors involved in neuroinflammation [e.g., complement component 1q (C1q)], metabolism disorder [e.g., P-S312-insulin receptor substrate-1 (IRS-1)], neurotrophic deficiency [e.g., hepatocyte growth factor (HGF)], vascular injury [e.g., vascular endothelial growth factor D (VEGF-D)], and autophagy-lysosomal system dysfunction (e.g., cathepsin D) have higher levels in NDEs of AD patients, supporting the usefulness of EV-based protein biomarkers as a clinical blood test for AD (Liu et al., 2022).

2. microRNAs in Extracellular Vesicles as Potential Biomarkers for Alzheimer's Disease. The analysis of proteins in EVs has shown great promise in developing biomarkers for AD, but accuracy and sensitivity could still be dependent upon the choice of method used for

their detection. In this regard, besides proteins, developing EV-based miRNA biomarkers holds a few advantages. Quantitative assessment of miRNAs using real-time PCR is much more sensitive than ELISA or western blotting, and it is cost effective, too. Furthermore, the analysis of miRNAs can also provide information about altered molecular pathways and possible druggable targets. Approximately 70% of experimentally detected miRNAs have been reported to be expressed in the CNS, and their expression is dynamically and precisely regulated during brain development and neuronal maturation (Nowak and Michlewski, 2013). Any aberrant change in these miRNAs may contribute to abnormal brain function and pathologies. The analysis of altered expression of several miRNAs in CSF, plasma, and brain tissue has shown promise as AD biomarkers to predict the disease progression and identify potential therapeutic targets (Swarbrick et al., 2019; Zhang et al., 2019b), although their brain cell type specificity remains in question. Moreover, analyzing the miRNAs' expression in biofluids may show a poor correlation with the brain tissue primarily because the circulatory miRNAs in biofluids are contributed by many different cell types in the body. Since EVs released from different brain cell types are loaded with miRNA cargo and can cross the blood-brain barrier to enter circulation, analyzing miRNAs' expression in various BDEs from blood holds promise as novel biomarkers for the diagnosis of AD.

Although all forms of nucleic acids, including DNA, mRNA, miRNAs, and long noncoding RNAs (lncRNAs), are detected in EVs, they are highly enriched in miRNAs (Huang et al., 2013). Several studies have profiled the expression of miRNAs in total EVs from plasma, serum, and CSF as well as in specific BDEs (Cheng et al., 2015; Lugli et al., 2015; Cha et al., 2019; Upadhyay et al., 2020; Sproviero et al., 2021; Vandendriessche et al., 2022; Kumar et al., 2023). The expression analysis of several miRNAs, like miR-193b, miR-223, miR-135a, miR-384, miR-23a, miR-100, and others, in plasma or serum samples has shown the potential to discriminate AD patients from healthy controls (Vandendriessche et al., 2022). In a comprehensive profiling of cell-free miRNAs in CSF and blood using next-generation deep sequencing, Burgos et al. (2014) reported differential expression of 41 miRNAs in the CSF and 20 miRNAs in the serum of AD patients compared with healthy controls. Most studies analyzing the expression of circulatory miRNA in different biofluids and brain tissues have shown low expression of miRNAs in AD condition but with poor consistency and lack of reproducibility (Burgos et al., 2014; Kiko et al., 2014; Denk et al., 2015; Riancho et al., 2017). Moreover, most studies reported a poor correlation between the expression of miRNAs in the brain tissues and biofluids but improved consistency with EVs. For example, miR-9, a brain-specific miRNA highly expressed in the brain to regulate several cellular and developmental processes (Kapsimali et al.,

2007; Coolen et al., 2013; Millan, 2017), has shown higher expression levels in late-stage AD in brain tissues compared with controls (Lukiw, 2012; Chen et al., 2021b). The expression of miR-9-5p has been found to be reduced in the CSF, but interestingly, analysis of miR-9-5p expression in exosomes enriched from CSF showed an inverse expression pattern, in agreement with data from AD brain tissue, showing high expression in CSF-enriched exosomes in AD subjects (Riancho et al., 2017). In a comparative study of miRNA fingerprinting from frontal cortex homogenate EVs and serum-derived EVs of AD and control subjects, Cheng et al. (2020) reported upregulated expression of disease-associated miRNAs in BDEs, which correlated well with miRNA profiles obtained from matching total brain homogenate. However, the expression of miR-486-3p was found to be enriched in both AD and control brain tissue-derived EVs, suggesting that this miRNA may be regularly packaged into brain tissue-derived EVs during disease or physiologic states and play an important role in CNS development and cell motility. Furthermore, miRNA changes in the brain tissue and brain tissue-derived EVs were also correlated with those detected in peripheral EVs collected from the serum of AD subjects, and a panel of miRNA was suggested as a liquid brain biopsy (Cheng et al., 2020). Similarly, the assessment of dysregulated miRNAs in AD brain tissue and plasma NDEs confirmed the decreased expression of miR-132 and miR-212 both in the brain and plasma NDEs of AD individuals, showing diagnostic utility in separating controls from MCI and AD (Cha et al., 2019). Analysis of miRNAs in NDEs isolated from the plasma of 40 AD and 40 healthy individuals showed increased expression of miR-23a-3p, miR-223-3p, and miR-190a-5p, whereas decreased expression of miR-100-3p, in NDEs of AD individuals compared with controls (Serpente et al., 2020). Furthermore, the analysis of miRNA expression in NDEs isolated from the plasma of AD and healthy individuals via next-generation sequencing revealed let-7e as the most dysregulated (increased) miRNA in AD, showing excellent diagnostic potential (Durur et al., 2022).

Although AD research has traditionally been focused on neuronal dysfunction, growing evidence suggests that changes in the cell types involved in maintaining the integrity of the neural system also contribute to AD pathology (De Strooper and Karran, 2016; Henstridge et al., 2019). Therefore, analyzing the role of specific non-neuronal cell types in the CNS could provide a holistic understanding of the distinct and chronological cascades as well as novel molecular biomarkers associated with AD pathogenesis. Moreover, the cross communication of different cells in the brain microenvironment is mediated through the transfer of miRNAs via EVs. Different BDEs loaded with miRNAs establish crosstalk among brain cells and could also affect the normal physiologic function of the recipient cells in the brain and peripheral system

(Morel et al., 2013; Bahrini et al., 2015). For example, miRNAs loaded in NDEs have been shown to affect astrocytes, microglia, and neurovascular integrity (Morel et al., 2013; Bahrini et al., 2015; Xu et al., 2017). Similarly, under inflammatory conditions, alterations in miRNAs cargos (like miR-125a-5p, miR-16-5p, and miR-34a) have been reported in ADEs, which affects the synaptic stability of target neurons and their ability to support neuron survival or exacerbate neurodegeneration (Mao et al., 2015; Chaudhuri et al., 2018). To establish the alterations in miRNAs expressions in different BDEs, we performed comprehensive profiling of eight key miRNAs (miR-9-5p, miR-29a-5p, miR-106b-5p, miR-107, miR-125b-5p, miR-132-5p, miR-135b-5p, and miR-210-3p) in NDEs, ADEs, MDEs, ODEs, PDEs, and EDEs simultaneously isolated from the plasma of control individuals and individuals at different stages of dementia (Kumar et al., 2023). We observed that different miRNAs showed altered expression in various BDEs and exhibited excellent diagnostic potential in identifying dementia status. Interestingly, we reported that the expression of hypoxia-specific miRNA, miR-210-3p, increased in NDEs and ADEs in prodromal dementia, possibly due to vascular dysfunction. This observation is supported by the finding that hypoxia induced the expression of miR-132 in mouse and human brain microvascular endothelial cells, which, in turn, caused disruption to the blood-brain barrier (Burek et al., 2019; Kumar et al., 2023). Moreover, the expression of miR-132 and miR-135b in PDEs and EDEs could distinguish overall dementia from control individuals (Kumar et al., 2023).

3. Lipid Cargo of Extracellular Vesicles as Novel Biomarkers for Alzheimer's Disease. In addition to profiling proteins and miRNA cargos, lipid analysis of EVs, predominantly isolated from brain tissue, has also provided evidence of dysregulation in AD samples, which can be attributed to lipid imbalance in AD (Wang et al., 2012; Cohn et al., 2021; Su et al., 2021). A multiomics analysis of microglial (CD11b-positive) EVs from cryopreserved human brain tissue and the parietal cortex of four late-stage AD (Braak V and/or VI) and three age-matched normal/low pathology cases revealed dysregulation of 594 lipids identified by targeted lipidomics. The study presented an overall proinflammatory lipid profile, endolysosomal dysfunction, and a significant AD-associated decrease in the levels of docosahexaenoic acid (DHA)-containing polyunsaturated lipids (Cohn et al., 2021).

Together, to achieve a more comprehensive understanding of AD pathogenesis, biomarker development, and the identification of novel avenues for patient stratification and therapy, it is essential to analyze dynamic changes in proteins, lipids, and miRNAs expression across different brain cell types. Since BDEs offer a unique tool to simultaneously study such molecular alterations in peripheral circulation, these EVs could be a potential adjuvant

biomarker to support the existing methods for AD diagnosis and prognosis.

B. Parkinson's Disease

PD is the second most prevalent age-associated neurodegenerative disorder after AD, caused by the selective destruction of dopaminergic neurons, resulting in unintended or uncontrollable body movements (Jankovic, 2008; Massano and Bhatia, 2012). Clinically, PD is characterized by four fundamental features, which can be grouped under the acronym TRAP: tremor at rest, rigidity, akinesia (or bradykinesia), and postural instability (Jankovic, 2008). Additionally, PD is associated with other nonmotor symptoms, such as disturbed sleep, disturbed autonomic function, psychiatric problems, and constipation. Despite several studies suggesting that both genetic and environmental factors contribute to the etiology of PD, the clear pathologic and etiological features of the disease remain unclear (Warner and Schapira, 2003). The diagnosis of PD is primarily based on neurological and physical examinations and sometimes medical and family history. However, PD is often not diagnosed until the advanced stage, when more than 80% of the dopaminergic neurons have degenerated. Moreover, the low fidelity of neurological examination in PD diagnosis necessitates the development of better biomarkers for accurate and early prediction of the disease. Furthermore, understanding the cellular and molecular changes underlying the degenerative processes and the development of novel biomarkers is crucial for improved disease management. Therefore, in recent years, there have been increasing efforts to develop liquid biopsy-based biomarkers using multiomics approaches for the early diagnosis of PD (Miller and O'Callaghan, 2015). However, like other neuropsychologic diseases, these blood-based biomarkers lack brain cell specificity.

1. Protein Cargo of Extracellular Vesicles as Promising Biomarkers for Parkinson's Disease. In the context of neurodegenerative diseases, including PD, EVs could be useful in multiple ways. For example, EVs could facilitate the expulsion of pathologic proteoforms and restrict their accumulation within the cells. However, the pathologic cargo of BDEs, when received by the other brain cells, can affect their normal physiologic function and contribute to the spread of the pathology. α -Synuclein, a presynaptic neuronal protein, is a major contributor to the pathogenesis of PD, mainly through its aberrant secreted soluble oligomeric conformations, and has been implicated as a genetic defect leading to PD (Stefanis, 2012). The analysis of α -synuclein levels in plasma or serum has yielded inconsistent outcomes and is inadequate as a biomarker for detecting PD, primarily due to the contribution of peripherally derived α -synuclein. Despite these limitations, α -synuclein is still by far the most studied biomarker for PD (Malek et al., 2014).

Compared with peripheral biofluids, the levels of α -synuclein in CSF is considered a more useful

biomarker for PD (Shi et al., 2014). However, collecting CSF is painful and not feasible for repeated measures over time. Therefore, plasma BDEs offer a unique opportunity to develop PD-related biomarkers. A study by Shi et al. (2014) demonstrated the presence of α -synuclein in plasma NDEs and reported approximately a two-fold higher concentration of α -synuclein in individuals with PD compared with age- and sex-matched healthy controls. However, this study used the isolation of NDEs directly from plasma using biotin-tagged L1CAM antibody, which could limit the isolation of NDEs due to the higher soluble form of L1CAM compared with membrane-bound L1CAM present on NDEs surface. Consequently, further studies employed a two-step method involving the isolation of total EVs from plasma followed by immunocapturing of NDEs. Niu et al. (2020) reported increased levels of α -synuclein in plasma NDEs in patients with early-stage PD. In a 22-month follow-up, they observed a longitudinal increase in α -synuclein level, which was associated with a higher risk for motor symptom progression in PD. Furthermore, the level of α -synuclein in NDEs correlated with Movement Disorders Society Unified Parkinson's Disease Rating Scale III scores, Non-Motor Symptom Questionnaire scores, and Sniffin' Sticks 16-item test scores of patients with PD. A large cross-sectional study involving 664 serum samples collected from multiple cohorts investigated individuals with various neurodegenerative disorders, including rapid eye movement sleep disorder, PD, dementia with Lewy bodies, multiple system atrophy (MSA), frontotemporal dementia, progressive supranuclear palsy, corticobasal syndrome, and controls. Longitudinal analysis from PD and control individuals demonstrated an overall increase in mean NDEs α -synuclein levels in prodromal and clinical PD when compared with MSA, controls, or other neurodegenerative diseases. More importantly, α -synuclein in NDEs was found to be elevated at an early stage and remained elevated with disease progression (Jiang et al., 2020).

Since the differential diagnosis of PD with atypical syndrome is challenging due to overlapping pathologic features, the analysis of plasma NDE for α -synuclein and aggregated tau was shown to efficiently distinguish PD from atypical Parkinsonian syndromes with high sensitivity and specificity (Meloni et al., 2023). In addition to NDEs, an increased level of α -synuclein was also shown in ODEs isolated from the plasma or serum of PD and MSA using the anti-MOG marker compared with healthy controls (Dutta et al., 2021). Interestingly, α -synuclein levels were found to be higher in NDEs compared with ODEs in the PD group, whereas the opposite trend was reported for MSA (Dutta et al., 2021). Contrary to these reports, Si et al. (2019) analyzed the levels of α -synuclein in serum-derived NDE from early-stage PD individuals and subdivided them into tremor-dominant and non-tremor-dominant groups, along with

individuals with essential tremor and healthy controls. Lower levels of α -synuclein were reported in the PD group compared with the essential tremor and healthy controls. However, among the PD groups, the levels were lower in the non-tremor-dominant group than in the tremor-dominant group, suggesting the biomarker potential of α -synuclein in NDE toward identifying PD from essential tremor and healthy controls as well as identifying different motor types in PD. Moreover, a recent study by Kluge et al. (2022) analyzed plasma NDEs for levels of α -synuclein from 30 PD patients and 50 healthy controls but did not find any significant difference between the two groups.

α -Synuclein is also known to interact with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, causing synaptic dysfunction. Analysis of NDEs for levels of oligomeric α -synuclein and the presynaptic SNARE complex proteins, including syntaxin-1A (STX-1A), vesicle-associated membrane protein-2 (VAMP-2), and SNAP-25, demonstrated higher levels of α -synuclein, whereas significantly lower levels of STX-1A and VAMP-2 were observed in PD patients compared with healthy controls (Agliardi et al., 2021). In addition to α -synuclein, cortical amyloid deposition is a common feature in PD dementia (PDD). Analysis of platelet-derived EVs, using nano-scale flow cytometry with fluorescence-labeled CD62P and A β 1-42 antibodies, showed a higher concentration of the A β 1-42 in the PDD group compared with the control. This has been proposed as a diagnostic and prognostic biomarker for PDD patients (Wang et al., 2023). Furthermore, considering the contribution of inflammation in the pathogenesis of PD, a recent study by Chan et al. (2023) profiled the levels of various cytokines in plasma EVs of PD patients compared with healthy controls. They reported significant changes in the levels of interleukin (IL)-1 β , tumor necrosis factor- α (TNF- α), and IL-6, which were found to be significantly associated with changes in the severity of postural instability, gait disturbance, and cognition between baseline and the 1-year follow-up. Similarly, comprehensive profiling of plasma EVs' surface markers related to inflammatory and immune cells from PD, matched healthy controls, MSA, and atypical parkinsonism with tauopathies, using flow cytometric multiplex bead-based platform, revealed that PD and MSA patients displayed a greater pool of overexpressed immune markers compared with atypical Parkinsonism with tauopathies (Vacchi et al., 2020). Additionally, DJ-1 (protein deglycase, also known as Parkinson's disease protein 7), a highly conserved dimer protein associated with inflammatory disorders involving PD, was found to be highly expressed in plasma NDEs of PD patients compared with healthy controls, suggesting its potential as a biomarker along with α -synuclein in PD (Zhao et al., 2019).

Mass spectrometry analysis of serum EVs from PD patients and healthy controls identified 14 proteins

that were significantly different in mild and severe PD patients compared with controls. Among these, pigmented epithelium-derived factor, afamin, and apolipoprotein D and J were significantly increased in PD patients, whereas the expression of seven proteins, including complement C1q and protein immunoglobulin λ variable 1-33 (IGLV1-33), were decreased in PD patients (Jiang et al., 2019). Interestingly, a recent mass spectrometry analysis of EVs isolated from urine samples of PD patients with and without mutations in the leucine-rich repeat kinase 2 (LRRK2) gene, as well as idiopathic PD patients, identified 4476 unique proteins and 2680 unique phosphoproteins. Many of these proteins are involved in key PD pathways, including autophagy, and were found to be elevated (Hadisuraya et al., 2023).

2. microRNAs in Extracellular Vesicles as Potential Biomarkers for Parkinson's Disease. Apart from protein-based biomarkers, numerous studies have suggested alterations in the miRNAs in EVs isolated from biofluids for biomarker studies in PD (Dutta et al., 2023). miRNAs mediate the regulation of almost all PD-related genes and are involved in the pathogenesis of PD, making them potential biomarkers. The analysis of miRNAs as biomarkers for PD has been performed in several biofluids, including CSF, plasma, serum, and brain tissue (Cao et al., 2017; Dos Santos et al., 2018; He et al., 2021; Valencia et al., 2022). In a study analyzing EVs derived from CSF of PD and age-correlated controls, using next-generation small-RNA sequencing, Caldi Gomes et al. (2021) presented the complete and quantitative microRNAome. They detected a total of 688 miRNAs, with 22 miRNAs showing differential expression in PD subjects compared with controls, the majority of which were upregulated in PD. Using a machine-learning approach, they suggested an iterative signature involving miR-126-5p, miR-99a-5p, and miR-501-3p, which could differentiate PD and control samples (Caldi Gomes et al., 2021). Another study analyzing miRNA expression in CSF exosomes using a TaqMan low-density array for human miRNA panel and further validation of differentially expressed miRNAs using quantitative PCR (qPCR) revealed reduced expression of 11 miRNAs and increased expression of 16 miRNAs in PD patients compared with healthy controls. Further analysis of these dysregulated miRNAs in an independent cohort identified miR-1 and miR-19b-3p as significantly reduced, whereas miR-153, miR-409-3p, miR-10a-5p, and let-7g-3p were significantly overexpressed in CSF exosome from the PD group (Gui et al., 2015). Similarly, the analysis of serum EVs identified several miRNAs that showed differential expression patterns between PD and controls. Several miRNAs, like miR-34a-5p, miR-155-5p, miR-24, and miR-195, were found to be significantly elevated, whereas miR-146a, miR-19b, and miR-125b were reported to be

reduced in the serum EVs of PD patients compared with healthy controls (Cao et al., 2017; Caggiu et al., 2018; Fan et al., 2020; Grossi et al., 2021). Interestingly, the reduced expression of miR-125a-5p correlated with the increased expression of lncRNA brain-derived neurotrophic factor (BDNF)-antisense and was suggested as a potential therapeutic target for PD (Fan et al., 2020).

The analysis of miRNAs' expression in plasma EVs revealed increased expression of miR-331-5p, miR-30c-2-3p, and miR-411-5p as well as reduced expression of miR-505, miR-15b-5p, miR-138-5p, miR-338-3p, miR-106b-3p, miR-431-5p, and miR-146a-5p in PD patients compared with healthy controls. Among these miRNAs, miR-331, miR-505, and the combination of miR-15b-5p, miR-30c-2-3p, miR-138-5p, and miR-106b-3p showed valuable diagnostic potential as identified by the area under the curve (Yao et al., 2018; Xie et al., 2022). In a study by Wang et al. (2020), next-generation sequencing was performed on plasma exosome samples from PD patients and age- and sex-matched healthy individuals, identifying 15 upregulated and 24 downregulated exosomal lncRNAs in the PD group. Importantly, the expression of lnc-MKRN2-42:1 was positively correlated with the Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale III score for PD patients. Similarly, the level of lnc-POU3F3 in the plasma NDE showed a positive correlation with motor dysfunction or nonmotor symptoms in PD patients (Zou et al., 2020).

3. Lipid and Carbohydrate Cargo of Extracellular Vesicles as a Novel Source for Biomarkers for Alzheimer's Disease. Beyond protein and miRNA-based biomarkers, Gualerzi et al. (2019) studied the biochemistry of EVs using Raman spectroscopy and identified significant biochemical differences in lipids and saccharides of PD patients from controls. The authors suggested that after validation, the Raman spectrometry approach for characterizing EVs could be developed into a reliable, automatable, and sensitive method for stratifying PD patients.

Overall, these studies have demonstrated the potential utility of EVs in developing less invasive and reliable biomarkers for PD.

C. Schizophrenia

SCZ is a complex, multifactorial psychiatric disorder that mainly occurs in late adolescence and early childhood. It is characterized by symptoms such as hallucinations, delusions, apathy, anhedonia, and cognitive deficits. This disorder shows a heritability rate of approximately 80% (Lichtenstein et al., 2009; Correll and Howes, 2021; Sun and Chen, 2023). Despite numerous attempts to study the brain pathology of SCZ, no specific pathologic changes have been definitively identified in post-mortem brains, unlike other psychiatric disorders, such as AD and PD. The diagnosis of SCZ primarily relies on a relatively subjective assessment of symptoms, medical and family history, and clinical interview-based tools

(Hany et al., 2023). Unfortunately, there are no objective biomarkers available, leading to a high rate of misdiagnosis, particularly with psychotic bipolar disorder (Wang et al., 2022). However, few reports in consensus suggested that cortical pathology could be a fundamental feature of SCZ [reviewed in Sun and Chen (2023)]. There is some evidence suggesting the molecular and cellular etiology of SCZ, including aberrant connectivity in glutamatergic, GABAergic, dopaminergic, serotonergic neurotransmission, and other neuronal networks. These abnormalities create an imbalance in excitation and inhibition and play a pivotal role in SCZ (Yizhar et al., 2011; Lisman, 2012; Gao and Penzes, 2015; Liu et al., 2021; Sun and Chen, 2023). Additionally, synaptic disturbance (synaptopathy) has been implicated in the pathogenesis and/or pathophysiology of SCZ (Hayashi-Takagi, 2017; Obi-Nagata et al., 2019). Dystrobrevin binding protein 1 (DTNBP1), which encodes dysbindin, is a known risk gene for SCZ. Dysbindin serves as a synaptic protein by forming multiple complexes and regulating the transport of neurotransmitters and receptors (Straub et al., 2002; Ghiani and Dell'Angelica, 2011). The aggregation of dysbindin-1B can affect neuronal viability and can be transferred among primary cortical neurons via exosomes with high efficiency (Zhu et al., 2015). Moreover, studies have demonstrated the long-distance propagation of dysbindin-1B aggregates from fimbria (site of injection of exosomes containing dysbindin-1B) to the molecular layer of the entire hippocampus, suggesting the exosomes-mediated propagation of neurotoxic proteins and their accumulation in the brain of SCZ mice (Zhu et al., 2015).

1. Protein Cargo of Extracellular Vesicles as Potential Biomarkers for Schizophrenia. Cognitive dysfunction is widely accepted as a fundamental characteristic of SCZ that typically manifests from the onset of the disorder and significantly contributes to disability (Bora et al., 2010; Guo et al., 2019). The higher glucose levels in the brain, identified using magnetic resonance spectroscopy, and lower levels of neuronal insulin resistance biomarkers in NDEs have been suggested as potential biomarkers and a measure of memory impairment for SCZ (Wijtenburg et al., 2019). Although the involvement of A β and tau pathology in cognitive impairment during AD pathogenesis is well established, their role in SCZ remains unclear. A study conducted by Lee et al. (2021) analyzed the levels of A β 1-42, A β 1-40, and p-T181-tau in plasma-derived NDEs and ADEs from control individuals and those with SCZ. The study found significantly higher levels of ADE A β 1-42 in SCZ patients, whereas other exosomal markers showed no significant difference between the two groups. Additionally, higher levels of ADE p-T181-tau were associated with worse executive function. Furthermore, neuroinflammation is a well established feature in a subset of individuals with SCZ, with a significant increase in inflammatory markers. The elevated

expression of the astrocyte marker glial fibrillary acidic protein (GFAP) is known to be associated with increased astrocytic activation and inflammation in the CNS. Interestingly, a significantly higher concentration of GFAP was observed in plasma EVs of individuals with SCZ compared with controls, suggesting a possible astrocyte pathology (Ranganathan et al., 2022).

2. Extracellular Vesicle-Derived microRNAs as Potential Biomarkers for Schizophrenia. Since the analysis of post-mortem brain tissue did not reflect any significant and distinct phenotypic changes in the brains of individuals with SCZ, establishing protein-based biomarkers may be challenging. As a result, parallel research efforts have focused on analyzing the expression of miRNAs in EVs for biomarker development. The first genome-wide analysis of miRNA expression profiling in serum exosomes, conducted in 49 drug-free, first-episode SCZ patients and 46 controls (divided into training and testing groups), identified several miRNAs, including hsa-miR-206, hsa-miR-145-5p, hsa-miR-133a-3p, hsa-miR-144-5p, hsa-miR-144-3p, and hsa-miR-184, that exhibited more than a twofold alteration in SCZ patients. These miRNAs were predicted to regulate protein glycosylation and pathways associated with neurotransmitter receptor and dendrite (spine) development. Additionally, 11 miRNAs were suggested to differentiate between SCZ patients and control individuals with high specificity and sensitivity. Further validation of these differentially expressed miRNAs in 100 SCZ patients and 100 controls using qPCR identified increased expression of miR-206 levels in blood exosomes of SCZ patients, which was consistent with the downregulation of its target, BDNF, in the blood (Du et al., 2019). Another study analyzed miRNA expression in EVs isolated from frozen post-mortem prefrontal cortices of SCZ patients using a Luminex FLEXMAP 3D microfluidic device and validated the findings through qPCR. This analysis revealed significantly increased expression of miR-497 in SCZ samples compared with control samples (Banigan et al., 2013). Similarly, miR-223, an exosome-secreted miRNA, targets N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits to regulate neuronal function. Overexpression of miR-223 in the orbitofrontal cortex of SCZ and unaffected control individuals was identified at the mature miRNA level of SCZ and compared with control (Harrasz et al., 2012; Amoah et al., 2020). Interestingly, miR-223 was primarily enriched in cultured mouse astrocytes (as opposed to neurons) and in their exosomes. When co-cultured with rat cortical neurons, these ADE increased neuronal miR-223 expression and downregulated *Gria2* and *Grin2b* levels (Amoah et al., 2020).

Circular RNAs (circRNAs), which function as miRNA sponges, play a crucial role in gene regulation at various levels, including the post-translational level, by sequestering proteins (Chen, 2020). Aberrant expression of circRNAs in the dorsolateral prefrontal

cortex of SCZ patients was found to be positively correlated with the age of SCZ onset (Liu et al., 2019), suggesting the potential of EV circRNAs as early biomarkers for SCZ (Guo et al., 2023). Tan et al. (2021) conducted the first comparison of alterations in plasma exosomal circRNAs between SCZ patients and matched healthy controls using high-throughput sequencing. They proposed that 44 plasma exosomal circRNAs exhibited differential expression in SCZ patients compared with healthy controls. Further validation of these differentially expressed circRNAs through qPCR confirmed the differential expression of four circRNAs, which contained binding sites for many microRNAs and were predicted to play a crucial role in metabolic process, stress response, and histone ubiquitination (Tan et al., 2021). Additionally, the expression of lncRNAs was also analyzed in the serum exosomes of SCZ patients and control individuals. Guo et al. (2022) analyzed the expression of 15 lncRNAs in the serum exosomes from 152 subjects to evaluate their diagnostic potential for SCZ by generating receiver operating characteristics curves. They found that the expression of lncRNAs MIAT was increased, whereas PVT1 was decreased, in SCZ patients compared with the control group.

3. Metabolites in Extracellular Vesicles as Novel Biomarker for Schizophrenia. In addition to protein and RNA-based EV biomarkers, there has been at least one study performed that profiled the exosome-derived metabolites in patients with SCZ and healthy controls using ultra-performance liquid chromatography–tandem mass spectrometry. This study involved a large cohort of 385 SCZ patients and 332 healthy controls. The analysis revealed 25 perturbed metabolites in SCZ patients compared with healthy controls, demonstrating high diagnostic performance. Bioinformatics analysis of these metabolites identified enrichment in pathways such as glycerophospholipid metabolism, which is known to be implicated in SCZ (Du et al., 2021).

Collectively, these studies highlight the potential utility of EV-based biomarkers in enhancing our understanding of SCZ pathology.

D. Bipolar Disorder

BD is characterized by recurring episodes of mania or hypomania alternating with depression. It ranks as the sixth leading cause of disability worldwide, affecting approximately 5% of the population and leading to serious consequences (Colombo et al., 2012). BD is widely recognized as a heritable disease, with numerous genetic loci identified through genome-wide association studies (Craddock and Sklar, 2013). Additionally, several factors, including childhood emotional abuse, stressful or traumatic life events, and interactions between multiple genetic, neurochemical, and environmental factors, have been linked to the development of BD (Rowland and Marwaha, 2018). The molecular mechanisms associated with the pathophysiology of BD involve

alterations in neurotropic signaling molecules, such as BDNF, nerve growth factor, neurotrophin-3 (T-3), and neurotrophin-4 (NT-4), along with oxidative stress, immune-inflammatory imbalance, mitochondrial dysfunction, and compromised hypothalamic-pituitary-adrenal axis (Scaini et al., 2020). Although analyzing the levels of these proteins in BDEs could serve as a potential biomarker for BD, supporting studies are still lacking.

1. Protein Cargo of Extracellular Vesicles as Potential Biomarkers for Bipolar Disorder. The dysregulation of insulin signaling is considered a characteristic feature of BD (Stahl et al., 2019) as evidenced by the high prevalence of metabolic disorders, such as type 2 diabetes mellitus and obesity, in individuals with BD (Vancampfort et al., 2015). NDEs isolated from the plasma of patients with bipolar depression were used to assess the treatment response of infliximab, a TNF- α antagonist known to affect insulin signaling. The levels of neuronal insulin signaling molecules, particularly pS312-IRS-1, as well as related canonical pathways [Akt, glycogen synthase kinase 3 β (GSK-3 β), and p70 ribosomal S6 kinase (p70S6K)] and alternative pathways (ERK1/2, JNK, and p38-MAPK), were evaluated in NDEs. Based on preliminary results, the authors reported that BD patients treated with infliximab showed concerted changes in NDE cargos related to the insulin signaling pathway and hypothesized this pathway as a relevant pathophysiological mechanism and a potential target for interventions in BD (Mansur et al., 2021).

2. Extracellular Vesicle-Derived microRNAs as Potential Biomarkers for Bipolar Disorder. In a preliminary investigation, the expression of miRNAs in plasma-derived EVs from 20 patients with type 1 BD and 21 age- and sex-matched healthy controls was examined. This analysis identified 33 miRNAs that were nominally altered in BD, including miRNAs that were previously reported (such as miR-133a, miR-29c, and miR-22) to be altered in post-mortem BD patients' brain tissue, primarily the anterior cingulate cortex and prefrontal cortex (Kim et al., 2010; Azevedo et al., 2016; Fries et al., 2019). Although detailed studies specifically analyzing different BDEs in the biofluids of BD patients are currently lacking, such studies in the future could be crucial in the development of novel biomarkers for BD cases.

3. Extracellular Vesicle Metabolites as Biomarkers for Bipolar Disorder. Alterations in the insulin signaling pathway can contribute to a series of metabolic dysfunctions. Several studies have indicated increased lactate levels and reduced intracellular pH in the brains of individuals with BD, which may be indicative of mitochondria dysfunction, impaired metabolism, change in morphology, increased mitochondria polymorphism, altered mitochondrial respiration, and glycolytic shift (Clausen et al., 2001; Scaini et al., 2016). Analysis of serum exosomes from BD patients using liquid chromatography-tandem mass spectrometry identified 26 differentially

expressed serum exosomal metabolites in patients with BD compared with healthy control, primarily related to sugar metabolism pathways. Further analysis revealed that 15 exosomal metabolites could accurately distinguish BD patients from controls, with 0.838 accuracy (95% confidence interval, 0.604–1.00) in the training set and 0.971 accuracy (95% confidence interval, 0.865–1.00) in the testing set (Du et al., 2022b).

E. Major Depressive Disorder

MDD is one of the most prevalent neuropsychiatric pathologies, characterized by a wide range of symptoms, including depressed mood, diminished interests, anhedonia, impaired cognitive function, fatigue or loss of energy, and vegetative symptoms, such as disturbed sleep or appetite (Otte et al., 2016; Bernaras et al., 2019). The clinical diagnosis of MDD is based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, which requires the presence of at least five of the aforementioned symptoms, of which one must have a depressed mood or anhedonia (Bains and Abdijadid, 2023). Etiologically, MDD is a multifactorial disorder involving biological, genetic, environmental, and social factors. However, due to symptom overlap with other psychiatric diseases and the absence of specific biomarkers, MDD is often misdiagnosed, which can impact the disease's progression and prognosis.

1. Protein Cargo as Potential Biomarkers for Major Depressive Disorder. Similar to bipolar disorder, dysfunction in insulin signaling and mitochondrial metabolism, leading to oxidative stress and increased production of reactive oxygen species, has been suggested as a pathologic feature of MDD (Brivio et al., 2022). Increased concentration of IRS-1 was reported in the plasma NDEs (L1CAM⁺ EVs) of individuals with MDD compared with age- and sex-matched controls. Furthermore, the increased concentration of IRS-1 in NDEs of MDD participants was found to be associated with suicidality and anhedonia (Nasca et al., 2021). In addition, a study by Goetzl et al. (2021) analyzed NDEs isolated from the plasma of individuals with MDD for the levels of 14 neuronal mitochondrial proteins involved in mitochondrial biogenesis, dynamics and function maintenance, mitochondrial energy metabolism, neuroprotection, and regulation of neuronal metabolism. The study suggested differential levels of most of these proteins in NDEs of individuals with MDD compared with age- and sex-matched healthy controls.

Several studies have also indicated a strong correlation between members of the fibroblast growth factor (FGF) system and depression (Deng et al., 2019), with significantly higher peripheral levels of FGF2, mainly secreted by astrocytes, in individuals with MDD compared with healthy controls (Wu et al., 2016). However, it remains to be assessed whether the higher peripheral FGF2 is loaded in ADEs, which could be an interesting parameter to investigate in individuals with

MDD. Similarly, the sigma-1 receptor (Sig-1R), an upstream regulator of endoplasmic reticulum stress and endoplasmic reticulum–mitochondrial signaling, was found to be significantly enriched in the plasma EVs in animal models of depression as well as in patients compared with healthy controls (Wang et al., 2021). Interestingly, injecting exosomes from depression mouse models or patients into lipopolysaccharide-challenged mice was reported to exert antidepressant-like effects and prevent neuroinflammation (Wang et al., 2021).

EVs have also been shown to be involved in the neuroinflammation process in MDD (Duarte-Silva et al., 2022). In an exploratory pilot case-control study, NDE-related blood-based biomarkers were analyzed using a novel sandwich immunoassay. In this assay, SNAP-25 antibody (neuronal marker) was used to capture NDEs, which were then analyzed for the levels of other markers related to neuroinflammation and synaptic functions along with a typical exosomal marker (CD81). Importantly, this study suggested that during the course of depression, certain proteins related to neuroinflammation (such as IL-34) and synaptic functions (such as synaptophysin) might be carried into peripheral blood via exosomes (Kuwano et al., 2018). Analyzing the expression of these proteins in NDEs could be useful for the objective clinical evaluation of MDD. Similarly, another study by Xie et al. (2023b) assessed the levels of inflammation-related markers in serum samples and ADEs of 70 individuals with MDD and 70 healthy matched controls. The serum samples from MDD subjects showed higher levels of TNF- α and IL-17A and a low level of IL-12p-70 compared with the healthy controls. Interestingly, in ADEs, the levels of all analyzed inflammatory markers (interferon- γ , IL-12p70, IL-1 β , IL-2, IL-4, IL-6, TNF- α , and IL-17A), except IL-10, were found to be elevated in MDD subjects. Previous studies have shown that reactive astrocytes induce inflammation in a mouse model through the NF- κ B pathway, leading to increased secretion of IL-6 and IL-1 β (Chen and Wang, 2017).

2. Extracellular Vesicle-Derived microRNAs as Biomarkers for Major Depressive Disorder. A small study analyzed the expression of miRNAs from plasma EVs of four treatment-resistant depression patients and four healthy subjects using miRNA sequencing. The study revealed a significantly dysregulated miRNA expression profile, with significant upregulation of miR-335-5p and downregulation of 1292-3p. Pathway analysis predicted that these differentially regulated miRNAs could affect postsynaptic density and axonogenesis as well as pathways related to axon formation and cell growth (Li et al., 2021).

Most studies investigating miRNA expression in EVs have been conducted in animal models of depression. The increased level of miR-207 was found in EVs isolated from the cultured natural killer cells from unstressed mice, which directly targeted Tril

(TLR4 interactor with leucine-rich repeats) and inhibited NF- κ B signaling in astrocytes, thereby reducing the release of proinflammatory cytokines (Li et al., 2020). Similarly, expression profiling of serum EVs in a chronic unpredictable mild stress-induced rat model and control rats, using high-throughput sequencing and subsequent expression validation by qPCR, revealed differential expression of several miRNAs, including miR-146a-5p, miR-122-5p, miR-133a-3p, miR-206-3p, miR-187-3p, and miR-1b (Fan et al., 2022). Moreover, an increased level of miR-146a-5p was shown in the CSF of chronic unpredictable mild stress rats compared with controls, and this miRNA was predicted to be closely associated with signaling pathways regulating the pluripotency of stem cells (Fan et al., 2022). Previous research suggested that miR-146a-5p is highly enriched in microglia, absent in hippocampal neurons, and expressed at low levels in astrocytes (Jovičić et al., 2013). Increased expression of miR-146a-5p in serum EVs was suggested to be primarily contributed by microglia, and MDE-mediated transfer of miR-146a-5p to neurons was shown to affect proliferation and differentiation of neuronal stem cells (Fan et al., 2022). Apart from cargo analysis of EVs, the size of plasma NDEs was also measured between control and MDD individuals, revealing smaller sizes of both total EVs and NDEs in MDD subjects compared with controls (Saeedi et al., 2021).

In conclusion, various EVs in the brain participate in signaling pathways related to inflammation, neurogenesis, synapse formation, and neuroprotection and have the potential to be developed as biomarkers of MDD.

F. Substance Use Disorders

EVs are emerging as useful tools in identifying molecular biomarkers associated with the pathophysiology of drug misuse and its associated side effects (Hu et al., 2012, 2018; Dominy et al., 2014; Momen-Heravi et al., 2015; Saha et al., 2016; Li et al., 2018a; Rao et al., 2018; Duan et al., 2019; Shahjin et al., 2019; Kodidela et al., 2020; Kumar et al., 2020; Chen et al., 2021a; Zhang et al., 2021). Chen et al. (2022) conducted a study on 120 patients with SUDs and reported stage-specific alterations in exosomal miRNA and neurotransmitters in the peripheral circulation. They successfully identified critical exosomal miRNAs associated with neurotransmitters and psychologic comorbidities. Notably, they suggested that plasma exosomal miRNAs, particularly has-miR-451a and has-miR-21-5p, could be useful in monitoring substance dependence and withdrawal. They also reported, in heroin and methamphetamine users, a relationship between exosomal markers and anxiety using the Hamilton-Anxiety scale. Dominy et al. (2014) identified unique proteins in the saliva of HIV⁺ heroin-dependent people that were associated with a neurocognitive disorder, and most of the identified proteins were associated with exosomes. In another example highlighting the

use of peripheral markers to identify clinical conditions, Darbinian et al. (2022) used blood from pregnant women who consumed alcohol and found significant correlations between the markers of fetal BDEs, isolated from maternal blood, and eye diameter of the fetus; the levels of myelin basic protein in fetal BDEs predicted fetal alcohol spectrum disorder.

Several preclinical studies have incorporated within-subjects designs to examine how substance use alters peripheral and central markers. For example, Li et al. (2018b) assessed the expression of miRNAs in serum exosomes and in the hippocampus of rats following methamphetamine exposure. They noted that the biological function of differentially expressed miRNAs' target genes correlated between serum exosomes and hippocampus. They also reported that rhynchophylline treatment could block the alterations in behavior and the expression of several differentially expressed miRNAs induced by methamphetamine exposure. In another study, the same group identified key miRNAs in serum exosomes associated with methamphetamine-dependent and also ketamine-dependent rats (Li et al., 2018a). Using brain-derived EVs, Koul et al. (2020) reported sex-specific differences in EV biogenesis, protein cargo signatures, and molecular pathways associated with long-term nicotine self-administration. Jarvis et al. (2020), using CD63-GFP^{fl+} exosome reporter mice, reported that cocaine self-administration strongly reduced the internalization of neuronal exosomes by astrocytes and microglia cells, which can be effectively reversed by extinction training. Hu et al. (2012) reported that astrocytes treated with morphine and HIV Tat secreted exosomes containing miR-29b that could be taken up by the neurons, resulting in neuronal death. This study also demonstrated that exosomal miR-29b was associated with morphine's in vivo effects. Toyama et al. (2017) identified several circulating miRNAs that were differentially expressed following oxycodone treatment in males and could serve as a surrogate of μ -opioid receptor signaling. Shahjin et al. (2019) identified distinct brain-derived EVs' miRNA signatures associated with in utero and postnatal oxycodone exposure. Our recent study also reported the usefulness of CSF-derived EVs in assessing the transgenerational adverse effects due to prenatal cocaine exposure in adult female and male rhesus monkeys (Rather et al., 2022). Proteomics analysis of CSF EVs suggested that prenatal cocaine exposure could potentially be associated with long-term neuroinflammation and an increased risk of neurodegenerative diseases.

In the field of SUD research, the role of EVs in understanding the interaction between neurons and glial cells has also been studied. Meng et al. (2020) reported that methamphetamine exposure increased the secretion of pathologic phosphorylated- α -synuclein in exosomes by neurons. When these exosomes were added to the astrocyte culture, phosphorylated- α -synuclein was

transferred to astrocytes and induced a proinflammatory response. Hu et al. (2018) reported that EVs derived from astrocytes exposed to morphine were taken up by microglial endosomes, leading, in turn, to activation of TLR7 with subsequent upregulation of lincRNA-Cox2 expression, ultimately resulting in impaired microglial phagocytosis. We have also recently reported the feasibility of isolating distinct brain cell-derived EVs (NDEs, ADEs, and MDEs) and characterized those to understand the effect of oxycodone self-administration on biomarkers of neurodegeneration in cynomolgus monkeys (Kumar et al., 2021a). We also reported that the treatment of NDEs (from monkeys self-administering oxycodone) induced glucocorticoid receptor localization to the nucleus in human astrocytes as well as induced proinflammatory effects in human THP1 monocytes in cell culture. Interestingly, Kumar et al. (2021b) reported that cocaine treatment significantly affected EV biogenesis, reducing the concentration of exosomes secreted by microglial cells in culture and affecting the loading of various cargo proteins. This study suggested that several CNS disorders associated with cocaine misuse could be due to the interruption of cell-to-cell communication mediated by exosomes. Barreto et al. (2022) also reported that cocaine exposure decreased the number of both neuronal early and late endosomes and exosomes in the brain of male mice but not female mice. Similarly, recently, D'Acunzo et al. (2023) reported that the generation and secretion of vesicles of mitochondrial origin (mitovesicles) could be affected by mitochondrial abnormalities induced by chronic cocaine exposure.

Overall, various drugs can affect EV secretion by neurons as well as other cell types in the brain, thereby affecting intercellular communication in the brain and contributing to substance use disorders. Furthermore, these studies suggest that EVs in biofluids could be helpful in identifying biomarkers associated with various stages of drug use and assessing the effects of behavioral and pharmacological interventions.

G. Human Immunodeficiency Virus–Associated Neurocognitive Disorder

HAND encompasses a range of cognitive, motor, and behavioral dysfunctions that affect a significant percentage of individuals living with HIV/AIDS. Even though there is a reduction in the rate of progression and severity of HAND-related symptoms due to effective antiretroviral therapy for HIV infection, the overall prevalence of HAND has not changed (Ditiatkovski et al., 2020). This suggests that pathologic changes associated with HAND continue to process even when viral replication is effectively suppressed by antiretroviral therapy.

EVs have emerged as crucial contributors to the pathogenesis of HAND (Ditiatkovski et al., 2020). EVs containing the HIV-1 protein Nef (exNef) have been found in the blood of individuals infected with HIV and undergoing antiretroviral therapy (Raymond

et al., 2011; Lee et al., 2016; Ferdin et al., 2018). Importantly, HIV proteins (Tat, gp-120, and Nef), which have the potential to induce neurotoxicity, have been reported as cargo within EVs (Kodidela et al., 2019). Ditiatkovski et al. (2020) reported that the modification of the neuronal cholesterol trafficking and lipid rafts by exNef (EVs from HEK293 cells transduced to express Nef) contributes to early stages of neurodegeneration and pathogenesis in HAND. This study showed that exNef were rapidly taken up by neural cells in cell culture, reducing the abundance of ABC transporter A1 (ABCA1) and cholesterol efflux and increasing the abundance of lipid rafts in neuronal plasma membranes. Importantly, exNef treatment caused the redistribution of APP and tau proteins to lipid rafts and increased the abundance of these proteins as well as that of A β 1-42. In vivo, treatment of C57BL/6 mice with either purified recombinant Nef or exNef reduced the abundance of ABCA1 and elevated the abundance of APP in brain tissue. Additionally, this study found a lower abundance of ABCA1 in the brain tissue of HIV-infected individuals diagnosed with HAND, whereas the abundance of lipid rafts was higher compared with HIV-negative individuals. Similarly, Sami Saribas et al. (2017) reported that exNef were released by primary human fetal astrocytes when transduced with an adenovirus construct to express Nef. Furthermore, exNef levels were enriched significantly when the cells were treated with autophagy activators (perifosine, tamoxifen, and MG-132) or inhibitors (LY294002 and wortmannin), suggesting the involvement of autophagy signaling in exNef release within EVs. Importantly, exNef were readily taken up by neurons, induced oxidative stress, and suppressed functional neuronal action potential assessed by multi-electrode array studies.

Interestingly, the characterization of total EVs and NDEs in the blood has been conducted to identify biomarkers that could be useful for diagnosing HAND (Pulliam et al., 2019) and monitoring its progression and/or the effects of treatment. Sun et al. (2017) reported that neuropsychologically impaired men, including both HIV-infected and noninfected men, had fewer NDEs but were enriched with high-mobility group box 1 (HMGB1), neurofilament light (NFL), and A β compared with neuropsychologically normal individuals. In a subsequent study, the same group characterized plasma NDEs to identify sex-based differences associated with HIV infection and cognitive impairment (Sun et al., 2019a). Through small RNA sequencing in total plasma EVs, they identified the upregulation of 11 miRNAs in individuals with lower neuropsychological performance compared with those with higher performance among people living with HIV (O'Meara et al., 2019). Several of the differentially expressed exosomal

miRNAs were predicted to be involved in inflammation and neurodegeneration pathways (O'Meara et al., 2019).

Overall, EVs contribute to HIV-induced neurodegeneration, and gaining a better understanding of the mechanisms underlying EV-mediated pathogenesis of HAND could provide valuable biomarkers and potential targets for therapeutic intervention.

H. Cancer-Related Neurodegeneration

Neurological disorders are not solely associated with genetic and physio-biochemical changes in the brain and its microenvironment. Conditions such as cardiovascular diseases, hypertension, type 2 diabetes, vascular dysfunction, and cancer can also impact normal brain functioning and cognition. The susceptibility of the CNS and peripheral nervous system to cancer and its treatment is well recognized (Giglio and Gilbert, 2010; Stone and DeAngelis, 2016). Cognitive decline is commonly observed in cancer patients, including those in remission (Pendergrass et al., 2018). Understandably, tumors in the brain or brain metastasis of non-CNS tumors can directly or indirectly affect different brain cells, leading to impairments in attention, memory, and executive function (Gehring et al., 2010; Aaronson et al., 2011). Additionally, tumors in non-CNS regions have been shown to impact cognitive status, including verbal memory, language, visual-spatial skills, executive function, and psychomotor function, even in newly diagnosed patients (Meyers et al., 2005; Ahles et al., 2008; Wefel et al., 2011). For example, women with breast cancer have been reported to experience cognitive issues related to attention impairment, learning, memory, and immediate and delayed word recall prior to treatment (Wefel et al., 2004; Hurria et al., 2006). Similarly, patients with small cell lung cancer were identified with cognitive deficits before receiving therapy, particularly in verbal memory, frontal lobe executive functions, and motor coordination (Meyers et al., 1995). These findings suggest that tumor cells could affect the cognitive status of cancer patients even before treatment.

Cancer treatment has also been shown to impact cognitive behavior and induce neurotoxicity (Giglio and Gilbert, 2010; Stone and DeAngelis, 2016; Lange et al., 2019). Efforts have been made to understand cancer- and cancer therapy-associated cognitive impairment and neurodegeneration, primarily relying on assessments of cognitive functioning and brain imaging measures. However, these approaches do not provide a comprehensive understanding of the etiology and pathophysiology of cognitive dysfunction and neurodegeneration in cancer patients. EVs could play a role in the communication between distant tumors and the brain, mediating processes such as the preparation of premetastatic niches in the brain microenvironment and influencing cognitive functions. Numerous studies have analyzed alterations in EV cargo and their release, due to

cancer and its treatment, and considering their capabilities to cross the blood-brain barrier, tumor-derived EVs can transmit their cargo to brain cells to affect their normal functioning. Although studies exploring whether these EVs could influence neurobiological functions, cognitive status, and behavior are lacking; and primarily focused on characterization of EVs for cancer diagnosis or prognosis. For example, high $A\beta$ expression has been associated with various cancers (Jin et al., 2017; Munir et al., 2021; Zayas-Santiago et al., 2022), which could be loaded in EVs, potentially seeding $A\beta$ in the brain and inducing amyloidopathy. Additionally, cancer cell-derived EVs can affect the functions of immune cells, including macrophages, T cells, and natural killer cells (Othman et al., 2019). Elevated levels of $TNF\alpha$ in EVs from colorectal cancer patient serum samples were reported, which correlated with aggressive features of colorectal cancer (Xie et al., 2023a). Also, elevated levels of $TNF\alpha$ were reported in ADEs of MDD subjects and have been shown to participate in cognitive dysfunction (Bortolato et al., 2015; Xie et al., 2023b). Further, EVs secreted by breast cancer cells exhibited higher levels of Annexin A2, which triggered macrophage-mediated activation of the p38 mitogen-activated protein kinases, $NF-\kappa B$, and signal transducer and activator of transcription 3 (STAT3) pathways, leading to increased proinflammatory cytokine production (Maji et al., 2017). Analyzing the cargo of EVs and BDEs for neurodegenerative and neuroinflammation markers, such as $A\beta$, total and p-tau, NFL, α -synuclein, proinflammatory interleukins, and specific miRNAs in the biofluids of cancer patients, could be an important indicator of neurological disorders.

V. Brain Cell-Derived Extracellular Vesicles' Usefulness in Predicting Treatment Response in Neurological Disorders

BDEs have shown promise in not only serving as biomarkers for the diagnosis of neurological disorders but also in assessing the response of treatments and interventions. Analyzing the early molecular changes that occur following the adoption of treatment or interventional plan aids in making timely decisions regarding the continuation of treatment or the need for an alternative approach. The response to treatments for neuropsychiatric disorders is typically observed through behavioral, cognitive, and phenotypic changes. However, these changes are often preceded by molecular alterations at the cellular level. EVs are suggested as snapshots of the cells or tissues in their healthy or specific pathophysiological state, and they have been regarded as windows into parental tissues. By analyzing the cargo of BDEs, a molecular understanding of the response of brain cells to a particular treatment regimen can be obtained.

Several studies have reported the beneficial effects of the ketogenic diet against Alzheimer's disease and related dementia (ADRD) (Reger et al., 2004; Krikorian et al., 2012; Jensen et al., 2020; Kraeuter et al., 2020; Lilamand et al., 2020). In a recent clinical pilot study, the effect of a high-fat, low-carbohydrate modified Mediterranean-ketogenic diet (MMKD) was compared with a low-fat American Heart Association diet on 11 cognitively normal older adults and nine adults with amnesic MCI (Neth et al., 2020). The outcomes of the study showed that MMKD was well tolerated and associated with increased levels of CSF $A\beta_{1-42}$ and decreased tau levels (Neth et al., 2020). To further understand the molecular effects of the MMKD intervention on ADRD biomarkers, NDEs were isolated from the plasma samples of the same individuals (Kumar et al., 2022). The cargo analysis of NDEs revealed that the MMKD intervention reduced the levels of $A\beta_{1-42}$, p-T181-tau, and NFL in participants with MCI (Kumar et al., 2022). Further analysis provided molecular insights and suggested that the MMKD intervention differentially targeted the glutamate receptors, including glutamate receptor ionotropic NMDA1, glutamate receptor ionotropic NMDA2A, glutamate receptor ionotropic NMDA2B, and glutamate receptor ionotropic AMPA type subunit 1 (Kumar et al., 2022). Moreover, the molecular measures of NDEs showed a strong correlation with corresponding clinical biomarkers in the CSF (Kumar et al., 2022). Furthermore, cerebrolysin, a peptidergic preparation, and donepezil, a cholinesterase inhibitor (approved for the symptomatic treatment of AD), are known to inhibit $A\beta$ levels, tau, and synaptic pathology. In a randomized clinical trial, the treatment response of these drugs individually and/or in combination was tested using plasma NDEs from mild to advanced AD patients compared with control subjects. The combination therapy with both drugs reduced the levels of $A\beta$ in NDEs compared with monotherapies, and NDEs' total tau, P-T181-tau, and P-S396-tau were decreased in cerebrolysin-treated patients compared with those on donepezil monotherapy (Alvarez et al., 2022). These findings suggest the potential utility of plasma NDEs as a tool in the development of novel preventive and therapeutic interventions for ADRD.

Another study showcased the utility of NDEs' biophysical measurements and cargo (miRNAs) analysis in assessing the response to ADT in patients with MDD, including both drug-responsive and nonresponsive individuals, as well as controls. Interestingly, a comparison of size before and after ADT revealed an increase in NDEs' size after 8 weeks of treatment, which displayed a significant inverse relationship with the Montgomery-Asberg Depression Rating Scale score. In addition to size, miRNA analysis of NDEs revealed changes in the expression of nine miRNAs following ADT, namely miR-423-3p, miR-191-5p, miR-486-5p, miR-30d-5p, miR-425-5p, miR-

25-3p, miR-21-5p, miR-335-5p, and miR-126-5p. Interestingly, the combination of miR-21-5p, miR-30d-5p, and miR-486-5p changes over the course of ADT was found to be associated with treatment response (Saeedi et al., 2021). To further validate the changes in miRNA expression in NDEs isolated using L1CAM as a marker, the authors isolated NDEs using another neuronal marker, SNAP-25, and consistently found altered expression of miR-486-5p and miR-30-5p in ADT responders compared with nonresponders (Saeedi et al., 2021).

In patients with MDD, altered expression of several proteins involved in mitochondrial biogenesis, dynamics, and energy metabolism were identified in plasma NDEs. The levels of these proteins were normalized in patients who responded to 8-week treatment with selective serotonin reuptake inhibitor but not in nonresponders, highlighting the importance of plasma NDEs in accessing the treatment response (Goetzl et al., 2021). Another study conducted by Mansur et al. (2021) investigated the potential of infliximab as a potential treatment of bipolar depression. NDEs' cargo analysis in plasma samples collected during a 12-week randomized, double-blind, placebo-controlled clinical trial revealed that responders to infliximab showed a significant increase in biomarkers of insulin alternate pathway (but not in canonical pathways) and increased phosphorylated JNK levels compared with infliximab nonresponders, placebo responders, and placebo nonresponders. Additionally, infliximab responders exhibited increased levels of phosphorylated ERK1/2 relative to placebo nonresponders (Mansur et al., 2021). Importantly, these changes in insulin alternate pathway biomarkers in plasma NDEs were proposed to mediate neurostructural changes as observed by MRI measures of brain volume and treatment-related changes in the dorsolateral prefrontal cortex volume (Mansur et al., 2021). Furthermore, reduced expression of DJ-1, a redox-sensitive protein with neuroprotective function, was observed at both protein and mRNA levels in blood EVs of patients with SCZ. The targeting miRNA of DJ-1, miR-203a-3p, showed higher expression in blood EVs of SCZ patients. Treatment with an antipsychotic drug, olanzapine, for 6 weeks increased DJ-1 level and attenuated miR203a-3p level in blood-derived exosomes, bringing those closer to the levels observed in controls (Tsoporis et al., 2022). The treatment of SCZ patients after resolution of the positive symptoms with antipsychotic medication was recommended for 2–5 years (Remington et al., 2017); accessing the treatment response as early as 6 weeks using NDE cargo measures will certainly help clinicians to decide the long-term treatment regimen. A similar analysis of miR-333-5p expression in plasma exosomes of patients with depression revealed elevated levels of miR-335-5p and decreased levels of miR-1292-3p in patients with treatment-resistant depression and predicted to affect postsynaptic density, axonogenesis, signaling pathway of axon formation, and

cell growths. Screening patients resistant to conventional treatment at the start of the therapy could help in adopting alternate approaches (Li et al., 2021). Moreover, analyzing the expression of these miRNAs and their targets not only has the potential to serve as novel biomarkers for improving the accuracy of diagnosis and effectiveness of treatment but could also provide specific targets for the development of personalized therapy for treatment-resistant depression.

Although limited studies are available, the existing literature supports the translational application of EVs in assessing the response to therapeutic interventions in various neurological disorders.

VI. Conclusion: “Boom or Bust”

In the past decade, numerous studies have demonstrated the potential of EVs as biomarkers for the diagnosis and assessment of cellular and molecular alterations related to various neurological disorders. Advances in the EV field have enabled the isolation of various subpopulations of BDEs from biofluids, offering the possibility to predict the pathophysiological state of brain cells. The isolation of BDEs from biofluids is less invasive compared with collecting CSF and more convenient and cost effective than neuroimaging modalities. This allows for repetitive measurements to track disease progression and treatment response over time. Analyzing the cargo of BDEs, including proteins, nucleic acids, and metabolites, in longitudinal studies can provide insights into progressive molecular changes specific to different types of brain cells, highlighting their contribution to various neuropsychiatric conditions. Moreover, BDE cargos were shown to be related to molecular alterations with treatment/intervention responses at early stages at the cellular level. The potential of BDEs for clinical utility in the context of different neurological diseases has been supported by many evidence-based laboratory studies, which certainly encourages more collaborative practice among laboratory scientists, industries, and clinicians to develop EV-based diagnosis/treatment of patients.

Despite the promising findings suggesting the usefulness of BDEs in neurological disorders, these vesicles are currently not approved for clinical use. However, the increasing number of ongoing clinical trials (Rezaie et al., 2022) focused on EVs for cancer management provides hope and may pave the way for their application in other disease conditions, including neurological disorders. Clinical studies analyzing the cargo of EVs, such as proteins and miRNAs, for prostate cancer (PCa) diagnosis have been completed or are in the clinical validation stage. The ExoDx Prostate (*IntelliScore*) test, which analyzes a three-gene RNA signature: V-ets erythroblastosis virus E26 oncogene homologs (ERG), prostate cancer antigen 3 (PCA3), and SAM pointed domain containing ETS transcription factor (SPDEF), in urine exosomes, known as “EPI score”, has been commercially

available in the United States since 2016 (Donovan et al., 2015; McKiernan et al., 2016; Tutrone et al., 2020). This test determines the risk of clinically significant PCa, aiming to help clinicians in making critical diagnostic and treatment decisions.

To fully exploit the tremendous potential of BDEs as liquid biopsy for neurological disorders, there is an urgent need to address the technical challenges associated with their isolation and analysis. The major and persistent challenge in the field is the CNS specificity of biomarkers used to isolate brain cell-specific EVs from blood samples. Earlier, we mentioned the conflicting reports regarding the use of L1CAM as a surface marker for isolating NDEs. Although there is a substantial body of literature supporting the usefulness of NDEs isolated using L1CAM, it is undeniable that L1CAM expression is not specific to neurons or the CNS and is also present as a cleaved secretory protein. As a result, extensive research efforts are focused on identifying CNS- and neuron-specific surface markers for NDEs as well as addressing similar challenges for other types of BDEs, such as PDEs and EDEs. In this context, characterizing EVs derived from brain tissues and induced pluripotent stem cell-derived or primary brain cells is proving to be a valuable approach for identifying specific and sensitive surface markers for BDEs (Muraoka et al., 2020; You et al., 2022, 2023).

Another challenge lies in isolating the needed quantity of BDEs from plasma samples, especially for assays that require relatively larger amounts of the samples, such as immunoblotting, metabolomics, or the assessment of multiple biomarkers. However, ongoing efforts to develop sensitive assays and implement multiplexing techniques are helpful in overcoming this challenge. Alongside this, continuous improvement in existing methods and the development of new approaches for isolation and analysis of BDEs are being developed.

Furthermore, when considering the clinical utility of BDEs in neurological disorders, as well as the utility of EVs in the diagnosis or prognosis of any disease, it is important to acknowledge that outcomes can be influenced by the methodology used to isolate the EVs. There is a wide range of methods employed for isolating total EVs from biological fluids, including ultracentrifugation, polymer-based precipitation, density gradient, filtration, and size exclusion chromatography, each with its own advantages as discussed in detail in many review articles (Konoshenko et al., 2018; Brennan et al., 2020; Sidhom et al., 2020). Additionally, the analysis of EV size and concentration can yield variable results depending on the method and instrument used for analysis (Kumar, 2021; Vogel et al., 2021). The variations in EV isolation and characterization methodologies make it challenging to compare and, at times, reproduce results. Efforts have been made to raise awareness of the impact of procedures for sample collection, EV isolation, and

characterization as well as the importance of in-depth reporting of methodology. Guidelines such as the Minimal Information for Studies of Extracellular Vesicles and EV-TRACK have been developed to assist researchers in these endeavors (Théry et al., 2018; Witwer et al., 2021). Similarly, useful guidelines are emerging for implementing relevant controls and good practices in the use of various methodologies such as flow cytometry, RNA sequencing, or real-time PCR for EV characterization (Welsh et al., 2020, 2023).

Moreover, in recent years, there have been efforts to develop tools and techniques specifically tailored to the EV field, operating at the nano level instead of relying on techniques and standards developed for characterization at the cell level. For example, several nanoflow cytometry tools, improved immunoassays, and sensitive sequencing-based protein analysis methods are being specifically designed for EV research. Such advancements have the potential to accelerate discoveries in the EV field.

Altogether, BDEs hold great promise as attractive and useful tools for biomarker development in a wide range of neurological disorders. To fully unlock this potential, it is crucial to carefully define and control key features and the complexity of EVs. In the near future, larger multisite prospective studies should be planned, ensuring meticulous collection and storage of biofluids, implementing standardized operating procedures for EV isolation and characterization, and employing robust data analysis techniques. By overcoming current challenges, BDEs have the potential to serve as valuable liquid biopsies, complementing existing clinical biomarkers and neuroimaging measures for enhanced diagnostic and prognostic capabilities in neurological disorders.

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Data Availability

There are no datasets in the manuscript.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Kumar, Nader, Deep.

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