The potential of extracellular vesicle lipid profiling in Alzheimer's disease

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Glossary

Aβ, amyloid-β
AD, Alzheimer's disease
APP, amyloid precursor protein
BDEV, brain derived extracellular vesicles
BMP, bis(monoacylglycerol)phosphate
CNS, central nervous system
CDR, clinical dementia rating
CSF, cerebrospinal fluid
DG, diglyceride
DHA, docosahexaenoic acid
EAL pathways, endosomal, autophagy, and lysosomal pathways
FFA, free fatty acid
GL, glycerolipids
GP, glycerophospholipids
HDL, high-density lipoproteins
LBD, Lewy body disorders
LBPA, lysobisphosphatidic acid
LDL/VLDL, low-density lipoprotein/very low-density lipoprotein
LPC, lysophosphatidylcholine
LPE, lysophosphatidylethanolamine
MCI, mild cognitive impairment
MG, monoglyceride
MISEV, minimal information for studies of extracellular vesicles
MMSE, mini-mental state examination
MuS, multiple sclerosis
NFT, neurofibrillary tangles
NIA-AA, National Institute on Aging and Alzheimer's Association
NMR, nuclear magnetic resonance
PC, glycerophosphocholine
PC-O, alkyl-ether glycerophosphocholine
PC-P, alkenyl-ether glycerophosphocholine or plasmalogen-PC
PE, glycerophosphoethanolamine
PE-O, alkyl-ether glycerophosphoethanolamine
PE-P, alkenyl-ether glycerophosphoethanolamine or plasmalogen-PE
PG, glycerophosphoglycerol
PS, glycerophosphoserine
PUFA, polyunsaturated fatty acids
S1P, sphinsosine-1-phosphate
SEC, size exclusion chromatography
sEV, small extracellular vesicles
SL, sterol lipids
SM, sphingomyelin
SP, sphingolipids
TG, triglyceride
TG-O, alkyl-ether triglyceride

Abstract

Over the past two decades, there has been increasing research into the molecular composition and function of small extracellular vesicles in the central nervous system. This is due in part to the recognition that small extracellular vesicles likely contribute to the pathogenesis of neurological diseases such as Alzheimer's disease, but also an understanding that small extracellular vesicles are a source of potential biomarkers. Small extracellular vesicles carry specific cargo that reflects their biogenesis and cellular origins, including proteins, RNAs and lipids. While the protein and RNA content of small extracellular vesicles in the central nervous system diseases have been studied extensively, our understanding of the lipidome of small extracellular vesicles in the central nervous system is still in its infancy.

Lipids play a significant role in maintaining central nervous system structure and function, and the dysregulation of lipid metabolism is known to occur in many neurological disorders, including Alzheimer's disease. Here we review what is currently known about lipid dyshomeostasis in Alzheimer's disease. We propose that small extracellular vesicle lipids may provide insight into the pathophysiology and progression of Alzheimer's disease and other neurological disorders, and, in the future perhaps, aid in disease monitoring and detection.

1. Introduction

Since their discovery more than 30 years ago, it has become clear that small extracellular vesicles (sEV) play a role in the pathogenesis of various neurological disorders [1-5]. A subset of sEV are exosomes. Exosomes are formed in the endocytic pathway and then secreted from parental cells carrying nucleic acids, proteins, and other metabolites enclosed in a lipid bilayer [6-9](**Figure 1**). sEV have a capacity for intercellular communication, inducing phenotypic and molecular alterations in recipient cells [10-13]. They can mediate important cellular processes and responses required for normal brain function and neuronal support in the central nervous system (CNS) [4, 13-26] but also contribute to disease pathogenesis [2, 4, 27-35]. sEV are found in the extracellular environment circulating in body fluids such as cerebrospinal fluid (CSF), blood, urine, and breast milk. The cargo packaged within sEV can reflect the physiological and pathological condition of their cellular origin, making them an excellent source of fluid-based biomarkers.

While the protein and RNA content and function of sEV has been subject to intense investigation, only a limited number of studies have been published on sEV lipids [26, 31, 36-42] and even fewer on CNS sEV lipids [43, 44]. In this review, we describe what is currently known about the lipid composition of EVs, with a focus on CNS derived sEV. Changes in lipid metabolism and lipid-regulating enzyme activity exists in many neurological disorders, including AD [45-51]. We provide a comprehensive summary of the known lipid changes in the brain, CSF, and blood in AD and their association with disease pathogenesis. We pose the question, 'could the lipid content of sEV provide insight into biological pathways and aid in the diagnosis of Alzheimer's disease (AD) or other neurological disorders?' and highlight the developments and challenges of sEV isolation for the purposes of lipid profiling.

2. What is known about sEV lipids?

Lipids, including fatty acyls, glycerophospholipids (GP), sphingolipids (SP), sterol lipids (SL), and glycerolipids (GL) among others [52, 53], are the building blocks for biological membranes and are critical for maintaining membrane structure and function, energy storage, and intercellular signaling [54, 55]. sEV possess a specific lipid signature relative to the cell membrane. It is lipids that are key to maintaining sEV morphology and enabling sEV (and their cargo) to travel in biofluids without degradation [56-58]. Typically, sEV are enriched in cholesterol, sphingomyelin (SM), ceramide, glycerophosphoserine (PS), ether glycerophosphoethanolamine (PE), lysophosphatidylethanolamine (LPE), and lysophosphatidylcholine (LPC) relative to their parental origin, with some variation noted for different cell and tissue types [6, 59-66]. The biogenesis and cargo sorting pathways of sEV are highly lipid regulated [41, 58, 67, 68]. Ceramide, cholesterol, and phosphatic acid (PA) are involved in sEV formation, vesicle transport and release (reviewed by Hessvick *et al.* [6]) while lysobisphosphatidic acid (LBPA, also known as bis(monoacylglycerol)phosphate, BMP) is thought to bind the protein ALIX, to regulate vesicle budding and membrane fusion [56-58, 69].

Only a handful of studies to date have examined the biological activity of sEV lipids [59, 70-72]. Extracellular vesicle PE and PS are known to participate in membrane dynamic modulation and facilitate sEV-cell membrane fusion. PE lipids are present on both leaflets of the sEV membrane in an asymmetric manner while the localization and dynamics of PS lipid reorganization within the membrane bilayer are still unclear [10, 60, 64, 73-75]. When PS

lipids localize to the sEV outer membrane leaflet, they are recognized by PS receptors (TIM1/4, Annexin 5) on recipient cells, facilitating sEV uptake/fusion and molecular transfer [24, 73, 74, 76].

Investigations into sEV lipids in disease are still in the discovery phase, and none have yet progressed to validation or clinical use. Most studies on human sEV lipid composition have come from the cancer field, of note colorectal, prostate, renal, and pancreatic cancer [61, 62, 77-80]. Some *in vitro* studies include Lydic *et al.* that characterized the lipid composition of a colorectal cancer cell LIM1215, and their derived sEV [61, 62], and reported an enrichment in total lipid content, a distinct sphingolipid profile, and alterations in fatty acyl chain length and saturation degree in sEV compared to the parental cells [62]. An in-depth lipidomic characterization of the metastatic prostate cancer cell, PC3, and their derived sEV by Llorente *et al*, reported that sEV are 8.4 times more enriched in lipids per mg protein compared to cells, specifically, glycosphingolipids, SM, cholesterol, and PS [61].

In recent years, a number of clinical studies have investigated the utility of peripheral sEV lipids as potential biomarkers. Urine sEV have been examined in prostate cancer, diabetic kidney disease, and non-alcoholic fatty liver diseases. Urinary sEV lipid species, including PS(18:1/18:1) and lactosylceramide(d18:1/16:0), were identified as being able to distinguish prostate cancer patients from healthy controls [79]. In another study, urinary sEV showed significant differences in glycerophosphocholine (PC), LPC, glycerophosphoinositolphosphate-2 (PIP2), diglyceride (DG), and ganglioside lipids that could distinguish diabetic nephropathy and diabetic mellitus patients [81]. More recently, Zhu *et al.* showed that a panel of urinary sEV lipids, composed of free fatty acids FFA(18:0), LPC(22:6/0:0), FFA(18:1), and phosphatidylinositol PI(16:0/18:1), could report on disease progression to non-alcoholic steatohepatitis, with an area under the curve of 92.3% [82].

The lipid content of vesicles in blood and bronchoalveolar lavage fluid (BAL) has been investigated, however whether these vesicles are sEV is unclear. A study examining serum vesicle lipids in pancreatic cancer found LPC(22:0), PE(16:0/18:1), and alkenyl-ether (plasmalogen-) containing PC(P-14:0/22:2) associated with disease stage and tumor diameter, with PE(16:0/18:1) correlating with survival rate [80]. Plasma vesicle eicosatrienoic acid (C20:3) has been proposed as a potential biomarker for severe acute pancreatitis [83] and plasma vesicle lipids are suggested to differentiate between early and late stage of non-small cell lung cancer [84]. Significant changes in glycerophosphoglycerol (PG), ceramide-phosphate, and ceramide have been reported in vesicles isolated from the BAL of asthmatics patients and SM(34:1) is thought to be increased in asthmatic patients exposed to secondhand smoke [85].

3. What is known about CNS derived sEV lipids?

Extensive evidence suggests altered lipid metabolism and abnormal activity of lipid regulating enzymes in the context of neurological disorders, including Alzheimer's disease (AD) [45-51], Parkinson's disease [86-91]; frontotemporal dementia [92]; multiple sclerosis (MuS) [43, 93], and Lewy body disorders (LBD) [44]. At the time of writing, only a handful of studies have reported the lipid profile of CNS derived sEV [43, 44, 94] and the potential function of sEV lipids or lipid-regulating proteins [26, 43, 44, 94-96]. Our group has shown that lipid dyshomeostasis in AD is also evident in sEV isolated from subject frontal cortex tissue and

that brain derived extracellular vesicles (BDEV) are enriched in PS and ether-PS lipids [94]. Pieragostino *et al* investigated CSF EV lipids of MuS patients with a particular focus on SP lipids, namely SM [43]. Acid sphingomyelinase, ASMase, a key enzyme in sphingolipid metabolism hypothesized to be involved in MuS, is also found enriched and active in MuS patient CSF EVs [43]. Another study carried out by Kurzawa-Akanbi *et al*. reported LBD CSF EVs were heavily loaded with ceramides, a characteristic of LBD [44]. From the few studies thus far, it is becoming apparent that the lipids and lipid-regulating proteins in sEV can report on the biological changes that occur as a consequence of the cellular impairments that characterize some neurological conditions [26, 44, 94-96].

Together these studies showed that sEV in the CNS have a similar lipid content to sEV from other tissues, but they are enriched in lipids pertinent to the physiological or pathological state of the CNS. Of note, these studies demonstrate the benefit of analysing sEV over gross tissue/CSF for enhancing lipid signals [43, 44, 94]. With improved detection of lipids will come greater insight into the biological/biochemical changes that occur as a cause or consequence of disease mechanisms, the role of sEV in disease progression and further understanding into whether sEV lipids drive pathology and or report on preclinical disease. CNS disorders hallmarked by lipid dysregulation [96, 97] including AD [45-51], are likely to benefit from the insight to be gained from profiling sEV lipids. Below, we provide an overview of what is currently known about lipid dysregulation in AD and suggest that sEV could serve as indicators of AD-associated lipid pathobiology and candidate biomarkers to aid in disease diagnosis.

4. Alzheimer's disease (AD)

AD is a neurodegenerative condition responsible for 60-80% of dementia cases worldwide [98]. Patients experience memory loss and changes in personality and behavior. Unfortunately, patients are generally diagnosed after the onset of clinical symptoms [99-101] and limited treatment options exist [102].

The cause of AD is multifactorial and although a variety of genetic, lifestyle, and environmental factors have been implicated, age is the number one risk factor [103-106]. Mutations in the amyloid precursor protein （APP） , presenilin-1 and presenilin-2 are associated with early onset familial AD [105]. The ApoE-ε4 allele is regarded as the major genetic risk factor for late-onset AD, with carriers of ApoE-ε4 having a higher risk of developing dementia than ε3 allele carriers and carriers with the protective ε2 allele [103-106]. Lifestyle and environmental risk factors that contribute to the likelihood of developing AD include diet, educational attainment, physical exercise, and brain injury, amongst others [101]. The importance of any one of these environmental factors in increasing or decreasing the risk of AD will differ from person to person.

Although an extensive array of factors in varied combinations may result in AD, two pathological hallmarks in the brain define the disease: amyloid-β (Aβ) plaques and neurofibrillary tangles (NFT). Aβ plaques accumulate outside neurons and are primarily composed of aggregated Aβ40/42 peptides generated from the cleavage of the APP [107, 108]. NFT, on the other hand, are intra-neuronal and primarily composed of hyper-phosphorylated tau protein [109]. Prior studies have revealed total-tau and phosphorylated tau are associated with cognitive decline in mild cognitive impairment (MCI) and AD [110-114]. In addition to Aβ plaques and NFT, a range of other AD pathological hallmarks include

neuro-inflammation, synaptic dysfunction [115], hypo-metabolism [116-119], oxidative stress [115, 120-125], brain atrophy [126, 127] and lipid dysregulation [50, 51, 128].

No single biochemical test can diagnose AD. The National Institute on Aging and Alzheimer's Association (NIA-AA) have emphasized diagnostic guidelines focusing on differential diagnosis of three stages of AD; preclinical [129], MCI due to AD [130], and dementia due to AD [131]. Current AD diagnosis consists of neuropsychological and pathophysiological assessments. Neuropsychological assessments, including the broadly accepted clinical dementia rating (CDR) [132] and the mini-mental state examination (MMSE) [133, 134], are employed to evaluate an individual's cognitive performance. These tests are also utilized to stage disease progression. Pathophysiological assessments include the detection of biomarkers, mainly Aβ40, Aβ42, total tau and phosphorylated tau (p-tau) species in CSF and blood, and imaging (PET and MRI) [135-141]. While biochemical measurements and imaging can be used to accurately diagnose dementia due to AD, they are not routinely performed due to factors such as resource accessibility and cost [99-101].

The pathophysiological process of AD occurs decades before the appearance of symptoms and clinical diagnosis [142-145]. This long 'preclinical' phase is an opportunity for therapeutic intervention; however early diagnosis (and available treatments) is required for this to occur. Recent years have seen considerable breakthroughs in detecting, identifying and quantitating Aβ species, total tau and p-tau species [114, 146-155] as well as protein markers, i.e. glial fibrillary acidic protein and neurofilament light protein [156, 157], in CSF and blood. However, several challenges remain, such as the variability in acceleration/deceleration rate of changes in molecules of interest, the complexity and the variable biomarker baselines among individuals, and the specificity of biomarkers. There remains an urgency to develop efficient and accurate blood-based biomarker strategies for clinical and pre-clinical AD diagnosis and to identify new therapeutic targets.

5. Disruption of lipid homeostasis in the brain in AD and association with disease pathogenesis.

Lipidomic studies, primarily on post-mortem tissues, suggest that dysregulation of lipid metabolism is a hallmark of AD [46-51, 128, 158-164] (see **Table 1** for a summary of published studies).

An overall decrease in AD in the GP lipid category has been reported in the temporal and frontal lobes [46, 165-167]. The majority of studies report decreased plasmalogen-PE and -PC levels in multiple cortical regions and cerebellum in MCI and end-stage AD [163, 165, 168-174], with one study reporting increased plasmalogen-PE in the superior-middle frontal gyrus and the superior temporal gyrus via nuclear magnetic resonance (NMR) [167]. For the GL lipid category, an overall increase in monoglyceride (MG) and DG is observed in MCI and AD post-mortem frontal cortex [163, 164], an increase of DG lipids is further evident in the recent study in the neocortex brains, accompanied with an increase of triglycerides (TG) lipids [174].

In the CNS, SP lipids are involved in signaling cascades, synaptic function, cholinergic function, signal transmission, and neuronal growth (axonal growth). The SM/ceramide cascade is impaired in AD [175] but there is little agreement between studies on the relative expression of SM and ceramide lipid species [47, 176]. The level of SM has depended on the brain region

examined [46, 167, 177] and could be attributed to the density of myelinated axons in white and grey matter [47, 178, 179]. An increase in ceremide is a consistent finding in the frontal cortex [176, 180], the grey matter of the frontotemporal cortex [47], and the middle frontal gyrus [177], with specificity in terms of fatty acyl chain composition [179]. There is evidence suggesting saturated ceramides are present in Aβ plaques in the superior temporal gyrus (Braak stage VI) [181]. Increased ceramide is linked to mitochondrial dysfunction, oxidative stress, neuronal apoptosis and Aß generation [162, 177, 182-184], which implicates a role for ceramides in disease pathogenesis. The enzymes involved in SM/ceramide pathways are dysregulated in AD, which is another possible explanation for the enhanced biosynthesis of ceramide [47, 176, 185]. Sphingosine-1-phosphate (S1P), a neuroprotectant against Aβ-induced apoptosis, is downregulated in AD and suggested to enhance apoptosis [47, 183, 186]. Sulfatide depletion has been reported in MCI (CDR 0.5) [178] and (Braak stage ≥ II) [165]. Degradation of sulfatides is suggested to cause hypo-myelination, resulting in neuronal dysfunction, shrinkage, and cholinergic dysfunction [47, 178, 186].

Ganglioside lipids, including GM2, GM3, GD3, and GM4, are increased in AD post-mortem tissue [46, 187, 188]. GD3 is regarded as pro-apoptotic [189, 190], suggesting a role in modulating cell death. The complex gangliosides, GT1b, GD1b, GD1a, and especially GM1, which tightly bind Aβ42 [191], are generally down-regulated in AD [188, 192]. Cholesterol, a major component of myelin sheaths and lipid rafts, is altered in AD [46, 177, 179, 193] with an increase in cholesterol proposed to enhance Aβ production and secretion [128, 194, 195] and contribute to memory impairment [195]. It is reported that cholesterol accelerates the binding of Aβ to GM1 [196], forming an Aβ-GM1 cluster that not only causes membrane damage but also seeds Aβ accumulation and induces oligomerization and fibril formation [197-201]. Aβ is well known for interacting with membranes during the aggregation of Aβ plaque, which disrupts membrane structure, alters membrane permeability, and causes cytotoxicity [97, 195, 202-209].

The brain contains polyunsaturated fatty acids (PUFA), cholesterol, and has a high oxygen level for energy consumption, making it susceptible to oxidative stress, and subsequent oxidative modification. Oxidative stress and lipid peroxidation occur in AD, but their contribution to disease progression (cause or consequence) remain unclear [120, 123, 210-213] [115, 121, 124, 212-216]. One of the main PUFA species, docosahexaenoic acid (DHA), is decreased in AD brain, CSF, and plasma [163, 171, 217]. DHA is capable of attenuating Aβ amyloidogenesis [217-222] making it neuroprotective. Peroxidation products including 4-hydroxyhexanal, 4-hydroxynonenal, neuroprostanes, neuroketals, isoprostanes, and oxysterols are increased in AD brain and CSF [123, 211, 218, 222-228]. Lipid peroxidation products play an active role in reactive oxygen species propagation, disruption of membrane integrity, protein-protein interactions, metabolism, and neurotransmission, and they promote Aß42 accumulation and neuroinflammation [120, 123, 218, 222, 223, 229-233].

Plasmalogens, a group of functional ether-containing lipids, are predominantly synthesized in peroxisomes and are abundant in the brain. Plasmalogens are characterized by a vinyl-ether linkage (the alkenyl or plasmalogen group) at the first hydroxyl moiety of the glycerol backbone, the *sn*-1 position (according to the stereospecific numbering system). The loss of peroxisomes, which participate in regulating metabolic and catabolic pathways, including lipid metabolism [234, 235], leads to dyshomeostasis of fatty acid and plasmalogen metabolism in

AD [172, 236]. Plasmalogens have multiple biological functions, including being scavengers of free radicals [237-241] and playing an active role in modulating membrane dynamics and enhancing membrane fusion [237, 240, 242-245] due to the hydroxyl moiety at the *sn-1* position and their ability to accommodate second messengers, namely the PUFAs, at the *sn*-2 position on the glycerol backbone [246, 247]. Amyloid-β has been suggested to interfere with alkyl-dihydroxyacetonephosphate-synthase expression, the rate-limiting enzyme involved in *de novo* synthesis of plasmalogens [169]. Plasmalogens exhibit a protective feature by suppressing amyloidogenesis and neuroinflammation induced by lipopolysaccharide in a mouse model [248] and PUFA-containing plasmalogens are suggested to attenuate nitric oxide production in microglia cells [249]. It has also been reported that PUFA-containing plasmalogens induce ferroptosis (a type of programmed cell death dependent on iron) that has been implicated in AD [214, 250-253]. Although the molecular mechanism and the biological function of plasmalogens in the brain are not fully understood, changes in peroxisome function and plasmalogen levels could be both biomarkers and therapeutic targets for AD [254].

6. Lipid changes in AD CSF and blood

Changes in the lipid content of CSF (**Table 2**) and blood (**Table 3**) occur in preclinical and clinical AD. In CSF, PC lipids and the PC substrates, phosphocholine and choline, are increased [255] and the levels of ceramide and SM lipids, as well as specific PC lipids positively correlate with CSF Aβ42, tau, and p-tau181 [161, 180, 256]. Kosicek *et al*. reported increases in multiple CSF SM species in MCI but no change in mild or moderate AD compared to cognitively normal controls [257, 258]. A significant reduction in sulfatide has also been reported in AD CSF [259], consistent with that reported in the brain [178, 186].

Serum biomarker discovery studies have identified specific lipids capable of distinguishing AD from healthy control individuals in discovery studies (**Table 3**). Saturated and short chain PC, LPC and a group of lipid peroxidation products are up-regulated, while PE, especially plasmalogen-PE, are decreased in AD serum [173, 260-263]. A longitudinal study spanning nine years showed that serum SM and ceramide levels had potential as predictive biomarkers for memory impairment [159] while in the Alzheimer's Disease Neuroimaging Initiative (ADNI) serum study, PUFA-TG negatively correlated with AD neuropathology and brain atrophy in MCI and AD patients compared to controls [264].

The majority of lipidomic studies have been performed on patient plasma (relative to CSF or serum). In plasma, a few PC lipids, mainly the PUFA containing species, are decreased in AD [265-267] with an increase in PC(40:4) reported by Proitsi *et al.* [268]. Ether lipids, mainly alkyl-ether PC/PE (PC-O and PE-O) and alkenyl-ether PC/PE (PC-P and PE-P), were down-regulated in The Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing (AIBL) and ADNI AD cohorts [269]. Dysregulation in PE and plasmalogen lipid metabolism are worth examining further to pinpoint if alterations in these pathways could serve as targets for therapeutic intervention, or if changes in these lipids are simply a consequence of the disease [270]. Alterations in the levels of plasma SM and ceramides were also reported [158, 269, 271]. Importantly, they are altered in MCI and associated with cognitive decline and hippocampal volume loss [160, 272]. In the AIBL and ADNI cohorts, ceramides containing different acyl chains correlated with AD, irrespective of their sphingoid base, with negative correlation observed in C22:0 and C24:0 species and positive correlation observed in C18:0,

C20:0 and C24:1 species [269]. The ratio of very long chain to long-chain ceramides, for example, C24:0/C16:0 and C20:0/C16:0, were inversely associated with the risk of developing incident dementia and AD [271]. However, the ratio of ceramides C24:0/C16:0 was also found to be negatively correlated with coronary artery disease and acute coronary syndromes in three different patient cohorts [273], indicating that lipid changes in blood can be attributed to multiple factors. The PUFA-TG species, especially, C22:6 containing TG(58:8), together with alkyl-triglyceride (TG-O) lipids, were negatively associated with AD in the AIBL and ADNI cohorts [269], consistent with findings in serum (ADNI) [264].

7. Could the lipid content of sEV provide insight into biological pathways and aid in the diagnosis of Alzheimer's disease or other neurological disorders?

The first report suggesting EVs may contribute to AD was published by Rajendran *et al* in 2006 [274]. Since this time, the field has expanded with numerous discovery studies on the function of EVs in AD and their potential as a source of protein and RNA biomarkers. The protein and RNA content and function of EVs in AD will not be reviewed here as it has been covered by others in detail [31, 275, 276].

While there are numerous studies on the protein and RNA content of sEV, there are few studies on sEV lipids in AD. Recently, our group undertook a comprehensive and semi-quantitative lipid profiling of sEV isolated from human post-mortem frontal cortex [94] using an established protocol to isolate BDEV [277]. We identified differentially abundant lipids in BDEV that distinguished AD from neurological control tissue. AD BDEV contained decreased PUFA-containing lipids, including PS(40:6), PE(40:6) and LPE(22:6) containing DHA, LPE(22:4) containing docosatetraenoic acid, and PC(38:4) and PE(38:4) lipids containing arachidonic acid, consistent with that observed in AD tissue [94]. Plasmalogen-PE lipids, including PE(P-36:2) and PE(P-38:4), were significantly upregulated in AD BDEV compared to controls [94]. This lipidomic data also suggested remodeling of the sphingolipid metabolism pathway in a *N*-acyl chain dependent manner [94].

Cohn *et al* used a similar isolation approach [277] to examine sEV in the parietal cortex in AD, specifically examining microglia derived sEV (CD11b enriched BDEVs) [95]. In agreement with our study, Cohn *et al* also reported a decrease in phospholipids harboring DHA in microglia BDEV. They additionally reported upregulation of the most abundant lipid species of LBPA and monohexosylceramide. LBPA is an endo-lysosomal specific lipid, its presence in sEV likely reflects impairments in the endo-lysosomal pathway [4, 5].

It is well known that sEV, specifically exosomes, are formed in the endocytic pathway and are packaged with proteins and lipids that almost exclusively come from the endosomal, autophagy, and lysosomal (EAL) pathways [5, 11]. Crosstalk between the exosome biogenesis and EAL pathways contributes to cellular homeostasis in the form of coordinated release of exosomes and modulation of their cargo depending on the needs of the cell. Alterations in the endosomal/autophagy/lysosomal (EAL) pathways are well-recognized early neuropathological features of AD, marked by prominent enlargement of endosomal compartments, progressive accumulation of autophagic vacuoles and lysosomal deficits [278-280]. Therefore, the composition of the released exosomes might provide insight into the interactions between EAL compartments and enables detection, outside the cell, of pathway specific changes in AD.

Studies by Cohn *et al* [95] and our team [94] suggest that sEV could be used as a tool for integrating the EAL pathways and identifying molecular species in the blood that originate from these intracellular pathways. Some of the developments and challenges that need to be overcome for the potential of clinical sEV profiling to be realized are outlined below.

8. Future developments and challenges

One of the main challenges associated with sEV isolation from plasma or serum is the removal of co-isolated lipoproteins. Due to their common physical features, namely density and particle size, lipoproteins are often co-purified with sEV when using currently available 'EV' isolation techniques or kits [281, 282]. Lipoproteins are rich in lipids, which, when co-isolated with sEV, confound the identification of sEV specific lipid profiles [283, 284]. This is one of the main reasons that a precise lipid profile of EVs in blood is still unresolved. Several groups intending to profile the lipid content of sEV in serum or plasma, have likely analyzed EVs in the presence of lipoproteins [285-288]. For example, Peterka *et al.* isolated plasma 'sEV' via polymer precipitation, a method known to co-isolate lipoproteins [286], and not surprisingly reported an approximately 55-82% increase in TG (mol% lipid abundance) in 'sEV' relative to plasma via different mass spectrometry platforms [289]. Cholesteryl ester and TG lipids are predominant in lipoproteins [284, 290]. Chen *et al* took a more stringent isolation approach, using serial ultracentrifugation and density gradient separation, however their isolation method most likely would have still co-isolated high-density lipoproteins (HDL), which have a similar density to sEV [287]. In another study that used a commercial precipitation kit, negligible ApoA1 and ApoB proteins were detected in plasma- and serum-derived sEV compared to HDL and low-density lipoprotein (LDL) enriched particles. However, only GP and SP lipid categories were reported and the differential cholesteryl esters and GL lipid data were not reported [288]. Size exclusion chromatography (SEC) [291, 292] and serial ultracentrifugation [293] have also been used to isolate vesicles in blood for the purposes of EV lipid profiling, however these techniques, are unable to separate EVs from HDL (using SEC alone) and other lipoproteins (using ultracentrifugation).

Of the studies published thus far on blood EVs, PS lipids have either not been detected, or are only present as a small percentage of the total lipid concentration [287-289, 291, 293]. However, PS lipids are known to be highly enriched in EVs isolated from other sources [56-58, 65, 94]. This discrepancy may relate to the source of EVs or the EV corona in plasma and serum [283, 294, 295]. The minimal information for studies of extracellular vesicles (MISEV) 2018 guidelines, suggest using apolipoprotein A1/A2 (major components in HDL), apolipoprotein B (major components LDL/VLDL), and albumin levels to demonstrate the efficiency of contaminant removal (lipoprotein and plasma proteins) from sEV preparations [296]. Removal of contaminates can be achieved when density gradient and size exclusion are used in tandem and while these methodologies together may reveal the true lipid content of sEV in blood, they are low throughput, so unsuitable for use in large scale discovery studies and clinical applications [286, 297]. Thus, new generation, high throughput products capable of enriching sEV from blood plasma and serum without co-isolation of contaminants are needed.

It has been suggested that sEV can cross the blood-brain barrier, possibly via transcytosis [291, 298, 299]. This provides the opportunity to profile the lipid content of BDEV in patient blood. This is of particular interest in CNS disorders characterized by impairments in lipid metabolism.

Capturing legitimate BDEV from blood, however, has proven difficult. Several groups have isolated and characterized neuronal, astrocytic, or microglial exosomes (NDE, ADE, MDE respectively) [300-306]. The isolation of these populations has been via the use of a commercial polymer precipitation kit followed by immuno-capture with cell type specific antibodies. Questions have arisen as to whether this technique isolates EVs of specific origin firstly because of lack of antibody specificity [307] and secondly the use of polymer precipitation which is widely known to isolate EVs of low purity. The field is currently reassessing targets for EV immuno-capture and exploring new methods to capture CNS cell type specific EVs from blood. The question will then be, 'are there sufficient numbers of the EV population of interest for downstream lipid analysis and detection of changes associated with disease or treatment?'

Advances in mass spectrometry are beginning to enable high-throughput, sensitive, comprehensive, and quantitative detection of lipid species from clinically relevant biological samples [62, 269]. With further technological advances, we envisage that detection of oxidized lipids will also become easier. As oxidative stress is a hallmark of several neurological diseases [120, 121, 124, 177, 213], we predict that comprehensive profiling of oxidized lipids will advance our understanding of disease mechanisms. In the future, mass spectrometry-based lipidomics will become a powerful tool to facilitate comprehensive clinical profiling for disease diagnosis [308] however its clinical application is currently limited for a number of reasons (see Meikle *et al* for a comprehensive review on the subject [308]). One reason is the complex nature and number of the lipids in biological fluids, particularly blood plasma and serum. Complexity reduction of clinical samples, such as blood, could be achieved by enriching for sEV to remove non-EV associated lipids. We have shown that sEV not only have a unique lipid signature, but they also provide improved detection of lipids of interest, relative to gross or more complex tissues [94].

9. Conclusion

There is great potential in sEV lipids, particularly in the aspect of diagnosing neurological disorders associated with lipid dyshomeostasis. To take full advantage of this potential, current limitations must be resolved. To overcome these limitations, future research needs to focus on developing high throughput products capable of enriching sEV from blood without co-isolation of contaminants, novel isolation methods to capture CNS cell type specific EVs, and the development of clinically applicable lipidomic platforms. Additionally, research should focus on understanding the role of sEV lipids in health and disease, as well as developing strategies to manipulate sEV lipids for therapeutic purposes. With the right combination of technological advances and scientific understanding, the potential offered by sEV lipids could be fully realized.

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Conflict of Interest
The authors have declared no conflict of interest.

Table 1 **| Lipid dysregulation in AD brain**

Lipids	Findings
Glycerophospholipids	
PE	• Reduction in AD superior temporal gyrus [167] and prefrontal cortex [46].
PI	• Reduction in AD superior temporal gyrus [167].
PA	• Reduction in AD superior temporal gyrus grey matter [167].
PS	• Reduction in AD inferior parietal lobule and in occipital cortex [167].
PG	• Reduction in AD superior temporal gyrus grey matter [167].
Plasmalogen-PE	• Plasmalogen-PE deficiency present in frontal, parietal, temporal and cerebellar white matter and grey matter in early stage of AD (CDR 0.5) with no further depletion in white matter in CDR 1, 2 and 3 samples while further depletion was observed with the progression of AD in grey matter in all examined brain regions except for cerebellar cortex [170]. • Deficiency of plasmalogen-PE to PE ratio in AD mid-temporal cortex and in cerebellar grey matter [168]. • Elevation of plasmalogen-PE in the AD superior-middle frontal gyrus and superior temporal gyrus [167].
Plasmalogen-PC	• Deficiency of plasmalogen-PC in stage V-VI (modest AD) prefrontal cortex but no alteration in plasmalogen-PE [171].
Fatty acyl chain length	• Down-regulation of long chain fatty acids (C>40) and increase in short chain (C=34) [46].
Sphingolipids	
SM	• Increased SM in AD inferior parietal lobule [167], cerebellar cortex [167] and entorhinal cortex [46]. • Increased SM in middle frontal gyrus (MFG) grey matter and no change in MFG white matter [179]. • Decreased SM in superior temporal cortex white matter in late stage, no change in early stage [178]. • Decreased soluble cytosolic SM and no change of membrane SM in AD frontotemporal grey matter [47]. • Decreased SM C24:0 in middle frontal gyrus [177].
Ceramides	• Increased soluble cytosolic ceramide in AD frontotemporal grey matter [47]; Increased ceramide in AD frontal cortex [180] • Increased ceramide C24:0 in AD middle frontal gyrus grey matter but not in white matter where ceramides C16:0, C22:0 and C24:1 were significantly down-regulated [179]. • Increased ceramides C18:0 and C24:0 in AD middle frontal gyrus [177].
Sphingosine and S1P	• Increased soluble cytosolic sphingosine and decreased soluble cytosolic S1P in AD frontotemporal grey matter while no change was observed in either sphingosine or S1P in membrane fraction [47]. • Decreased S1P/sphingosine ratio with increasing Braak stage in hippocampus and temporal gyrus [186].
Sulfatides	• Decreased sulfatide in early stage AD (CDR 0.5) cerebral and cerebellar grey and white matter [178], and in preclinical AD superior frontal gyrus [165].

Hexosyl-ceramide	• glucosylceramide and galactosylceramide were found to be increased in the prefrontal cortex [46].
Gangliosides	• Increased simple gangliosides GM2, GM3 and GM4 and decreased complex gangliosides GT1b, GD1b, GD1a and GM1 in AD frontal and parietal cortex [187, 192, 309].
	• Increased GM3, especially long chain GM3, i.e., GM3 (d18:0/24:0), GM3 (d18:1/22:0) and GM3 (d18:1/24:0) in AD entorhinal cortex [46].
	• Decreased GM1 and GD1a in AD temporal cortex grey matter [188]
Glycerolipids	
MG, DG and TG	• Increased pool of DG lipids in AD prefrontal cortex and selected triglyceride (TAG) species in AD entorhinal cortex [46].
	• Increased MG and DG but no significant changes in TG in frontal cortex [164]
	• Overall increase of DG and TG lipids in mild AD (Braak 3-4) and AD (Braak 5-6) compared to no cognitive impairment (Braak 0-2) neocortex [174].
Sterol lipids	
Cholesterol	• Increased cholesterol in AD cerebral cortex [193] and middle frontal gyrus grey matter [177, 179], with a trend of increase as disease progresses in frontal cortex [177].
	• No change observed in AD prefrontal cortex or entorhinal cortex [46].
Cholesterol esters	• Increased CE (C18:1) in AD middle frontal gyrus grey matter [179]

Table 2 | **Potential lipid biomarkers reported in CSF of AD patients**

Lipids	Findings
Glycerophospholipids	
PC metabolites	• Increased choline metabolites, phosphocholine, free choline and PC in AD CSF suggested PC breakdown in AD brain [255].
PC	• PC(32:0), PC(34:1), PC(36:1), PC(38:4) and PC(38:6) were significantly enhanced in CSF from patients with "AD-like pathology" compared to normal [256].
Sphingolipids	
SM	• Increased in pre-clinical patients compared to non-demented controls but no change in mild or moderate patients compared to controls [257, 258].
	• Significant increase of SM (d18:1_18:0) CSF level of patients displaying "AD-like pathology" [256].
	• All examined SM species were positively correlated with all Aβ species and total-tau [161].
Ceramides	• Ceramide C18:0 was positively correlated with all CSF Aβ38, Aβ40 and total-tau [161].
	• Increased ceramide levels in AD CSF compared to age matched neurological controls [180].
	• Ceramide in moderate (CDR 2) AD was significantly higher than that in mild (CDR 0.5-1) and severe (CDR 3) dementia [180].
Sulfatides	• Decreased in MCI patient due to incident dementia (CDR 0.5) [259].

Table 3 **| Potential lipid biomarkers reported in serum and plasma from AD patients**

Serum	
Lipids	**Findings**
Glycerophospholipids	
PC	• Increased saturated and short chain fatty acids containing PC lipids with decreased PUFA-PC [260].
LPC	• Increased LPC lipids [263].
Plasmalogen-PE	• Decreased plasmalogen-PE lipids and the depletion is correlated with disease progression [173, 260-262].
Sphingolipids	
SM and ceramides	• High level of SM and ceramide lipids is associated with memory impairment [159].
	• Decreased SM level [263].
lipid peroxidation products	• Increased oxidized PC, oxidized TG and F2-isoprostane [263].
Glycerolipids	
TG	• Negative correlation between PUFA- TG species with AD neuropathology and brain atrophy in MCI and AD patients compared to control in the ADNI study [264].
Plasma	
Lipids	**Findings**
Glycerophospholipids	
PC	• Various PC lipids, especially the PUFA containing PC species, (i.e., PC(16:0/20:5), PC(16:0/22:6) and PC(18:0/22:6)) were found to be decreased in MCI and AD [265-267].
	• Increased PC(40:4) in AD patients [268].
Ether lipids	• Decreased ether lipids, PC-O, PC-P, PE-O and PE-P in the AIBL and ADNI cohorts [269].
	• No change between AD and control but decreased plasmalogen-PE level was observed a year later in the same AD cohort [270].
Sphingolipids	
SM	• Decreased SM C22:1 and C24:1 [158].
Ceramides	• Increased ceramides C16:0 and C21:0 [158].
	• No change in ceramide level between AD vs control while lower levels of very long chain ceramides, C22:0 and C24:0, were found in MCI patients; Among MCI patients, higher level of ceramides C22:0 and C24:0 predicted further cognitive decline [272].
	• Ceramides C18:0, C20:0 and C24:1 had positive correlation with AD and negative correlation observed in ceramides C22:0 and C24:0 [269].

	• Ratios of ceramide C24:0/C16:0 and C20:0/C16:0, were inversely associated with the risk of developing incident dementia and AD [271].
SM and ceramides	• Increased ratio of ceramide to SM containing same fatty acyl chain in AD [158].
	• Increased SM/ceramide and dihydrosphingomyelin/dihydroceramide ratio predicted slower disease progression among AD patients [160].
Glycerolipids	
TG	• Decreased PUFA-TG, C22:6 containing TG(58:8) and TG-O species in AD patients [269].
	• Decreased TG(57:1) in AD patients [268].
MG and DG	• Increased MG and DG in MCI patients [164].
Free fatty acid	• A general increase of free fatty acids was observed in AD plasma [270].
Sterol lipids	
Cholesterol esters	• The level of long chain cholesteryl esters followed the trend of decrease from CTL to MCI and AD [310].

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Figure 1. A simplified illustration of the endocytic biogenesis pathway and lipid cargo of small extracellular vesicles (sEV).

A subset of sEV are referred to as exosomes. They are formed from invagination of the limiting endosomal membrane of the late endosome /multivesicular body (MVB). The MVB fuse with plasma membrane and releases the internalised vesicles as sEV into the extracellular environment. sEV lipid bilayer is key to maintaining vesicle morphology and enabling sEV (and their cargo) to travel in biofluids without degradation [56-58]. sEV are enriched in cholesterol, sphingomyelin (SM), ceramide, glycerophosphoserine (PS), ether glycerophosphoethanolamine (PE), lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) relative to the parental cell.