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Extracellular vesicles as mediators of cell-cell communication in ovarian cancer and beyond – A lipids focus

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ABSTRACT

Extracellular vesicles (EVs) are messengers that carry information in the form of proteins, lipids, and nucleic acids and are not only essential for intercellular communication but also play a critical role in the progression of various pathologies, including ovarian cancer. There has been recent substantial research characterising EV cargo, specifically, the lipid profile of EVs. Lipids are involved in formation and cargo sorting of EVs, their release and cellular uptake. Numerous lipidomic studies demonstrated the enrichment of specific classes of lipids in EVs derived from cancer cells suggesting that the EV associated lipids can potentially be employed as minimally invasive biomarkers for early diagnosis of various malignancies, including ovarian cancer. In this review, we aim to provide a general overview of the heterogeneity of EV, biogenesis, their lipid content, and function in cancer progression focussing on ovarian cancer.

1. Introduction

Extracellular vesicles (EVs) are released by multiple cell types and proclaimed to be novel regulators of intercellular communication[1]. EVs not only vary in their size, mode of biogenesis but also include number of subtypes including but not limited to exosomes, ectosomes,

Abbreviations: EVs, Extracellular vesicles; ARMMs, arrestin domain containing protein 1(ARRDC1-mediated microvesicles); ARRDC1, arrestin domain containing protein 1; ECM, Extracellular matrix; TSG101, tumour susceptibility gene 101; MVBs, multivesicular bodies; ILVs, intraluminal vesicles; ESCRT, endosomal sorting complex required for transport; MMP, matrix metalloproteinases; VEGF, vascular epithelium growth factor; HSP, heat shock protein; GPI, glucose phosphate isomerase; AST, aspartate transaminase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate; CK18, cytokeratin 18; sMB-Rs, shed midbody remnants; FDVs, filopodium derived vesicles; MIM, missing in metastasis; GANAB, neutral α-glucosidase AB; mTORC1, mammalian target of rapamycin complex; ADAM10, A Disintegrin and metalloproteinase domain-containing protein 10; ACE2, Angiotensin-converting enzyme 2; RACGAP1, Rac GTPase activating protein1; MKLP1, mitotic kinesin-like protein; PC-3, prostate cancer cell line, EOC, epithelial ovarian carcinoma cell line; EONT, normal immortalized ovary epithelial cell; OCAF, Ovarian cancer activating factor; ATX, autotaxin; FABP4, fatty acid binding protein 4; SCD1, stearoyl CoA desaturase 1; FAS, fatty acid synthase; HER2, human epidermal growth factor receptor 2; CA-125, cancer antigen 1; CRC, colorectal cancer; PSA, prostate specific antigen; TNBC, triple negative breast cancer; LT, leukotriene; HCC, Hepatocarcinoma; MM, multiple myeloma; ASM, acid sphingomyelins; SDF-1α, stromal cell-derived factor 1 alpha; CXCR4, C-X-C motif chemokine receptor 4; Akt, protein kinase B; BMP, bis(monoacylglycerol)phosphate; S1P, sphingosine 1-phosphate, PS, phosphatidylserine; DG, diacylglycerol; CHOL, cholesterol; SM, sphingomyelin; ChE, cholesterol ester; PLC, phospholipase C; LPAS, lysophosphatidic acid; PLA2, phospholipase A2; FA, fatty acyls; GL, glycerolipids; GP, glycerophospholipids; SP, sphingolipids; ST, sterol lipids; PR, prenol lipids; SL, saccharolipids; PK, polyketides; PA, phosphatidic acid; TAG, triacylglycerol; PLD, phospholipase D; LPE, Lysophosphatidylethanolamine; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositol; Gb3s, globotriosyl-ceramides; LPC, Lysophosphatidylcholines; Cers, ceramides; MG, monoacylglycerol; pPC, plasmenylcholine; pPE, plasmenylethanolamine; GM3, Gangliosides 3; ZyE, Zymosterol; LPS, lysophosphatidylserine; LPI, lysophosphatidylinositol; LPG, lysophosphatdylglycerol; TMEM16F, calcium-dependent transmembrane protein 16 F; Xkr8, Xk-related protein 8.

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large oncosomes, apoptotic bodies [2]. EV preparations often contain a mixture of vesicle types and their size, mode of biogenesis and unique markers can be used to categorize these vesicles, as recommended by the International Society of Extracellular Vesicles (ISEV) [3].

Eukaryotic cells utilize approximately 5% of their genes to generate a complex lipid repertoire suggesting its functional significance in various cellular processes and machinery[4]. Lipids form an essential component of EVs as they contribute to the maintenance of their structural integrity and morphology[5]. Despite significant recent effort to understand the lipid composition of EVs [6],the exact role that EV lipids play in normal or pathological states remains elusive. Thus, further research is needed not only to understand EV biogenesis or uptake, but also to establish the viability of EV lipid as minimally invasive disease biomarkers. Interestingly, lipids found within the EVs are distinct from the lipid composition of the plasma membrane from which those EVs are released, suggesting a separate process underlying EV biogenesis, and a specific role for EV lipids [6]. EV lipids act as signalling mediators by interacting with prostaglandin and phospholipases C and D to execute signalling cascades [7].

Lipid metabolism, including lipid anabolism and catabolism and lipid uptake by the extracellular environment are found to be altered in tumorigenesis[8]. Cancer cells upregulate their lipid metabolism machinery to meet the increased energy demand, support tumorigenesis, and suppress and/or evade immune responses[8]. Furthermore, ascites and adipocytes present in the omentum have been shown to contain lipid intermediates that promote tumour activity by downregulating the function of T-lymphocytes [9–12]. The data available for the shuttling of cancer specific molecules into EVs is minimal and thus there is a need to identify EV-associated lipids and proteins. While significant research describes the role of EV proteome in ovarian cancer progression [13,14],

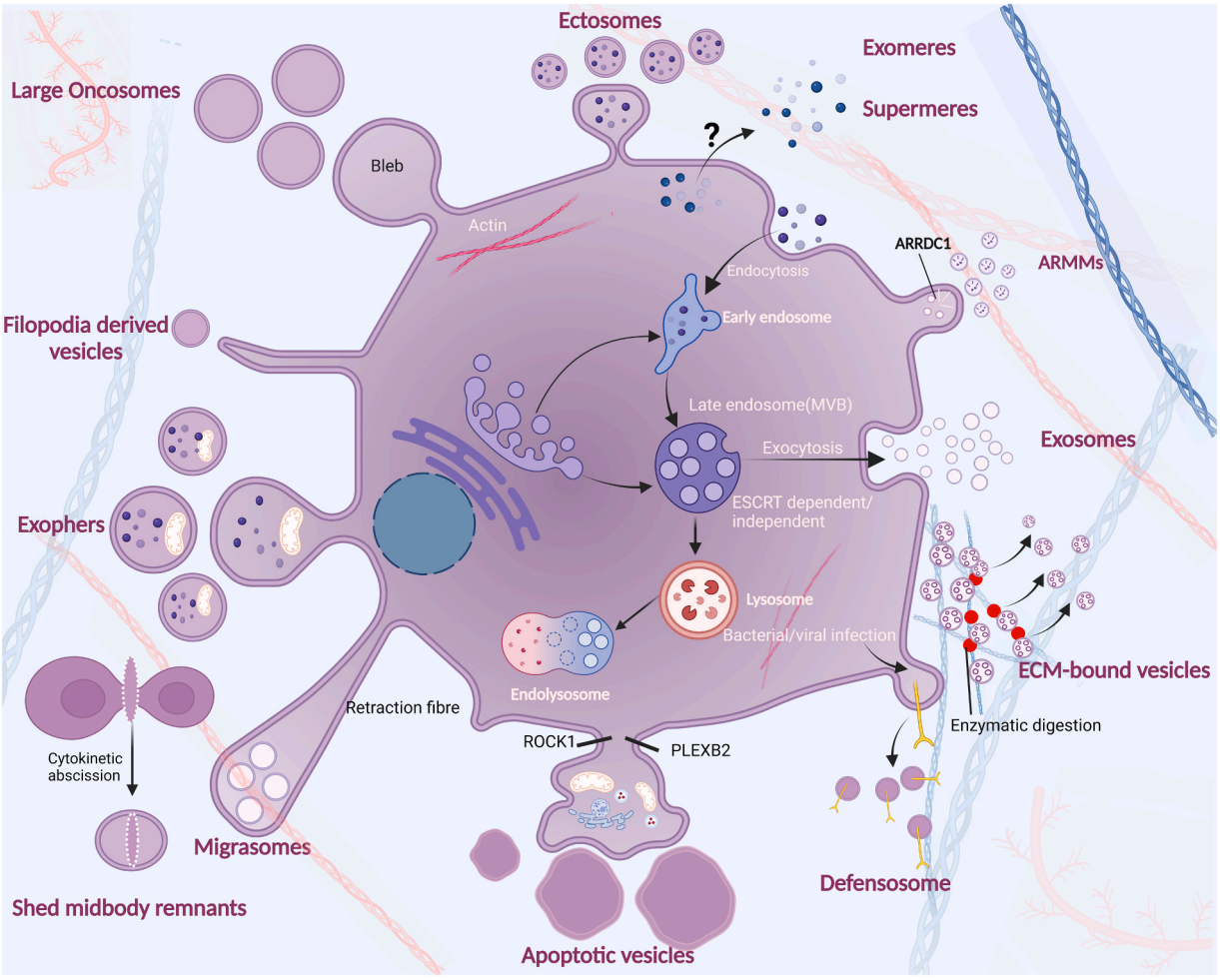

Fig. 1. Diversity in extracellular vesicles (EVs) population. EVs are classified based on their size and origin, broadly into two subtypes-small EVs (~<200 nm) and large EVs (~>200 nm). Small EVs include–exosomes (~30–150 nm), ARMMs (arrestin domain containing proteins (ARRDC1)-mediated microvesicles) (~<100 nm), defensosomes (~80 nm), and ECM-bound vesicles (~50–400 nm).Upon endocytosis, the early endosome matures into multivesicular bodies (MVBs) containing intraluminal vesicles(ILVs). MVBs can either undergo lysosomal degradation upon fusion with lysosomes or can be secreted into the extracellular space upon fusion with plasma membrane, and thus termed exosomes. The biogenesis of exosomes is mediated by ESCRT dependent or ESCRT independent pathways. The ARMMs are formed through plasma membrane budding driven by ARRDC. Defensosomes are produced by host cell upon bacterial or viral infections, contributing to the host defence mechanism. ECM-bound vesicles are produced upon enzymatic digestion of the extracellular matrix, and occurs during tissue decellularization. Large EVs (~>200 nm) include – ectosomes (~100–1000 nm), migrasomes (~500–3000 nm), apoptotic bodies (~1000–5000 nm), large oncosomes (~1000–10000 nm), exophers (~>3000 nm), shed midbody remnants (~300–1000 nm), and filopodia derived vesicles. Ectosomes are formed by direct blebbing from the plasma membrane. Migrasomes are released by migrating cells and its biogenesis involves generation of retraction fibres enriched in actin filaments.The apoptotic vesicles are produced as membrane blebs originating from apoptotic cells.ROCK1and plexins regulate the process of formation of apoptotic vesicles. Large oncosomes are formed via direct plasma membrane budding from amoeboid cells. Exopheres are released by cardiomyocytes to clear out the defective mitochondria and protein aggregates to maintain cardiac homeostasis. Shed body remnants are released as a result of cytokinetic abscission during cell division. Filopodia derived vesicles are released through mechanical scission of filopodia. Apart from small and large EVs, extracellular particles are also produced by the cells. They are subcategorized as exomeres(<50 nm) and supermeres (<50 nm), having an unknown mode of biogenesis and secretion. Created with BioRender.com.

the role of the EV lipidome in ovarian cancer remains underexplored.

Ovarian cancer is a common gynaecological cancer worldwide and leading cause of death from gynaecological carcinomas[15]. Due to the lack of reliable early diagnostic markers, approximately 50% of ovarian cancer patients are diagnosed at an advanced disease stage [16,17]. Thus, it is essential that research efforts should be focused on identifying a specific and sensitive biomarker for ovarian cancer detection in its earliest stages to improve ovarian cancer prognosis and enhancing treatment outcomes.

Therefore, this review will first focus on describing the different EV subtypes and their underlying pathways of biogenesis. Secondly, the dynamic changes observed in lipid metabolism during ovarian cancer progression will be discussed. Lastly, the review will elaborate on a subpopulation of EV lipids, and their role in ovarian and other types of cancer.

2. Extracellular vesicles (EVs)

EVs are lipid bilayer-delimited particles secreted from all living cells and found in a variety of biological fluids, such as plasma [18], urine [19], saliva[20], tear[21], breast milk[22], seminal fluid[23], amniotic fluid[24], and cerebrospinal fluid (CSF)[25]. EVs can be classified based on their biogenesis and size [26,27]. Broadly, as recommended by ISEV, EVs can be categorized into two main subtypes based on size-small EVs (<200 nm) and large EVs (>200 nm) (Fig. 1)(Table 1)[27].

2.1. Small extracellular vesicles (sEVs)

2.1.1. Exosomes

Exosomes are one of the most studied types of sEVs and originate from the endocytic pathway (Fig. 1)[28]. Exosome biogenesis begins with invagination of the plasma membrane leading to formation of clathrin-coated vesicles, which then form early endosomes (EE) in the cytoplasm[29]. Inward invagination of the endosomal membrane leads to the formation of intraluminal vesicles (ILVs). At this stage, proteins, nucleic acids, and lipids are selectively sorted and encapsulated into these ILVs [29]. Upon formation of multiple ILVs, the endosome is then termed a multivesicular body (MVB) [30]. The MVBs can then either fuse with lysosomes and undergo lysosomal degradation, or alternatively fuse with the cellular plasma membrane, leading to the release of the ILVs which are then termed as exosomes (Fig. 1)[29].

Exosomes mediate intercellular communication through transfer of various cargoes such as proteins, lipids, nucleic acids, and metabolites [31]. However, the parent cell determines the type of cargo that is sequestered in the exosomes[31]. Proteins that are enriched within exosomes include heat shock proteins (HSPs)- HSP60, HSP70, HSP90, tetraspanins (CD63, CD9, CD81), ALIX, TSG101,LAMP-2B, MHC I/II, annexins, and Rabs (Rab1B,7,11)(Fig. 2)(Table 1)[32]. These proteins such as annexins and Rabs have a role in MVB biogenesis. In addition, tetraspanins are involved in selective targeting to the recipient cells, whereas ALIX and TSG101 are MVB forming proteins and contribute to exosome release [33]. Nucleic acids are also found in abundance in exosomes, and include mtDNAs, mRNAs, non-coding RNAs, miRNAs, piRNAs, and circular RNA (Fig. 2). Nucleic acids within exosomes are often degraded which can still interfere with downstream protein synthesis in recipient cells [34].

Additionally, the exosomal surface is enriched with lipids including cholesterol (CHOL), sphingomyelin(SM), phospholipids, phosphotidylethanolamines (PE), polyglycerols, diglycerols, and glycosphingolipids, in contrast to the parent cells, suggesting the involvement of various lipids in cargo sorting and signalling [6,28,35,36]. The involvement of lipids in exosomal release has been demonstrated by secretion of flotillin-1, Lyn, and stomatin, extracellularly in conjunction with lipid raft domains present at the exosomal membrane [37]. Sphingosine 1-phosphate regulates exosomal multivesicular endosome maturation and involves cargo loading of CD63, CD81, and flotillin through the inhibitory G-protein-S1P receptor present on MVBs [38].

Multiple exosomal biogenesis mechanisms have been proposed that can be broadly categorized into endosomal sorting complex required for transport (ESCRT) dependent and ESCRT independent pathways [31]. ESCRT-dependent pathway involving ubiquitination of membrane protein cargo was the first known pathway [39]. The ESCRT-dependent pathway requires the ESCRT complex, which is comprised of four ESCRT subunits: 0, I, II, and III, and its accessory proteins that recognize ubiquitinated protein and sort them to the lumen of the multivesicular endosomes [40]. The ESCRT-0 (TSG101) and ESCRT-I selects the ubiquitinated cargoes and induces the recruitment of ESCRT-II/III and formation of ILVs [41]. Furthermore, protein cargoes undergo deubiquitylation prior to membrane budding and exosome shedding process [42]. A variation in the ESCRT-dependent pathway involving the formation of syndecan- syntenin complex, has also been identified in the regulation of exosomal biogenesis [43]. Syndecan is a heparan sulphate proteoglycan, which binds to the cytosolic adapter syntenin, and further interacts with ESCRT accessory protein ALIX, for intraluminal

Table 1
Subtypes of EVs and markers associated with them.

EV Subtypes	Size (Diameter)	Origin	Cargo	References
Small EVs (<200 nm)				
Exosomes	~30–150 nm	Multi-vesicular bodies (MVBs)	Alix, TSG101, HSP70, CD63, CD9, CD81, FLOT1/2	[26,29,30,89, 223–225]
ARMMMs (arrestin domain containing protein 1 (ARRDC1-mediated microvesicles)	~< 100 nm	PM budding (ARRDC1 driven)	ARDDC1, TSG101	[50]
ECM-bound vesicles	~50–400 nm	Enzymatic digestion of ECM	unknown	[226]
Defensosome	~80 nm	Unknown	ADAM10, ACE2	[52,53]
Large EVs (>200 nm)				
Apoptotic vesicles	~1000–5000 nm	PM blebbing from apoptotic cells	Phosphatidyl serine (PS), Annexin V	[77,79]
Large oncosomes	~1000–10000 nm	PM budding from amoeboid cancer cells	Cytokeratin 18 (CK18), GOT1	[85]
Ectosomes	~100–1000 nm	Direct budding from PM	Annexins (ANXA1, A2, V), CD40, integrins, selectins	[227–231]
Migrasomes	~500–3000 nm	Migrating cells	ITGα5/β1, WGA, TSPAN4	[67,69,70]
Shed midbody remnants	~300–1000 nm	Cytokinesis	RACGAP1, KIF23/MKLP1	[86]
Exophers	> 3000 nm	PM budding	Phosphatidylserine, LC3 and Tom20	[232]
Filopodium-derived vesicles	> 200 nm	Scission of filopodia	IRS4, RAC1	[88]
Extracellular Particles				
Exomeres	~< 50 nm	Unknown	ENO1, HSP90-β, GANAB, mTORC1, CALR, HEXB, APP, Argonaute 1–3, ST6Gal-I, AREG	[89,90]
Supermeres	~< 50 nm	Unknown	TGFβ1, ENO 1 and 2, HSPA13	[91]

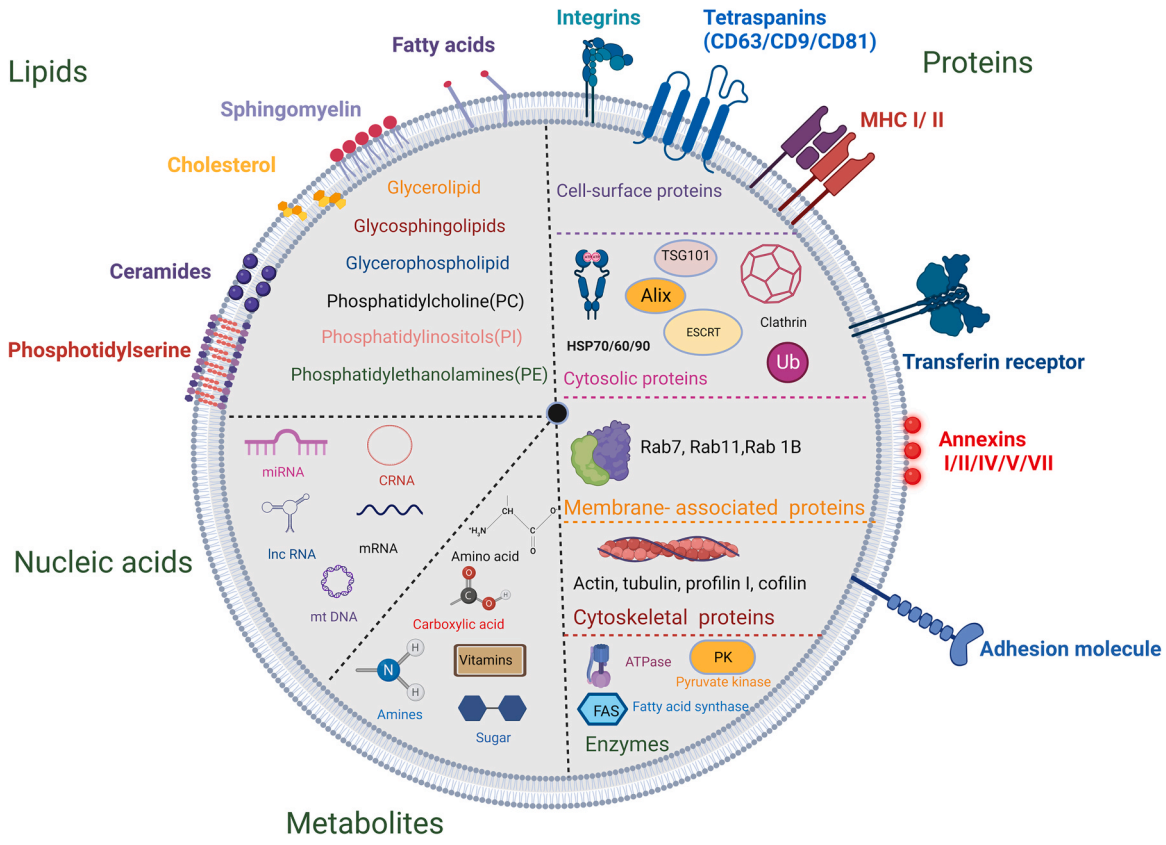

Fig. 2. The architecture and composition of exosomes: Exosomes are composed of a variety of molecules such as proteins, lipids, nucleic acids and metabolites. The exosomal proteins are subcategorized into cell-surface proteins, cytosolic proteins, membrane associated proteins, cytoskeletal proteins, and enzymes. The cell surface proteins of exosomes include tetraspanins (such as CD63, CD9 and CD81), MHCI/II. The cytosolic proteins present in the exosomes are heat shock protein (HSP70, HSP60, HSP90), clathrin, ALIX, ESCRT, TSG101and ubiquitin. The membrane associated proteins of exosomes are Rabs (Rab7, Rab11 and Rab1B), annexins (I/II/IV/V/VII). The cytoskeletal proteins include actin, tubulin, profilin1, cofilin and the enzymes present within the exosomes are pyruvate kinase, fatty acid synthase and ATPase. The major classes of lipids associated with exosomes are phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamines (PE), ceramides, sterol lipids, ganglioside (GM3), cholesterol, sphingomyelin (SM), glycosphingolipid, glycerophospholipid, glycerolipid and fatty acids. The nucleic acids present within the exosomes are miRNA, mRNA, lnc RNA and mtRNA. Vitamins, sugar, amines, carboxylic acids, amino acids are the metabolites present within the exosomes. Created with BioRender.com.

budding from the endosomal surface and shedding of exosomes [43].

In contrast, the ESCRT independent pathway involves the lipid microdomain in the formation of MVBs and is not yet completely understood [37,44]. The oligodendroglia precursor cells, deficient in functional ESCRT components, produce EVs enriched with Cers, SM, and CHOL [44]. Interestingly, MVBs produced through the ESCRT-independent pathway are larger in size and contain fewer ILVs of irregular morphology [45]. However, the ILVs formed through the ESCRT independent pathway begin with the assembly of SM and cholesterol in the lipid raft microdomain. The SM within the lipid raft undergoes hydrolytic cleavage by the neutral sphingomyelinase (nSMase) producing ceramides, which then trigger inward budding and forming ILVs. Inhibition of SM hydrolytic cleavage leads to a decrease in exosome production, highlighting the critical role of ceramides [44]. Further support for the ESCRT independent pathway is highlighted by a finding where the depletion of all four components of ESCRT, could generate MVEs carrying CD63[45]. Another EV biogenesis pathway can also be initiated by tetraspanins CD63, independently of ESCRTs and ceramides[46]. CD63 is required for ILV formation and its release and knockdown of ESCRTs or ceramides does not affect the release of ILV or sorting of cargoes [47]. Lately, ESCRT- independent ILV-formation pathway is found to be also controlled by Rab GTPase, Rab31, in ceramide dependent manner but independent of both ESCRTs and tetraspanins[48]. However, it is still ambiguous as to whether these pathways function concurrently in the cell or independently of each other [49].

2.1.2. *ARMMs, ECM-bound vesicles and defensosomes*

ARMMs (arrestin domain containing protein 1(ARRDC1)- mediated microvesicles), extracellular matrix (ECM) bound vesicles, and defensosomes are recently discovered small EVs (Table 1).

ARMMs (size <100 nm) are released by membrane budding, mediated by arrestin domain containing protein 1(ARRDC1) (Fig. 1). The late endosome resident protein TSG101 relocates to the plasma membrane and interacts with the conserved tetrapeptide PSAP motif of ARRDC1. It leads to the shedding of microvesicles containing TSG101, ARRDC1 and other proteins. However, these vesicles are distinct from exosomes as they are devoid of late endosomal markers [50].

Extracellular matrix (ECM)- bound vesicles are small EVs ranging ~50–400 nm, generated explicitly by enzymatic digestion of the extracellular matrix, when tissues undergo decellularization (Fig. 1). However, these ECM bound vesicles differ from EVs as they lack classical EV markers such as CD81, CD63, CD9, and Hsp70, suggesting a distinct population of extracellular vesicles. The presence of bioactive miRNA cargo within these ECM bound vesicles enables them to influence cellular behaviour similar to other EVs [51].

Defensosomes have been shown to play a crucial role in host defence mechanisms against bacterial and viral infections. These EVs are produced by host cells in response to infection by SARS-CoV2 [52], and *Staphylococcus aureus* [53], mediated by autophagy proteins. The autophagy protein ATG16L1 and other ATG proteins are involved in shielding against bacterial α-toxins by releasing ADAM10 rich defensosomes. Strikingly, in vitro experiments demonstrate that viral receptor

ACE2-enriched EVs can potentially inhibit viral infection by functioning as defensosomes. The bronchoalveolar lavage fluid of COVID-19 patients was observed to contain many EVs carrying ACE2. However, the role of these EVs in the recovery process from SARS-CoV-2 infection remains unclear [52,53]. The precise mechanism underlying defensosome biogenesis has not yet been fully elucidated.

2.2. Large extracellular vesicles (LEVs)

2.2.1. Ectosomes or microvesicles

Unlike exosomes, ectosome release does not involve the process of exocytosis, but rather it is a direct by-product of the plasma membrane blebbing, in the form of small vesicles, into the intercellular space (Fig. 1)(Table 1)[54]. Ectosome biogenesis involves multiple steps, including a)cell activation through agonists like cytokines, growth factors, and ATP, followed by an increase in intracellular calcium[55], b) changes in phospholipid composition (by aminophospholipid translocases and calcium dependent scramblase), and cytoskeletal reorganization (by activation of calcium dependent proteases like calpain and gelsolin) [56], and c)subsequent membrane blebbing with exposure of phosphatidylserine at the extracellular portion of the shed ectosomes [57]. Additionally, small GTPases (e.g.RhoA and ARF6) regulate ectosome release by altering the cytoskeletal complex, leading to increased ectosomal release from cancer cells [58–60].

Ectosomes carry a diverse cargo, such as matrix metalloproteinases (MMP), vascular epithelium growth factor (VEGF), and epidermal growth factor receptor (EGFR), implicating a role in cellular growth, angiogenesis, and remodelling of the extracellular matrix for tissue homeostasis [61]. Interestingly, tumour-derived ectosomes have the potential to promote cancer progression via interaction with adjacent or distant cells [62]. They also play a role in the transfer of oncogenic phenotypes [62], multidrug resistance [63], suppression of immune response [64], induction of thrombotic activity [65], angiogenesis [61], and tumour invasion and metastasis[66].

2.2.2. Migrasomes

Migrasomes are oval-shaped membrane-bound large vesicles released from migrating cells (Fig. 1) [67]. Biogenesis of migrasomes occurs with the formation of retraction fibres enriched with actin filaments, and these fibres eventually disassociate from the back end of the migrating cells [67,68]. Once detached from the cell body, vesicles are released into the extracellular space or taken up by the surrounding cells [67]. Migrasome biogenesis is based on actin filament polymerisation, and microdomain formation composed of cholesterol and tetraspanin4 (TSPAN4), as well as an abundance of integrins [27,67,69]. In addition, a specific lectin named wheat-germ agglutinin (WGA) is enriched in migrasomes and has a specific binding affinity for sialic acid and N-acetyl-D-glucosamine [70,71]. Hence, TSPAN4, integrins α5/β1, and WGA might serve as potential markers for migrasomes (Table 1).

2.2.3. Apoptotic bodies

Apoptotic bodies are released in the form of membrane blebs from apoptotic cells(Fig. 1)(Table 1) [72] and carry cargoes, ranging from fragmented nucleic acids to intact organelles [73]. During initiation of apoptosis, membrane lipids undergo a rearrangement process that switches phosphatidylserine(PS) from the cytoplasmic surface to the exoplasmic surface of the cell membrane.Once present on exoplasmic surface, PS provides the “eat me” signal to the phagocytes for recognition and engulfment [74–76], and binds to annexin. Therefore, annexin V represents a probe for detection and quantification of apoptotic cells [77]. Likewise, apoptotic bodies derived from apoptotic cells also present PS at their outer membrane marked them for clearance by macrophages via interacting with CD36 as a phagocytic receptor [72,78]. In early-stage carcinomas including ovarian and breast cancer, PS is displayed on tumour cells outer membrane and shown to secrete PS-positive exosomes in bloodstream,can be detected via ELISA based methods, indicating its potential as an early tumour biomarker [79,80].

2.2.4. Large oncosomes

Large oncosomes are secreted as membrane blebs by the ameboid tumour cells (Fig. 1)[81]. The presence of large oncosomes in cancer tissues and plasma samples of late-stage cancer patients is correlated with cancer metastasis and disease severity [81]. Large oncosomes are loaded with oncogenic cargoes and have the potential to transform the target cell phenotype through the horizontal transfer of oncogenes [82,83]. They contain miR-1227, miR-125, caveolin, ARF6, and metalloproteinases, highlighting their role in tumour metastasis [81,84]. Proteomic studies of large oncosomes identified diverse protein populations related to metabolic processes, namely glucose phosphate isomerase (GPI), heat shock 70-kDa protein 5(HSPA5), aspartate transaminase (AST), lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1). Interestingly, cytokeratin 18 (CK18) was found to be significantly enriched in large oncosomes and identified in plasma and tissue samples from human prostate cancer. Thus, CK18 is recognized as a marker for tumour derived large oncosomes [85].

2.2.5. Shed midbody remnants (sMB-Rs), exophers and filopodium derived vesicles (FDVs)

The shed midbody remnants (sMB-Rs) are a subtype of large EVs generated through cytokinetic abscission during the final stages of cell division (Fig. 1). Protein markers of the sMB-Rs include core cytokinetic protein MKLP1 and RACGAP1(Table 1). These vesicles are found to be distinct from other extracellular vesicles, and biogenesis mechanisms and cargo are variable for sMB-Rs [86].

Exophers are a new subtype of large EVs that are released from cardiomyocytes (Fig. 1). Exophers are secreted to eliminate defective mitochondria and protein aggregates during cardiac stress, to maintain the cardiac homeostasis, suggesting a non-canonical route of clearing abnormal mitochondria from cardiomyocytes. The exophers are further uptaken and absorbed by macrophages present around cardiomyocytes, facilitated by phagocytic receptor Mertk [87]. However, the precise mechanism of biogenesis of exophers is not yet fully understood.

Filopodium derived vesicles (FDVs) are a recently discovered subtype of large EVs, produced by mechanical scission of filopodia (Figure1). The interaction of I-BAR protein missing in metastasis (MIM) with the lipid membrane is found to be crucial for generation of cellular protrusions and facilitates formation of these vesicles through mechanical scission of filopodia. The absence of markers such as CD63 and PS in FDVs sets them apart from EVs that are currently recognized. Interestingly, unavailability of MIM induces enhanced production of small EVs through endocytic internalization of FDV cargo, implying a compensatory mechanism. FDV cargo gets transferred to multivesicular bodies and is packaged into small EVs. MIM seems to be responsible for loading specific cargo such as Rac1 and IRS4 into FDVs, that aid in cell migration[88].

3. Extracellular particles (EPs)

3.1. Exomeres

Exomeres are the most recently discovered extracellular particle and have a role in cancer growth(Fig. 1). However, biogenesis and secretion mechanism of exomeres remains unclear. Initially exomeres were considered to lack a lipid bilayer membrane and only surrounded by the protein corona with devoid of ESCRT components. Therefore, it was considered that they originate either from the plasma membrane or via the endocytic trafficking pathway [89]. However, recent study has revealed that few lipids are indeed associated with exomeres but not as prominent as other EVs. Interestingly, exomeres displayed the enrichment of ceramides and triglycerides while compared to exosomes, indicating their role in transportation of these lipids to recipient cells

[89].

Furthermore, exomeres are abundant in proteins ENO1, HSP90-β, neutral α-glucosidase AB (GANAB), mammalian target of rapamycin complex 1 (mTORC1), calreticulin (CALR), and hexosamindase B (HEXB)(Table 1). These markers are associated with the mitochondria, ER, and cytoskeletal proteins. Together, these findings indicate that the uptake of these exomeres by the recipient cells may alter the process of glycosylation and cellular metabolism [89]. Additionally, exomeres are highly enriched in Argonaute1–3 and amyloid precursor protein (APP), proteins implicated in Alzheimer's disease [90]. The presence of β-galactoside α2,6-sialytransferase 1 (ST6Gal-I), and amphiregulin (AREG) cargoes in exomeres may implicate its role in cancer metastasis [90].

3.2. Supermeres

The supermeres and exomeres are closely related EVs which cannot be adequately distinguished by conventional microscopic techniques such as atomic force microscopy (AFM) and electron microscopy. However, fluid AFM can be employed to determine the structural and morphological differences between supermeres and exomeres. Interestingly, in vivo supermeres are more efficiently taken up by target cells compared to small EVs and exomeres [91]. Enriched cargo in supermeres include amyloid precursor protein (APP), glypican1(GPC1), TGF-β1, MET, argonaute-2, extracellular RNA(miR-1246), ACE2, and PCSK9 (Table 1). Cancer derived supermeres displayed unique functional impacts on normal recipient cells such as upregulation of lactate secretion, transfer of chemoresistance, and changes in liver metabolism [91].

It is important to note that there exists some overlap in size, density, biogenesis pathway, and markers of EVs types discussed posing an inherent challenge in distinguishing each subtype from each other.

4. Lipids

Lipids are a class of organic compounds comprised of fatty acid chains or their derivatives [92] that are insoluble in water and soluble in organic solvents [93]. They can be classified into eight primary categories, fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides(PK) [94]. These lipid classes have different chemical structural backbones that influence their biophysical characteristics and physiological functions [95]. Lipids are not only crucial for building the cell membrane matrix but also play an important role in energy storage, membrane trafficking, maintaining cellular architecture, and forming distinct sub compartments in the membrane to control cellular functions [96].

Asymmetricity in the lipid bilayer of the plasma membrane is unique and requires P-type ATPases for the generation of a large amount of ATP to maintain asymmetricity [4,97]. Lipids such as phosphatidylcholines (PC) and SP constitute the exoplasmic leaflet whereas the cytosolic leaflet is decorated with phosphatidylserines (PS), phosphatidylethanolamines(PE), phosphoinositides (PI) along with cholesterol present in both plasma membrane leaflets[96].

Characterization of lipids has been challenging compared to other well sequenced biomolecules such as proteins and nucleic acids [95]. Chromatographic techniques were initially employed to study the lipid classes, but were inadequate in resolving complex lipid species [98]. High resolution mass spectrometry for lipids is an emerging technique as it detects the alteration in the structure and function of lipids in cells, tissue, or biological fluid as observed in pathological conditions such as cancer, diabetes, obesity, fatty liver disease, and neurological disease [95].

Lipids also have an important role in regulating the endocytic pathway by contributing to MVB generation. Upon maturation of early endosomes to late endosomes, the availability of ST and PS decreases but bis(monoacylglycerol) phosphate (BMP) increases substantially[99]. The bis(monoacylglycerol) phosphate (BMP), an anionic phospholipid, is also involved in the formation of MVBs, fusion and hydrolysis of membrane SP [100,101]. The specific phosphoinositides present on the plasma membrane (PtdIns(4,5)P_2), early endosome (PtdIns3P), late endosome (PtdIns(3,5)P_2), and TGN (PtdIns4P) recognize the endocytic membrane, and subsequently recruit proteins from the cytoplasmic compartment that are required for vesicular trafficking[4102]. Numerous lipid mediators have been identified in the regulation of signalling and recognition machineries, and they function by interacting with specific proteins [102].

4.1. Changes in lipid metabolism in ovarian cancer

Altered lipid metabolism has been observed in cancer patients[103]. For instance, it has been demonstrated that cancerous cells exhibit anomalous utilization and absorption of lipids[104]. This reflects rapid cell division, increased β-oxidation of fatty acids, and enhanced cell proliferation in ovarian cancer progression due to the upregulation of the PI3-kinase mediated activity involving oncogene PIK3CA [105,106]. Altered metabolic pathways involving lipid uptake, lipogenesis and fatty acid oxidation have been observed in ovarian cancer progression[8]. The enhanced lipid uptake in ovarian cancer cells is mediated via receptor CD36 while fatty acid binding protein 4 (FABP4) acts as a lipid shuttle between adipocytes and ovarian cancer cells contributing to ovarian cancer metastasis[10,107,108]. Additionally, ovarian cancer stem cells demonstrated increased lipogenesis moderated via stearoyl CoA desaturase 1(SCD1) [109].

Furthermore, increased levels of lipogenic enzyme, fatty acid synthase (FAS) has been observed in an ovarian neoplasm[110]. In line with this study, immunohistochemical analysis of high-grade serous, endometrioid, and mucinous carcinomas show the focal or multifocal (positive) staining of FAS whereas ovarian adenomas display minimal or absent FAS staining. The multifocal FAS immunohistochemical staining is indicative of poor prognosis in ovarian cancer patients[110]. Increased FAS regulates localization, expression, and cellular functions of the oncogenic protein, such as human epidermal growth factor receptor 2 (HER2) in the case of ovarian carcinomas[111]. The inhibition of FAS either pharmacologically or by RNA-mediated silencing of apoptotic pathway in HER2 overexpressing cells, demonstrates FAS as a molecular sensor of cellular energy. Furthermore, it provides the rationale behind therapeutic targeting of FAS in HER-2 overexpressed ovarian tumours[111]. Cytotoxicity in human ovarian cancer cells has been observed upon selective inhibition of FAS through activation of adenosine monophosphate activated protein kinase (AMPK). Thus, FAS is considered as a potential pharmacological target for anticancer therapy[112]. A novel FAS inhibitor such as FAS31 does not exhibit cytotoxicity in normal tissue in vivo but shows apoptosis in a human cancer xenograft model [113].

Phospholipid enzymatic pathways also play an important role in the development of ovarian cancer. In particular, phosphocholine contributes $>$ 70% of the total choline presence in human epithelial ovarian carcinoma (EOC) cell lines [114]. The intracellular level of phosphocholine is found to be 3–8 fold higher in human EOC cell lines compared to normal immortalized ovary epithelial cells(EONT) [114]. The phosphatidylcholine hydrolysis pathways were also upregulated as evidenced by a five-fold increase of phosphatidylcholine-specific phospholipase C (PLC)/phospholipase D (PLD) activity, contributing to phosphocholine enrichment [114]. The phospholipids exist in several forms in human EOC and are identified as phospholipase A2 (PLA2) [115], lysophosphatidic acid (LPA) [116], phospholipase D(PLD) [117], and autotaxin [118].

LPA were found to be abundant in ascites of ovarian cancer patients with variability in fatty acid side chains and termed as ovarian cancer activating factor (OCAF) [116]. It triggers the rapid synthesis of DNA and proliferation of ovarian cancer cells [116]. Moreover, LPA has a

stimulatory effect on cell migration and the release of interleukin-8 (IL-8), contributing to cancer metastasis and angiogenesis [116]. LPA is also involved in cell adhesion, proliferation and migration, apoptosis inhibition, stimulation of platelet aggregation, transformation of cellular morphology, and smooth muscle contraction [119–123]. Notably, it is also a mitogen [124] and contributes to the invasive phenotype in hepatoma cells at mesothelial layer [125]. LPA indeed promotes proliferation of breast and ovarian cancer cells, even under growth factor-deprived conditions [126]. LPA signalling in ovarian oncogenesis by mediating the expression of vascular endothelial growth factor (VEGF) by increasing LPA_2 and LPA_3 receptor expression [127]. Even in the absence of hypoxia-inducible factor 1, LPA triggers expression of VEGF, thereby activating transcription factors c-Myc and Sp-1 for tumour angiogenesis to progress [128].

Phospholipase A2 (PLA2) enzymes are involved in the generation of LPA in ovarian cancer cells [115]. Calcium-independent PLA2 has a significant effect on proliferation and cell migration of ovarian cancer cells whereas cytosolic PLA2 has an opposite effect [115]. In the absence of other growth factors, suppression of calcium independent PLA2 enzymatic activity led to arrest of cells in S and G2/M phases of the cell cycle [129]. External supply of LPA or other growth factors releases the cells from S-phase, however, cells in the G2/M phase remain ceased during inhibition of calcium independent PLA2 [129]. This in turn increases apoptosis slightly and indicates that ovarian cancer cells are highly sensitive towards enzymatic activity of PLA2, and thus controls tumorigenicity. Phospholipase D (PLD) in ovarian cancer cells is involved in the formation of phosphatidic acids that are one of the source components for the generation of LPA [130]. The OVCAR-3 cell line showed upregulation of PLD activity which is mediated via the integrin receptor signalling cascade. Blocking of PLD leads to inhibition of integrin mediated Rac translocation and metastasis in OVCAR-3 cells [117].

The autotaxin (ATX) protein controls lysophospholipase D enzymatic activity and participates in hydrolysis of lysophosphatidylcholine (LPC), to produce LPA that acts as a mitogen and growth factor for the tumour [131]. LPA, while acting through LPA4 receptor, increases VEGF and VEGF receptor 2 expression which can also induce ATX via positive-feedback loop. The antisense morpholino oligomer mediated suppression of ATX secretion in ovarian cancer cell line, SKOV-3, diminished cell migration responses to LPA, LPC, ATX, and VEGF [118]. Therefore, FAS LPAS, LPA, and ATX might be excellent targets for ovarian cancer therapy considering modulation at lipid metabolism level, such as production of LPA.

Another class of lipids, sphingolipids and glycosphingolipids also have been implicated an in the pathogenesis of variety of cancers including ovarian cancer [132,133]. Ceramides are central lipid molecules in sphingolipid synthesis pathway, it generates sphingosine through diacylation by ceramidases. Further, sphingosine undergoes phosphorylation involving sphingosine kinase (SphK) 1 and SphK2 to form sphingosine-1-phosphate (S1P), which has been shown to enhance cell survival [134]. In contrast, ceramides alone are known proapoptotic lipids, shown to have inhibitory effects on tumor growth in human carcinoma cells [135]. Interestingly, the isoform SphK1 are found to be actively expressed in human ovarian cancer cells and presented resistance to the chemotherapeutic drug N-(4-hydroxypheny) retinamide (4-HRP) [136]. The functional contrast of S1P and ceramide indicates that the ratio between ceramides and S1P may underlie cancer progression. Ceramides are also involved in the synthesis of glycosphingolipids including sulphatides which were also elevated in ovarian cancer tissue along with corresponding mRNA expression of enzymes required for sulphatides synthesis [133,137]. However, the precise role of sulphatides in ovarian cancer progression remains ambiguous.

Apart from FAS and phospholipids, many other lipogenic enzymes are found to be dysregulated in ovarian cancer, namely, Stearoyl CoA desaturase (SCDI), ATP-citrate lyase, Acetyl CoA carboxylase, Mitochondrial elongation factor 2(MIEF2), and Diacylglycerol O-acyltransferase I (DGAT1) [8].

4.2. Lipidomic profile analysis in ovarian cancer patients

The large-scale profiling of metabolic alterations in 448 EOC patients shows that lysophosphatidylethanolamines(LPEs) and LPCs are enriched with CerP (d18:1/12:0) in localized EOC representing early stage of ovarian cancer (Fig. 3). Expression of these lipids was also decreased in metastatic EOC representing advance stages of disease. This suggests exploitation of phospholipids by the proliferating cancer cells in an attempt to preserve membrane integrity [138,139]. LPE was also elevated in ascites from ovarian cancer patients [126,140] and seems to modulate effects on cellular signalling in ovarian cancer through the upregulation of Ca^{2+} ions within the cell [140]. LPE triggers chemotactic migration and proliferation of SKOV-3 cells [141]. The lipidomic profile analysis of the tumour and plasma samples from metastatic high grade serous ovarian carcinoma showed consistent enrichment of lipids such as specific ceramides and triacylglycerol (TAG) containing short/medium FA side chains [142]. The Cer (d18:1/16:0), Cer (d18:1/18:0), Cer (d18:1/20:0), Cer (d18:1/24:1) were elevated in ovarian cancer patients with advanced disease while Cer 23:0 and 24:0 fatty acids were found to be diminished in advanced stage ovarian cancer. Notably, this ceramide profile of advanced ovarian cancer had similarity with coronary artery disease[103]. This suggests an increased risk of development of ischemic stroke in ovarian cancer patients. Therefore, incidence of stroke in women can also be an indicator of ovarian cancer disease [103,143,144]. Strikingly, most of the other lipid classes such as cholesteryl esters (CEs), diacylglycerols (DGs), SMs, sphingosine phosphates (SPs), PCs, PEs, PIs, globotriosyl-ceramides (Gb3s), lactosylceramides, S1P and cerebrosides were found to be diminished in these patients [103].

A recent study demonstrated a decrease in phospholipids like PCs, LPCs, PIs, Cers and SMs in early-stage I/II and late-stage III/IV ovarian cancer patients [142]. Specifically, there was an increase in ceramides with fatty acyl (FA) side chains (FA 18:0,20:0 and 24:1), and a concomitant decrease in ceramides containing FA 24:0. These lipid alterations are evident from stage I and increase as disease progresses [142].

There is also an alteration in lipid profiles between histological subtypes of EOC, suggesting a correlation between lipid metabolism and development of histological subtypes. Serous carcinomas showed greater levels of lipid alterations [142], whereas TAG (18:1/18:1/20:4) and Cer (18:1/18:0) were abundant in mucinous and endometrioid tumours, with a decrease in all other types of lipid classes. In addition, the combination of lipid expression with cancer antigen – 125 (CA-125) levels in stage I/II ovarian cancer enhanced the diagnostic value of CA-125 [142]. Whilst CA-125 is currently used for detection for ovarian cancer, it has low sensitivity and specificity [145,146]. Thus, a lipidomics approach could provide an avenue for improved ovarian cancer detection.

Recent studies have revealed the enrichment of sphingolipids and concomitant downregulation of the glycerophospholipids (namely plasmalogens, PCs and PEs) in EOC patients compared to control (patients with benign ovarian tumor and uterine fibroid) phosphatidylcholine PC – PC (38:4), PC (35:5). PC (34:3) and SM (d18:1/17:0) and SM (d18:0/16:1), along with CA-125, were considered for the diagnosis of ovarian cancer. The predictive performance of CA-125 in combination with five diagnostic lipids showed improved predictive accuracy compared to CA-125 alone, AUC 0.821 and 0.865, for EOC patients and benign ovarian tumour/ uterine fibroid patients respectively [147].

In addition, lipidomic analysis of ovarian cancer tissue showed PC (32:3), PC (34:1), and PC (36:2) enrichment which was similar to lipid profile seen in breast cancer tissue, suggesting a similar mechanism underlying lipid alterations in both ovarian and breast cancer [147, 148]. Furthermore, there is a significant decrease in all types of GP in EOC patients compared to control except for PC (33:5) and PC (34:3).

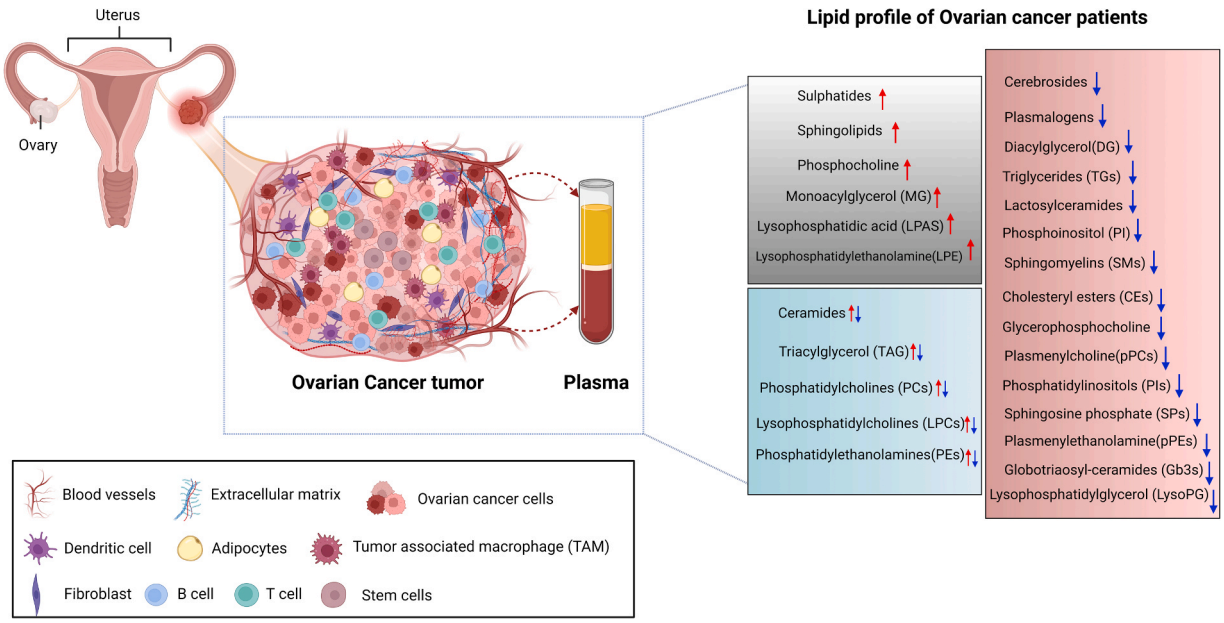

Fig. 3. Summary of lipid profile of ovarian cancer in patient samples.Ovarian cancer tumors generally originate from ovaries or fallopian tubes. The ovarian cancer tumor microenvironment contains immune cells (B and T lymphocytes, dendritic cells and TAMs), adipocytes, cancer associated fibroblasts, cancer stem cells, ovarian cancer cells, blood vessels and extracellular matrix (ECM), required for tumor growth and metastasis. Alteration in lipid profiles were found in ovarian tumor and plasma samples of patients having ovarian cancer as summarized in figure above.Significant increase in sulphatides, sphingolipids, phosphocholine, monoacylglycerol, LPAS, LPE are observed in ovarian cancer patient samples. The ceramides, TAG, PC, LPC, PE presented variable patterns in ovarian cancer patients. Consistent decrease in specific lipids such as cerebrosides, plasmalogens, lactosylceramides, DG, TGs, PI, SM, CE, pPC, SP, pPE, Gb3s, LysoPG were noted. Created with BioRender.com.

The sphingolipids were prominent in EOC patients but SM (d18:2/14:0) were not. Moreover, an increase in monoacylglycerol (MG) and diacylglycerol (DG) in EOC patients compared to patients with uterine fibroids and benign ovarian tumours was noted [147].

Interestingly, plasma metabolic profile studies have identified LPCs in potentially differentiating the stages of EOC [138,149,150]. In particular, the downregulation of three distinct LPCs: LPC (14:0), LPC (18:2) and LPC (20:3) from stage I-IV EOC patients indicates that an alteration in LPC lipid metabolism is associated with severity of disease [147]. It is likely that plasma LPC in EOC patients is downregulated as there is an abundance of phospholipase D, which converts LPC to LPA [147,151].

One of the major issues in the management of EOC is the high relapse rate after cytoreductive surgery and chemotherapy-based treatment. More than 60% of the relapses occurs within five years in patients with advanced stages of ovarian cancer and overall estimated survival rate of 25–30% [152,153]. Recent lipidomic research reveals that plasma lipid profile cannot only discriminate between patients with and without EOC recurrence but also between early and late ovarian cancer relapses [154]. Lysophosphatdylglycerol: LPG (20:5) has emerged as a potential biomarker in predicting EOC recurrences. The decrease in LPG (20:5) was evident in EOC patients with recurrence when compared to patients without recurrence [154]. LPG contributes to two distinct signalling pathways, the first involves the pertussis toxin (PTX) sensitive G-protein dependent (GPCR dependent) calcium increase, and the second involves PTX sensitive G-protein- independent ERK and AKT activation in human ovarian cancer cell lines. These signalling pathways culminate into the development and progression of EOC [154,155]. Furthermore, a drop in plasma TGs may also predict early EOC recurrence, dysregulation in glucose homeostasis and other various metabolic syndromes [154].

Concomitant downregulation of PCs–plasmenylcholine (pPCs) and plasmenylethanolamine (pPEs) in patients experiencing EOC recurrence corresponds to the elevation of oxidative stress and progression of EOC. Other than PC, TGs, and LPG downregulation of three specific sphingolipids has been demonstrated, SM(d18:1/14:0), SM(d18:2/14:0) and Cer(d18:1/23:0), indicating the defects in ceramide production and sphingolipid metabolic pathways contributing to cancer cell survival and chemotherapeutic resistance [154,156]. Decreased levels of phosphoinositol (PI) in patients with EOC recurrence compared to patients without recurrence could be explained by the altered PI3K/AKT cascade in ovarian cancer giving rise to cisplatin resistance and relapse [154,157,158]. Therefore, it is likely that homeostatic crosstalk or conversions occur between GP and SP during EOC relapses [154,159].

4.3. *Lipids in EV biogenesis*

Lipids not only constitute physical structure of EVs and protect their cargo, but also have a significant role in EV biogenesis and release [160]. Despite enormous chemical variation among lipid classes, only few lipids are associated with EVs[160]. However, more than 1900 lipid species have been identified in EVs derived from different cells, species, and bodily fluid [6,161–163]. EV-associated lipids are variable, depending on cell of origin, selective loading of specific lipids, and their enrichment [164,165].

This section will discuss the involvement of specific sets of lipids in EV biogenesis and release. Different classes of lipids play various roles in the EV biogenesis pathway, including cargo sorting, exosome formation and their stabilization, or secretion into the extracellular milieu [166]. The major types of lipids shown to be involved in exosome biogenesis are cholesterol [167], ceramides [44], sphingosine 1-phosphate (S1P) [38], ether lipids [168], diacylglycerol(DG) [169], phosphatidylinositol 3-phosphate [170], Bis (monoacyl-glycerol) phosphate [171], cardiolipin [164], and phosphatidylinositol-3,5-biphosphate [172] (Fig. 4).

It is evident that ESCRT machinery are required for the formation of the MVBs, and specific cargo sequestration into EVs [173]. Hydrophobic cholesterols contribute to fluidity and microdomain formation in cell membrane and are also involved in self-clustering of the endosomal sorting complex required for transport (ESCRT)-II machinery [167]. They also form ordered microdomains in the membrane in a cholesterol dependent manner [167]. This event provides favourable conditions for

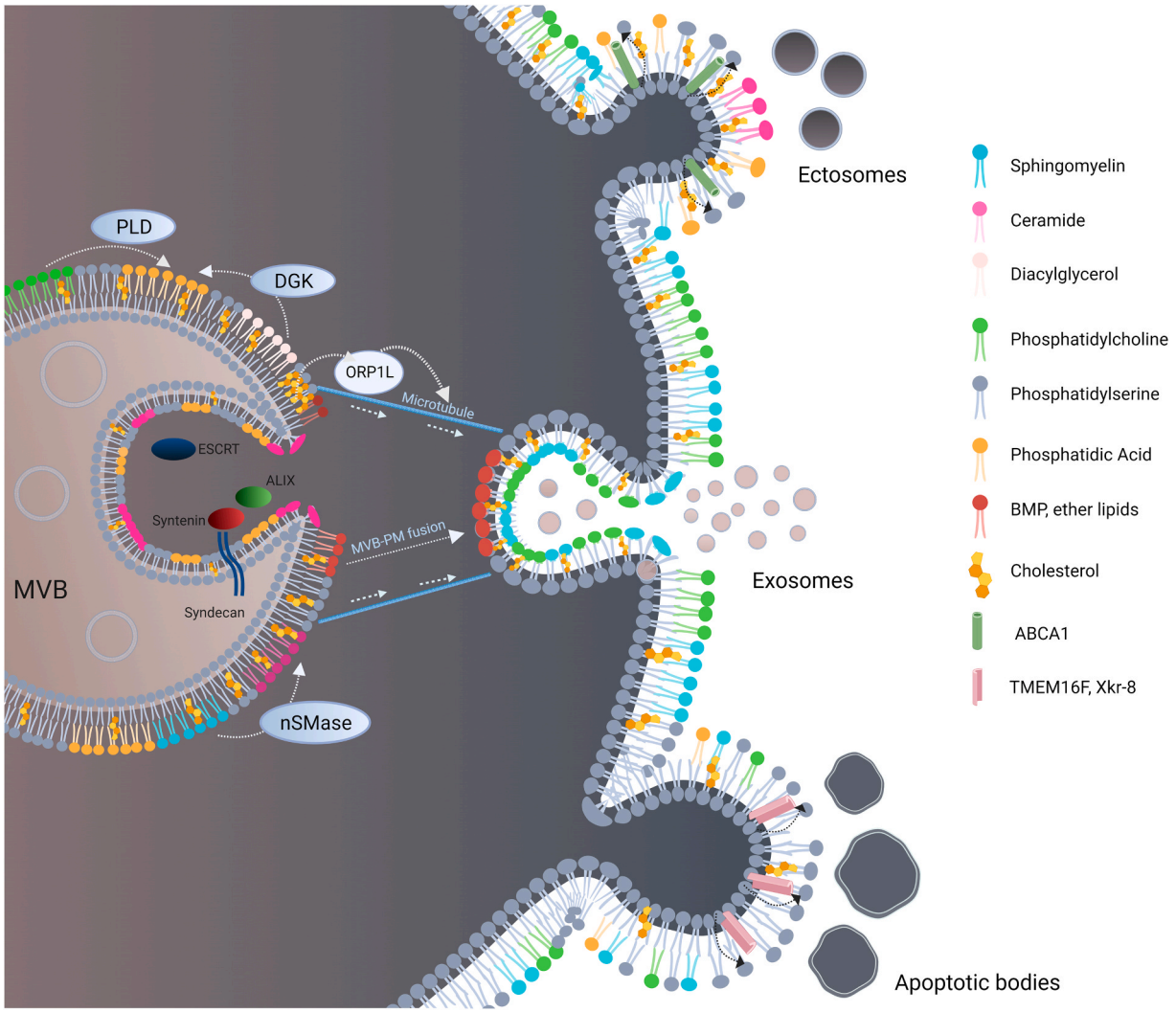

Fig. 4. Lipids in EV biogenesis and release. Membrane resident cholesterols provide favourable condition for the self-clustering of ESCRT components in MVBs, interact with ORP1L to regulate the movement of MVBs along microtubules and promote MVBs-PM fusion and secretion of exosomes. Bis(monoacyl-glycero) phosphate (BMP) and ether lipids have fusogenic properties that reinforce the fusion of MVBs-PM and exosomal release. The production of ceramides from sphingomyelin is mediated by activity of neutral sphingomyelinase (nSMase) which induces spontaneous negative curvature of MVBs in an ESCRT-independent manner. Phosphatidic acid can be generated from diacylglycerol and phosphatidylcholine by activity of diacylglycerol kinases (DGK) and phospholipase D (PLD) respectively. In addition, the interaction between phosphatidic acid and syntenin induces recruitment of syndecan followed by CD63 and ALIX contributing to ILV budding. Ectosome and apoptotic body formation require exoplasmic translocation of phosphatidylserine mediated by floppase such as ABCA1 and scramblases such as TMEM16F and Xkr-8 respectively.Created with BioRender.com.

MVB-PM fusion and PM budding for the generation of exosomes [167]. In addition to that, cholesterol interacts with oxysterol-binding protein ORP1L and regulates movement of late endosomes along motor proteins during EV biogenesis [174].

Ceramides are also an important lipid class, contributing to ESCRT-independent EV biogenesis [44]. It is generated from the substrate SM after excision of phosphocholine through sphingomyelinase activity, which leads to the sudden invagination of the ceramides containing MVB membrane and subsequent formation of negative curvature of ILVs [44]. It has been reported that the enzyme sphingomyelinase also induces ceramide formation by interacting with phosphatidic acid (PA) and thereby assists ILV production in an ESCRT-independent manner [175]. Similar to ceramides, PA is a simple phospholipid also contributing to the negative curvature of the membrane [176]. PA can be produced from substrate DG and PC through the activity of enzymes phospholipase D(PLD) and diacylglycerol kinase(DGK), respectively [177]. Furthermore, the head group of PA enables the interaction of PA with the proteins at lysine and arginine residues [178]. The interaction between PA and syntenin initiates recruitment of syndecan followed by CD63 and ALIX, ultimately leading to formation of ILVs at the membrane [179]. Likewise, other lipid-controlled mechanisms associated with maturation of MVBs involves ESCRT independent cargo sorting via activation of inhibitory G-protein coupled receptors such as sphingosine-1-phosphate (S1P) receptors by continuous supply of sphingolipid sphingosine 1-phosphate (S1P) [38]. Additionally, DG is involved in recruitment of cytosolic proteins to the cell membrane and their stabilization, followed by interaction with cytoskeletal proteins assisting in EV budding and fusion [169].

Ether lipids are a less discussed class of membrane lipids, but interestingly found to be highly enriched in exosomes derived from several sources including mast cells, PC-3 cells and platelets, mostly in the form of PC and PE lipids [160]. PE ether is uniquely enriched in exosomes derived from urine, HIV particles and nematodes and may contribute to stability of exosomes. However, it requires further investigation to comprehend its function [160,180–182]. Most importantly, ether lipids are involved in regulation of protein cargo sorting during formation of

MVBs and EV release. The formation of MVBs and ILVs per MVB were reduced when PC-3 cells treated with ether lipid precursor (hexadecylglycerol), indicating enhanced mature MVBs-plasma membrane fusion [168].

The phosphatidylinositol 3 -phosphate is shown to be involved in sorting of cargoes and formation of EVs[170,183]. Underlying mechanisms involve phosphatidylinositol 3 -phosphate interaction with Hrs protein for initiation of cargo sorting to the endosomal compartment [183], and interaction with ESCRT 0 to facilitate recruitment of other subunits ESCRT-I,II and III on to the membrane during formation of EVs [170]. The phosphatidylinositol-3,5-biphosphate are known to regulate fusion of lysosomes with MVBs and autophagosomes during exosome release [172].

Bis(monoacyl-glycero) phosphate(BMP) is one of the endolysosomal anionic phospholipids, which acts as a cofactor for the lysosomal sphingomyelin metabolism, contributing to EV formation and secretion through binding with Hsp70, thereby stabilising lysosomal structures [171]. Concomitantly, the BMP containing endosomal membrane interacts with Alix and other factors, possibly ESCRT proteins, for the inward fission process [100]. Cardiolipin is a unique phospholipid found in the inner mitochondrial membrane that provides negative curvature to the membrane. Exosomes derived from human bone marrow derived mesenchymal stem cells (MSC) and Huh7 cells have a higher concentration of cardiolipins, indicating their active loading to EVs, presumably stabilising exosomal structures [184].

Recent studies have demonstrated variation in lipid composition of exosomes and ectosomes (microvesicles) derived from different cell types including human bone marrow derived mesenchymal, glioblastoma and hepatocarcinoma cells [164]. Microvesicles contained high levels of ceramides and SMs, whereas exosomes contained abundant glycolipids and free fatty acids, suggesting role of specific class of lipids in generation of subtypes of EVs [164]. A study involving lipidomic profile analysis of healthy plasma donors demonstrated that the microvesicles are enriched in PA whereas exosomes showed the enrichment of SM. However, the level of PE,PI+PG showed slight enrichment in microvesicles compared to the exosomes whereas levels of PC and LPE+LPC were slightly higher in exosomes [185]. Ectosome biogenesis involves exoplasmic translocation of PS as discussed earlier in Section 2.2.1 and is reportedly mediated via floppase named as ATP binding cassette transporter 1(ABCA1), causing structural derangement of phospholipids at plasma membrane [186]. It has been suggested that PS relocation from inner to outer membrane leaflet is more prominent at the site of ectosome shedding and vesicles released are enriched with cholesterol [66] (Fig. 4).

As discussed in Section 2.2.3, the formation of apoptotic bodies is a consequence of alteration in membrane asymmetry of apoptotic cell through translocation of PS from inner to outer leaflet mediated via scramblases such as TMEM16F and Xkr-8 [76] (Fig. 4). As a result, the apoptotic bodies are found to be enriched in PS, providing signals for phagocytic clearance.

4.4. The role of EV-associated lipids in cell-to-cell communication and cancer progression

EV-associated lipids are shown to be involved in signalling pathways acting as first and second messengers [187]. Further, EVs fuse with the target cell, the EV-associated lipids alter the viscosity/fluidity of the cell membrane, suggesting an important role in intercellular communication [188]. Exosome associated lipids show homogeneity with lipid rafts and possess higher order lipids leading to higher resistance to detergents compared to other EV classes [160]. A recent in-depth EV lipid mapping study concluded that several factors such as charges on head groups, degree of saturation, fatty acid length, along with head group identity, are crucial for spontaneous curvature of membrane and lipid assortment into EVs [164]. Lipidomic studies in the pancreatic PC-3 cell line demonstrated that lipid species such as CHOL, SM, PS, and glycosphingolipids were found to be two to three-fold higher in exosomes compared to parent cells [189]. In contrast, phosphatidylcholine (PC) and phosphoinositol (PI) were less abundant in PC-3 cell derived exosomes but surprisingly, percentage of PE remained same [189]. On the other hand, exosomes derived from oligodendroglial precursor (oli-neu) cell line showed the enrichment of ceramides (Cer) and lower accumulation of SM, unlike PC-3 cells [44]. The very long chain SM (SM d18:1/24:0 and SM d18:1/24:1) and (PS18:0/18:1) were found to be enriched in PC-3 derived exosomes in a similar proportion as in the parent PC-3 cells [189]. This finding suggested that very long chain SM from the outer leaflet and PS from the inner leaflet are sequestered into EVs together. The transmembrane coupling event is entitled “hand shaking between two membrane leaflets”, occurring via interaction of the fatty acid chain from the outer and inner membrane leaflets [6,189]. The study of urinary exosomes lipid composition in prostate cancer patients showed a significant rise in lipid species LacCer d18:1/16:1 whereas PS 18:1/16:0 was found to be elevated in the control group [190]. Consistent with this study, another lipidomic analysis of urinary exosomes obtained from prostate cancer patients showed enrichment of most lipid groups, compared to the control [191]. The enrichment of lipids was greater in small EVs compared to large EVs.In contrast, depletion of neutral lipids such as triacylglycerol (TG), diacylglycerol (DG), and Cholesterol Ester (ChE), in PC-derived urinary EVs have been found (independent of size), suggesting more energy exploitation by the prostate cancer cells to survive and proliferate [191].

Furthermore, emerging evidence shows EV lipids as a potential non-invasive biomarker for disease.The lipid arrangement in the EV membrane provides structural stability, thus identifying EVs as efficient drug delivery vehicles. This was demonstrated through the survival of the anthrax toxin encapsulated within EVs in the blood circulation when injected intravenously [192].

EV lipids and their derivatives can interact with recipient cells, making them a crucial mediator of disease development. For example, it has been demonstrated that the exosomes derived from CSF of multiple sclerosis patients contained high amount of sphingomyelinase, that convert SM into ceramides, promoting damage of axonal structure and dysfunctional mitochondria production [193]. EV interaction with recipient cells occurs through several mechanisms, including but not limited to 1) direct fusion with the plasma membrane of recipient cells, 2) receptor-mediated interaction, and 3) internalization by the recipient cell such as through clathrin or caveolin-mediated endocytosis, phagocytosis and macropinocytosis [194,195]. Recent studies have demonstrated an additional mode of internalization of EVs, which is mediated through cholesterol [196]. It has been suggested that the abundance of membrane lipids improves the fluidity of the membrane and thus could possibly enhance EV internalization [197]. Lipid raft, cholesterol enriched microdomains at the cell membrane are known to play a significant role in EV internalization and depletion of cholesterol from the cell membrane leads to inhibition of EV uptake by the recipient cell [198] The Scavenger receptor type B-1(SR-B1) receptors present in lipid rafts utilize high density lipoproteins (HDL) as a ligand, and the interaction of SR-B1with HDL activates cholesterol efflux and blocks cellular uptake of EVs[198]. This suggests that cholesterol has a significant role in EV uptake.

Furthermore, enrichment of lipids in healthy cell membrane provides a favourable environment for the uptake of cancer derived EVs, thereby contributing to malignant cell transformation [199,200]. To better understand the role of EV lipids in cancer progression, a recent study employed synthetic lipid enriched nanoparticles (lacking proteins and nucleic acids) which stimulated the NF-κB/SDF-1α axis in pancreatic cancer cells and induced binding of secreted chemokine SDF-1α to its receptor CXCR4 at the cell surface to activate the Akt cell survival pathway[201]. This suggests that EV lipids have a role in cancer development and progression, chemotherapeutic resistance, and invasiveness.

5. Dynamic changes in EV-lipids in cancer progression

5.1. EV-lipid profile in ovarian cancer

Dynamic changes in the lipid profile of EVs has been observed in ovarian cancer progression. Notably, the lipoprotein lipase, an enzyme that has a crucial role in regulation of plasma lipid levels by cleaving triglyceride portion of lipoproteins, was significantly upregulated in SKOV-3 derived exosomes [202]. This could explain the alterations in plasma lipid species occurring in case of benign, borderline, and malignant ovarian cancer tumours [142,154]. To investigate the role of EV lipids in the context of ovarian cancer, a lipidomic study was performed on exosomes derived from SKOV-3 cells and the human ovarian surface epithelial cell line (Fig. 5), and found that SKOV-3 derived exosomes were enriched in cholesterol ester (ChE), Gangliosides 3 (GM3), zymosterol (ZyE), acylcarnitines (AcCa), and specific lysophosphatidic acids (LPS, LPG, LPI, and LPC)[202]. Interestingly, SKOV-3 derived exosomes were less abundant in ceramides (Cer), Phospholipids (PS,PI, PG,PE), SM,CerG3, and DG[202]. The high concentration of lysophosphatidic acids in ovarian cancer suggests that these lipids may play a crucial role in the progression of ovarian cancer, consistent with previous research that has linked lysophosphatidic acids to various types of cancers (including gastric cancer, ovarian cancer, colorectal cancer, and renal cell carcinoma), through differing mechanisms of pathogenesis [141,203–205]. The abundance of ChE and its precursor zymosterol in ovarian cancer derived exosomes suggests that cholesterol homeostasis is aberrantly regulated in ovarian cancer, which has also been observed in other types of cancer [206]. Most recent study shows that SKOV-3 treated with cholesterol-lowering agent, atorvastatin, led to reduction in overall production of EVs but enhanced secretion of cholesterol enriched small EVs while decreasing intracellular lipid accumulation. In particular, EVs derived from atorvastatin treated SKOV-3 showed enrichment in lipid classes includes sterols, phosphorylated esters, ceramides and DG [207]. Since there exist a crosstalk between adipocytes and ovarian cancer cells, leading to omental and peritoneal spread of ovarian cancer [107], it is reasonable to predict that cholesterol-lowering agents might functions in disrupting the communication between adipocyte and ovarian cancer cell, preventing ovarian cancer proliferation and enhancing treatment outcomes.

5.2. Alterations in EV lipids in other types of cancers

It is evident that individual cell types have an idiosyncratic lipid profile, and that released EVs may also retain some of these unique characteristics. EVs derived from each type of cancer present a unique lipid profile, indicating its potential as a diagnostic biomarker. This section will discuss the lipid profile of EVs observed in common cancer types, which are also summarized in Table 2.

5.2.1. Colorectal cancer (CRC)

Lipid profiling of CRC- derived exosomes show enrichment of sphingolipids, glycerolipids, sterol lipids along with decreased levels of glycerophospholipids when compared to the parent cell [208]. This suggests that the generation of EVs and their release requires specific sorting of lipid classes/subclasses with varying length of fatty acid chain and degree of unsaturation plays a critical role in CRC progression [208]. The decrease in PC,PE,PI (34:1) with increase in PC,PE,PI (38:4) in exosomes was observed in CRC patients compared to healthy individuals, indicating the switching from 34:1 containing lipid species to 38:4-containing lipid species [209]. A later study employed a more specific targeted LC-MS/MS approach, which provided a cluster of lipid signature differentiating between healthy, non-metastatic and metastatic CRC patients. Exosomes derived from plasma of CRC (metastatic and non-metastatic) patients when compared to healthy individuals showed marked enrichment of specific lipid classes including PC, PE, SM, Ceramides (Cer). Notably, lipid subclasses including PC 34:1, PE 36:2, SM d18:1/16:0, HexCer d18:1/24:0,d18:1/24:1 and Cer d18:1/24:1 were found to be higher in exosomes derived from metastatic colorectal cancer patients. Exosomes derived from non-metastatic patients exhibited a noteworthy elevation in the level of a single type of lipid named PS 18:1/18:0 when compared to exosomes derived from healthy individuals, thereby distinguishing the two groups [210]. However, the molecular mechanism involved in lipid segregation and regulation of lipid composition in CRC remains to be elucidated.

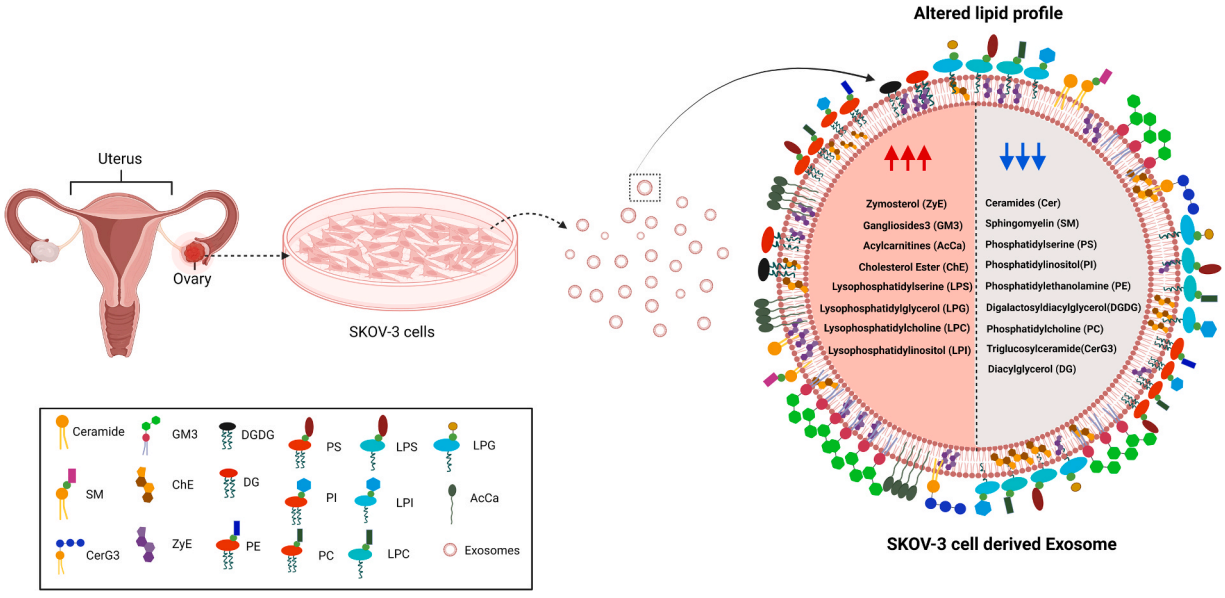

Fig. 5. Schematic diagram showing lipid profile of ovarian cancer cell line derived EVs. Ovarian cancer cells secrete EVs that carry significant information in the form of bioactive molecules such as proteins, lipids, and nucleic acids. Abundance of lipid classes associated with EVs derived from ovarian cancer cell line (SKOV-3) are summarized in the figure above. Lipid classes ZyE, GM3, AcCa,ChE, LPI, LPS, LPG and LPC were found to be enriched in EVs derived from the SKOV-3 cell line with an associated decrease in lipid classes Cer,SM,PS,PI,PE,DGDG,PC,CerG3 and DG compared to ovarian surface epithelial cells.Created with BioRender.com.

Table 2
Characteristics EV lipids as a potential diagnostic biomarker in cancer pathology.

Type of Cancer	EV source	EV subtype	Lipid biomarkers	Lipidomic method	References
Ovarian Cancer	SKOV-3 cell line	Exosome	LPI,LPS,LPG,LPC,GM3,ChE and ZyE	UHPLC, Q-Exactive mass spectrometer	[202]
Colorectal Cancer	Human colorectal cancer cell line	Exosome	Sphingolipids, glycolipids, sterol lipids	High-resolution "shotgun" and targeted tandem mass spectrometry	[208]
	Plasma from hyperplastic polyp, adenomatous, polyp and colorectal cancer patients (including invasive neoplasia and hereditary non-polyposis)	Exosome	PC 38:4, PE 38:4, PI 38:4	Triple quadrupole mass spectrometer, hybrid quadrupole-orbitrap mass spectrometer	[209]
	Plasma from metastatic and non-metastatic colorectal cancer patients	Exosome	PC 34:1, PE 36:2, SM d18:1/16:0, HexCer d18:1/24:0,d18:1/24:1 and Cer d18:1/24:1	Liquid chromatography-mass spectrometry (LC-MS) and targeted lipidomic analysis	[210]
	Plasma from colorectal cancer patients	Exosome	Glycolipids, phospholipids, fatty acids and sphingolipids	Quadrupole Time-of-flight Mass spectrometry (LC-qTOF-MS)	[233]
Prostate Cancer	PC-3 cell line	Exosome	Phosphatidylserine (PS 18:0/18:1), sphingomyelin, glycosphingolipids and cholesterol	Shotgun analysis on hybrid triple quadrupole/linear ion trap mass spectrometer and reverse phase ultra-high-pressure liquid chromatography (UHPLC)	[189]
	Non-tumorigenic and tumorigenic cell line	Exosome	Glycerophospholipids, sphingolipids and sterol lipids	Targeted lipidomic analysis	[211]
	Urine from prostate cancer patients	Exosome	PS 18:1/18:1 and lactosylceramide (d18:1/16:0)	High throughput mass spectrometry quantitative lipidomic analysis	[190]
	Urine from prostate cancer patients	Exosome	PG 22:6/22:6.	nUPLC-ESI-MS/MS (Nanoflow ultrahigh performance liquid chromatography-electrospray ionization-tandem mass spectrometry)	[191]
Breast Cancer	High and low metastatic triple negative breast cancer (TNBC) cell line	Exosome	Unsaturated diacyl-glycerol (DG) 18:1–20:2	Supercritical fluid chromatography fast scanning triple -quadrupole mass spectrometry	[161]
Pancreatic Cancer	Plasma from pancreatic cancer patients	Exosome	LysoPC 22:0, plasmenyl-PC 36:0; PC(P-14:0/22:2,PE 34:1; PE(16:0/18:1)	LC-DDA-MS based untargeted lipidomic analysis	[217]
Non-small cell lung Cancer	Plasma from lung cancer patients	Exosome	SM, cholesterol esters, PC, LPC and triacylglycerides	Ultra-high-resolution Fourier transform mass spectrometry	[219]
Hepatocellular carcinoma (HCC)	Huh7(Hepatocellular carcinoma cell line)	Exosome	Cardiolipins, saturated fatty acids and Lyso derivatives of PS,PI and PG	Electrospray ionization mass spectrometry	[184]
	Plasma from cirrhosis patients with and without HCC	Exosome	Sphingosines, lysophosphatidylserine and (O-acyl)− 1-hydroxy fatty acids and dilysocardiolipins	Untargeted lipidomics using ultra-high-resolution mass spectrometry	[222]
Glioblastoma	U87 glioblastoma cell line	Exosome	Sphingomyelin and lyso-phosphatidylethanolamines.	Electrospray ionization mass spectrometry	[184]
		Microvesicle	Phosphatidylserine (PS)		

5.2.2. Prostate cancer

Various lipidomic approaches have been employed to investigate alterations in the EV lipid composition in prostate cancer. Shotgun and targeted lipidomic analysis of exosomes derived from prostate cancer cell line, PC-3, exhibited a significant enrichment of PS,SM, glycosphingolipids and cholesterol [189]. In a follow-up study, targeted lipid profiling approach showed that exosomes derived from tumorigenic cells had a higher abundance of glycerophospholipids, sphingolipids and sterol lipids compared to non-tumorigenic cell derived exosomes [211]. In addition, exosomes derived from non-tumorigenic cells were found to be abundant in prenol lipids, fatty acids, and glycerolipids [211]. Therefore, above differences in lipid signatures of tumorigenic and non-tumorigenic prostate cancer cells can be useful in diagnosis and prognosis of prostate cancer. Increased plasma prostate-specific antigen (PSA) levels can indicate prostate cancer, their low specificity is a diagnostic concern [212,213].

Certain exosome lipids, PS 18:1/18:1 and lactosylceramide (d18:1/16:0), in urinary exosomes of prostate cancer patients, might be able to further enhance the diagnostic accuracy compared to PSA alone [214]. A recent lipidomic analysis using urinary exosomes in prostate cancer patients, showed significant increase in PG 22:6/22:6 with concomitant decrease in neutral lipids such as DAG,TAG and ChE was found, suggesting a shift towards increased consumption of energy by prostate cancer cells [191].

5.2.3. Breast cancer

Increasing evidence suggests that EVs originating from breast cancer cells could facilitate the formation of a conducive microenvironment for growth, invasion and spread of cancer [215]. Interestingly, EVs derived from human mesenchymal stem/stromal cells (hMSCs) have been found to promote breast cancer cell proliferation through their secretions. Of particular interest, the exosomes derived from hMSCs are enriched in bioactive lipids such as SM, DAG and ceramides. This indicates that ceramide pathway is likely involved in production of these EVs and that the lipid composition of EVs, specifically the presence of lipid raft domain plays a significant role in promoting breast tumour growth [216]. Recent targeted lipidomic analysis demonstrated the difference between lipid profile of low and high metastatic triple negative breast cancer (TNBC) cell lines. Exosomes derived from high metastatic TNBC were enriched in unsaturated diacylglycerol (DAG) 18:1–20:2, while the exosomes from low metastatic TNBC contained DG 18:0–18:1, suggesting specific DG lipid species are selectively loaded onto exosomes. Accumulation of DGs in exosomes can trigger protein kinase D signalling cascade in endothelial cells, promoting angiogenesis and tumour progression [161]. However, the mechanism of selective loading of lipids in EVs originating from breast cancer cells requires additional investigation to uncover diagnostic biomarkers.

5.2.4. Pancreatic cancer

A recent study revealed a correlation between the lipid profile of EVs in pancreatic cancer patients and their clinicopathological features, including tumour stage and size, lymphocyte count, serum level of tumour markers (CA19–9 and CA242), which can distinguish pancreatic cancer patients from healthy individuals [217]. The abundance of

LysoPC 22:0 and plasmenyl-PC 36:0; PC(P-14:0/22:2) in exosomes derived from plasma samples of pancreatic cancer patients were found to be associated with clinicopathological characteristics [217].

Additionally, the lipid PE 34:1; PE(16:0/18:1) was identified to be not only associated with clinicopathological features, but also with overall survival of patients [217]. However, to identify reliable lipid signatures for early detection of pancreatic cancer and its underlying mechanisms, a large cohort of each stage of patients is required. Research also suggests a link between obesity and risk of pancreatic cancer. Adipocytes exposed to pancreatic cancer derived EV showed not only a decrease in adipocyte triglycerides level but also upregulation of inflammatory cytokine such as IL-6 which enhances lipolysis [218].

5.2.5. *Lung cancer*

Exosomes originating from non-small cell lung cancers have been found to contain high levels of SM, cholesterol esters, PC, LysoPC and triacylglycerides and can potentially differentiate between healthy individuals and those with lung cancer [219]. The exosomes derived from pleural exudates of non-small cell lung cancer patients were shown to promote metastasis mediated by leukotriene (LT) machinery [220]. Leukotrienes are lipid mediators derived from arachidonic acid in the 5-lipoxygenase pathway, and have significant role in inflammation [221]. Exosomes derived from pleural exudates and lung cancer cells, had an abundance of gamma-glutamyl transpeptidase 1 (GGT-1) and were able to transform LTC_4 into its pro-metastatic metabolite LTD_4, increasing the level 100 times more than their endogenous cysteinyl LTs [220]. The lung cancer cells as well as the EVs and monocytic cells found in pleural exudates, work together to produce significant quantities of LTD_4,which in turn promotes the migration and survival of cancer cells that was counterbalanced by antagonist of CysLT1 [220]. The antagonist of CysLT1 (montelukast) not only inhibits the migration of cancer cells, but also induces apoptosis, supporting its use as a therapeutic option for lung cancer [220]. However, further research is needed to confirm whether using CysLT1 antagonists can be effective treatment to reduce the risk of metastasis in lung cancer patients.

5.2.6. *Hepatocarcinoma and Glioblastoma*

One of the lipidomic analysis conducted with exosomes derived from Huh7 (hepatocellular carcinoma cells) showed accumulation of cardiolipins, saturated fatty acids and Lyso derivatives of PS, PI and PG in exosomes and concomitant decrease in diacyl and triacylglycerols in exosomes. The active selection of cardiolipins in exosomes may contribute to their stability and negative curvature, while free fatty acids and lysophosphatidyl derivatives could promote positive curvature in exosomes [184]. Untargeted lipidomics was performed on exosomes derived from plasma of patients with cirrhosis, with and without HCC, to identify novel biomarkers for monitoring HCC in individuals. Interestingly, exosomes obtained from individuals with HCC had an abundance of sphingosines, lysophosphatidylserine and (O-acyl)-1-hydroxy fatty acids and dilysocardiolipins, as indicated by logistic regression analysis [222].

Lipid profile of exosomes derived from U87 glioblastoma cell line revealed a unique pair of lipid signature enriching in exosomes that are SM and LPE. Along with that, microvesicles derived from U87 cells showed high abundance of PS compared to its parent cell [184].

6. Conclusions

Lipids are as not only an essential structural component of EVs but are also crucial for intercellular communication and can reprogram recipient cells throughout disease progression. In the past few years, EV-lipids have been receiving increasing interest since they can be employed as minimally invasive biomarkers, thus facilitating early diagnosis, improving prognosis, and providing personalized treatment options. Moreover, EV lipid profiling has emerged as a novel approach to assess the lipid alterations in EVs that could provide valuable insight into cancer pathogenesis and metastasis. A lipidomic approach has the potential to serve as a straightforward and cost-effective method for patient stratification for further diagnostic evaluation. However, further research is required to validate findings in EVs derived from samples of benign, borderline, and stage 1-IV cancer patients, to obtain a lipid fingerprint that can aid in early diagnosis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Associate Professor Salomon is the head of the Translational Extracellular Vesicles in Obstetrics and Gynae-Oncology Group, NHMRC Investigator Fellow (EL2) and is considered the worldwide authority on biomarkers for complications of pregnancies and ovarian cancer (165 publications, H index 49, i10-index = 118). In the last 10 years, Associate Professor Salomon's primary research and commercialisation activities have focused on the identification and validation of biomarkers, and development of In Vitro Multivariate Index Assays for clinically relevant complications (including ovarian cancers, and obstetrical syndromes) and their translation into clinical applications. He is a pioneer on investigation the release of extracellular vesicles (EVs) by the placenta and tumour cells and their utility as a biomarker for a wide range of pregnancy complications and ovarian cancer (OC). Prior to his research program, the field had little understanding of the changes in circulating EVs and their content across gestation and in OC progression. In pioneering this research, his program recruited, and collected biological samples, from over 20,000 participants in multiples studies in the USA, India, Chile, UK, and Australia in the last 10 years. He has optimised methods to isolate total and placenta and tumour-derived EVs present in circulation, and profiled their content by quantitative proteomic analysis, and miRNA sequencing; identifying for the first time, molecules within EVs associated with different complications of pregnancies, and at early stages of oncogenic transformation in OC. One of the most significant contributions of his research program has been the development of a test for early detection of ovarian cancer, OCRF-7, that displayed a classification efficiency of 98%. These outcomes of the research program provide a novel conceptual basis, and evidence for translation, resulting in changes in clinical practice and management.