



An emerging focus on lipids in extracellular vesicles

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ABSTRACT

Extracellular vesicles contain a lipid bilayer membrane that protects the encapsulated material, such as proteins, nucleic acids, lipids and metabolites, from the extracellular environment. These vesicles are released from cells via different mechanisms. During recent years extracellular vesicles have been studied as possible biomarkers for different diseases, as biological nanoparticles for drug delivery, and in basic studies as a tool to understand the structure of biological membranes and the mechanisms involved in vesicular trafficking. Lipids are essential molecular components of extracellular vesicles, but at the moment our knowledge about the lipid composition and the function of lipids in these vesicles is limited. However, the interest of the research community in these molecules is increasing as their role in extracellular vesicles is starting to be acknowledged. In this review, we will present the status of the field and describe what is needed to bring it forward.

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Contents

1.	Introduction	309
1.1.	Extracellular vesicles	309
1.2.	Lipids in cellular membranes	309
1.3.	The asymmetry of cellular membranes	309
2.	Methodological aspects	310
2.1.	Methodological aspects in the preparation of EVs	310
2.2.	Pre-analytical considerations for lipid analysis	311
2.3.	Lipid analyses of EVs and evaluation of results	311
3.	Lipid composition of EVs	312
3.1.	Lipid composition of EVs released by cells in vitro	312
3.2.	Lipidomic studies of EVs released in vivo or from tissues	314
3.3.	BMP and PIPs in EVs	314
3.4.	Distribution of CHOL and PS in the membrane leaflets of EVs	315
3.5.	Lipid species in EVs	315
4.	Role of lipids in EV formation and function	315
4.1.	Role of lipids and lipid modifying enzymes in the formation and release of EVs	315
4.1.1.	Phospholipids	316
4.1.2.	Sphingolipids	316
4.1.3.	Cholesterol	317
4.2.	Secretion of EVs: effect on lipid composition in cells	317

Abbreviations: BMP, bis(monoacylglycerol)phosphate; CE, cholesterol ester; CHOL, cholesterol; CL, cardiolipin; Cer, ceramide; DAG, diacylglycerol; ESCRT, endosomal sorting complex required for transport; EVs, extracellular vesicles; ILV, intraluminal vesicles; MAG, monoacylglycerol; mβCD, methyl-β-cyclodextrin; MVB, multivesicular body; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PI3P, Phosphatidylinositol-3-phosphate; PI(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; PI3K, phosphoinositide 3-kinase; PIPs, phosphoinositides; PLD2, phospholipase D2; PS, phosphatidylserine; S1P, sphingosine 1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase; SphK, sphingosine kinase; TAG, triacylglycerol; TLC, thin layer chromatography.

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5. Clinical uses of EVs	318
5.1. The use of lipids in EVs as biomarkers	318
5.1.1. Lipids in urinary EVs as biomarkers	318
5.1.2. Lipids in EVs from other biofluids as biomarkers	318
5.2. EVs in drug delivery	318
6. Conclusion and future perspectives	318
Acknowledgements	319
References	319

1. Introduction

1.1. Extracellular vesicles

The ability of cells to secrete vesicles surrounded by a lipid bilayer appears to be ubiquitous since this process has been observed in animals, bacteria and plants [1]. The scientific community agrees to a large extent that these vesicles should be referred to as extracellular vesicles (EVs) [2,3]. During the last 10 years the interest in these vesicles has boosted due to their role in intercellular communication, in cellular disposal, and as a potential source of biomarkers and drug carriers [4–9] (Fig. 1).

There are different populations of EVs that overlap to some extent in physical characteristics (size, density), cellular origin, molecular composition and/or function [10]. Traditionally, it has been common to separate EVs in exosomes, microvesicles and apoptotic bodies based on their origin and mechanism of formation. The endocytic activity of the cell generates endosomes. These endosomes transform during a process of maturation in multivesicular bodies (MVBs), also called late endosomes, by invagination of the endosomal limiting membrane and the formation of intraluminal vesicles (ILVs). MVBs can then be directed to lysosomes for degradation or to the plasma membrane, where after a fusion event release their vesicles, known then as exosomes, to the extracellular environment (Fig. 1). In contrast, microvesicles bud directly from the cell membrane (Fig. 1). Moreover, cells undergoing apoptosis can also form large (over 1000 nm in diameter) EVs called apoptotic bodies [11]. Exosomes and microvesicles have been considered to have different sizes: exosomes being the smallest ones (30–150 nm in diameter), and microvesicles being the largest ones (100–1000 nm in diameter) [12]. These two EV populations were earlier separated by centrifugation at 10,000 g (microvesicles) and at 100,000 g (exosomes) [13,14]. However, recent studies have challenged this picture. For example, it has been suggested that a population of microvesicles may also be pelleted at 100,000 g [15], and that the 100,000 g pellet can also contain other structures, for example lipoproteins or material resulting from the fusion of amphisomes or autophagosomes with the plasma membrane (Fig. 1) [16,17]. Moreover, classical exosome markers have also been detected in other types of EVs [18].

Since at the moment there is not any good method to achieve a complete separation of EVs based on their mechanism of formation, it has been suggested that they could be classified based on their size as large and small EVs [2,18], or that the term exosomes should be used to name small vesicles originating both from MVBs and the plasma membrane [19]. One would expect that vesicles originating from the plasma membrane by endocytosis and being formed as ILVs within MVBs before being excreted from cells, and small vesicles released directly from the plasma membrane should have several similarities in their protein and lipid content. We think it is important to make further efforts in developing isolation and analytical methods that allow the characterization of these vesicle populations as precisely as possible, and discriminate between the various types of EVs. This is essential in order to understand the mechanisms involved in the release of different types of EVs from cells and their specific functions. By giving the same name to vesicles of different origin, one may simplify the publication of a given data set, but at the same time delay the progress of the

field. In the present article, we mainly keep the nomenclature used by the authors of the publications we refer to.

1.2. Lipids in cellular membranes

Cells contain several thousands of lipid species, and today it is possible to quantify approximately 1000 of these in a sample by using mass spectrometry (MS) [20–22]. The different structures of lipids are used to group them into different classes, e.g. glycerophospholipids, sphingolipids and cholesterol (CHOL), and variations in the number of carbon atoms and double bonds give rise to different species within a lipid class (Table 1 and Fig. 2). Although most glycerophospholipids contain ester-bound fatty acyl chains, ether-linked hydrophobic chains also are common in all tissues [23–25], but ether lipids have received much less attention than the acyl-containing phospholipids. The fatty acyl groups in mammalian phospholipids often contain 16 or 18 carbon atoms either with zero or a few double bonds in *cis* configuration, and the known unsaturated fatty acyl groups of these phospholipids are 16:1, 18:1, 18:2 and 18:3. For some phospholipid classes also longer and polyunsaturated fatty acyl groups such as C20:4 (arachidonic acid) and C22:6 (docosahexanoic acid; DHA) are common in the *sn*-2 position [26].

Glycerophospholipids are synthesized by addition of fatty acids to glycerol, whereas sphingolipids are synthesized in a different way starting with serine. Sphingolipids are composed of a sphingosine base and an N-amidated fatty acyl group, where the N atom originates from serine (Fig. 2) [26,27]. The distribution of the N-amidated fatty acyl groups of sphingolipids, and especially of glycosphingolipids, is very different from that of glycerophospholipids, as the N-amidated fatty acyl groups often have a “bimodal” species distribution with mainly N-amidated C16:0 as the shortest species and C22–C24 as the longest species [28–31]. In the cell lines we have analyzed, the major acyl groups of the glycosphingolipid species are C16:0, C24:0 and C24:1 [32,33]. Remarkably, C24:1 is the main unsaturated N-amidated fatty acyl group that is present in glycosphingolipid species (this species may constitute more than 50% in some classes).

Although the reason for such a bimodal distribution is not known, a possible explanation is that C16 and C18 hydrophobic chains are able to reach approximately to the middle of the membrane bilayer, whereas C24 chains should be able to reach deeper into the opposite layer and thus give a stronger interaction between the two membrane leaflets [34].

1.3. The asymmetry of cellular membranes

The cellular membrane bilayers have an asymmetric distribution of lipids in the two leaflets. This asymmetric distribution is best studied for the plasma membrane, where almost all sphingolipids and phosphatidylcholine (PC) are present in the outer leaflet, whereas almost all the other phospholipids are present in the cytosolic leaflet, i.e. phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). The distribution of CHOL in the plasma membrane has been a subject of debate for a long time, and this issue is discussed later. The intracellular distribution of lipids has also been investigated for many years, including the estimation of the amount of the different

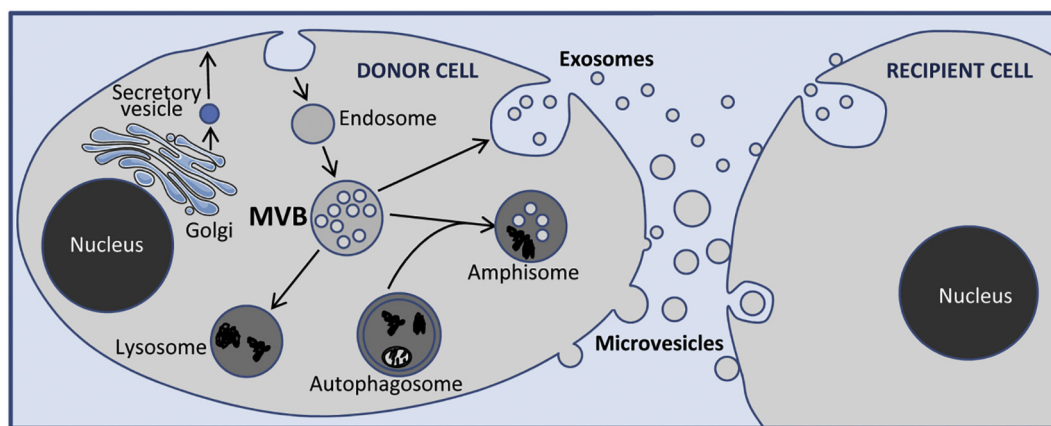


Fig. 1. EVs and EV-based model of cell-to-cell communication. Exosomes are released after fusion of multivesicular bodies (MVBs) with the plasma membrane, and microvesicles by direct budding from the plasma membrane. EVs can function as trash cans to remove unwanted material from donor cells, and in intercellular communication by binding to the plasma membrane and/or being taken up by recipient cells. Several studies indicate that organelles of the autophagy pathway (amphisomes, autophagosomes) also can fuse with the plasma membrane and release their material to the extracellular environment.

classes found in various intracellular membranes [20]. It should be mentioned that it is still not possible to isolate different intracellular vesicles/membranes from mammalian cells with a sufficient degree of purity to obtain good data for their lipid composition. Thus, there is still much to learn about the intracellular distribution of the different lipid classes, including the amounts of the various species that are present in the different intracellular membranes. For more information about the diversity of lipids in cellular membranes we refer to a recent review by Harayama and Riezman [26]. Although some EVs have been shown to contain internal membrane structures in their lumen by electron microscopy, most of them only contain one lipid bilayer [35,36]. EVs are therefore very interesting to study in order to learn more about the structure and composition of biological membranes. Due to their mechanism of formation, the distribution of lipids in the outer and inner membrane leaflet of exosomes is expected to be similar to the plasma membrane.

2. Methodological aspects

2.1. Methodological aspects in the preparation of EVs

EVs are isolated from cells grown in culture and from different biological fluids. Isolation of EVs from biological fluids is most challenging. For example, human serum/plasma contains lipoproteins that can co-isolate with EVs [17], and these EVs can be released from different circulating and non-circulating cells. EVs can be purified using different methods based on their physical and/or molecular characteristics. All methods have advantages and disadvantages, and the choice of method will depend on the purpose of the study. In any case, it is important to characterize the samples as good as possible [2] and, as we write below, quantitative lipid analyses can give valuable information about the purity of EV preparations.

Sequential ultracentrifugation (100,000–120,000 g for at least 60 min) has been the most popular way to purify exosomes from conditioned media and biofluids, and recently it was demonstrated that further separation of EVs by density gradient centrifugation results in vesicles with different molecular content [15,18].

Several groups have used immunocapture methods to obtain EVs with a given epitope on the surface [15,18]. This may give a more homogenous preparation, but it may also exclude other vesicles having the same origin but containing other surface-bound proteins, or insufficient amount of the epitope to get captured. It is also important to consider whether vesicles with a different protein pattern and/or densities could originate from MVBs, and not only from different types of organelles/membranes [6]. In fact, although annexins, in particular Annexin A1, have recently been suggested as markers of small EVs released by budding from the plasma membrane, these proteins also have been associated with MVB/late endosomes [37].

Size exclusion chromatography is also used for the purification of vesicles. This method is useful for removal of soluble proteins in the EV preparations, but not to separate exosomes and microvesicles based on their size or to remove lipoproteins. This is because the size exclusion material with the largest pores (Sephacrose CL-2B), according to the manufacturer, can separate proteins in the range 70–40,000 kDa, which fits with an exclusion of particles larger than 50–60 nm in diameter. Thus, if the use of these size exclusion columns results in separation of different types of EVs, it is probably due to different affinities between the column material and the vesicles, i.e. not due to a size-based separation. Moreover, one should also be aware of the risk of EVs being retained (lost) on the column when using chromatographic approaches.

Table 1
Most common lipid classes in biological membranes.

Lipid class/abbreviation	R1	R2	Headgroup
Phosphatidylcholine/PC	FA ^a	FA	Choline
Lysophosphatidylcholine /LPC ^b	FA	H	
Ether-linked PC: PC O, PC P ^c	Alkyl, alkenyl	FA	
Phosphatidylserine/PS	FA	FA	Serine
Phosphatidylethanolamine/PE	FA	FA	Ethanolamine
Phosphatidylinositol/PI	FA	FA	Inositol
Phosphatidylglycerol/PG	FA	FA	Glycerol
Phosphatidic acid/PA	FA	FA	H
Ceramide/Cer	LCB ^d	FA	H
Sphingomyelin/SM	LCB	FA	Phosphocholine
Glycosphingolipids/GSL ^e	LCB	FA	Carbohydrates
Cholesterol/CHOL	Structure shown in Fig. 1		
Cholesterol ester/CE	OH group of CHOL esterified with FA		

^a FA = Fatty acyl chain.

^b Lysolipids may be present in all classes listed in this table (except for cholesterol), but are for simplicity shown for PC only.

^c Ether-linked lipids may be present in all glycerophospholipid classes shown (PC, PS, PE, PI, PG and PA), but are for simplicity shown for PC only. Ether lipids with an alkyl group are abbreviated as exemplified for PC O and ether lipids with an alkenyl group are abbreviated PC P (the alkenyl ether phospholipids are often called plamalogen).

^d LCB: Long-chain base (see Fig. 1).

^e GSLs contain many different classes with a large variation in the carbohydrate structures as shown in [31].

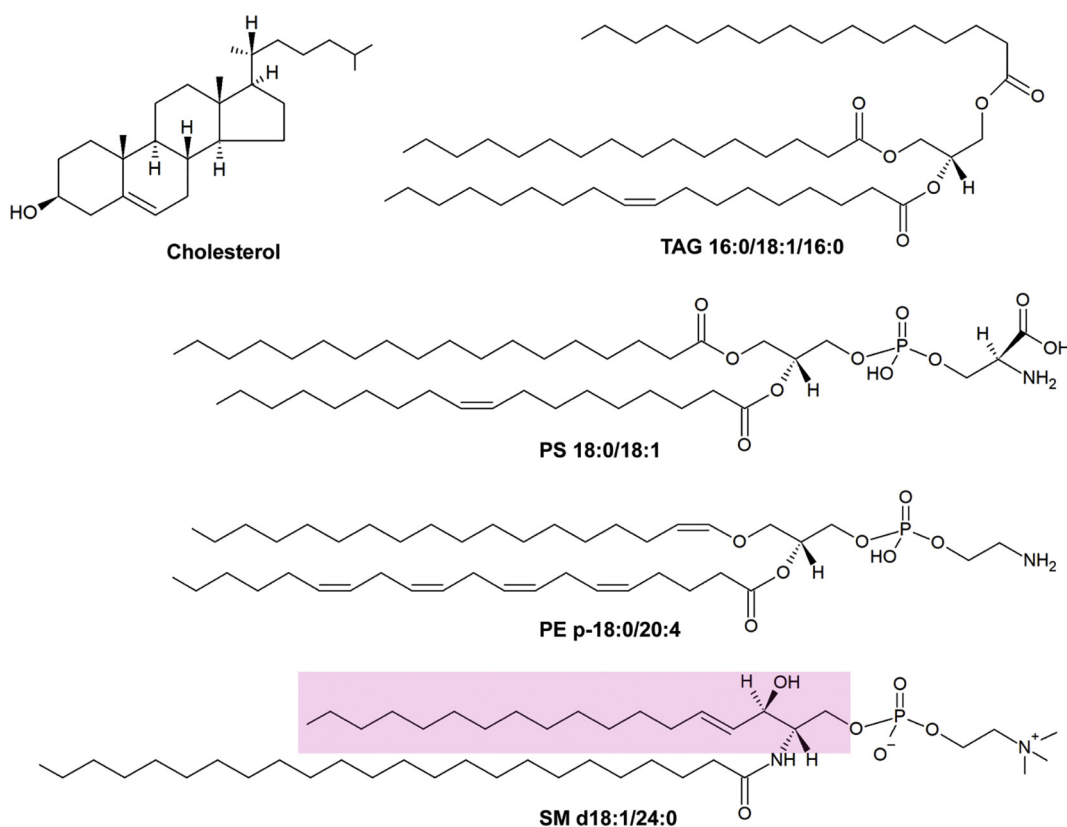


Fig. 2. Illustration of some lipid structures. Cholesterol is shown up to the left. The other structures are from top to the bottom: TAG 16:0/18:1/16:0, the glycerophospholipid PS 18:0/18:1, the alkenyl ether glycerophospholipid (plasmalogen) PE P 18:0/20:4 and the sphingolipid SM d18:1/24:0. The sphingosine part of the SM molecule is marked in pink. The shown 18:1 and 20:4 structures are the acyl groups of oleic acid and arachidonic acid respectively. The structures have been made by using the structure drawing tools available at Lipid Maps (<https://lipidmaps.org/>).

Other methods for purification of EVs include field-flow fractionation techniques and ultrafiltration. Although ultrafiltration has the advantage of being rapid and does not require special or expensive instrumentation, it may cause problems due to adsorption of EVs to the filter, and also possibly cause vesicle deformation or even destruction. Several commercial kits are now available for isolation of EVs, e.g. based on polymer-based precipitation. In our opinion, kits should be used with care, especially when the basic principle and composition of the reagents in the kit are not commonly available. Moreover, information about how the kit may influence the different analytical methods often used to characterize EVs, such as MS, should be provided by the manufacturers. A more extensive description of these and additional methods for purification and analysis of EVs can be found in recent reviews [38,39].

2.2. Pre-analytical considerations for lipid analysis

Numerous pre-analytical factors can influence lipid determinations, and they should be considered specifically for the type of sample that is going to be analyzed. It is then useful to have guidelines to minimize the impact of pre-analytical variables in lipidomic analyses [40]. As a general rule, pre-analytical parameters should be carefully reported for the correct interpretation of the results, and pre-analytical processing steps should be minimized to reduce error sources and variability. Regardless of sample origin, lipid extraction and storage conditions are important pre-analytical variables to be considered. Concerning lipid degradation, and in particular the oxidation of unsaturated fatty acid chains, the addition of antioxidants and storage at low temperature (-80°C or lower) and under an inert gas (e.g. argon) may limit lipid oxidation. So far the impact of different pre-analytical variables in the EV lipidome has not been thoroughly investigated, but it is expected that

many pre-analytical aspects that apply for lipid analyses of blood plasma [40] also apply for lipid analyses of EVs.

2.3. Lipid analyses of EVs and evaluation of results

It has been proposed to use methods such as the sulfovanilin assay, fluorescent dyes that incorporate into membrane bilayers (e.g. dialkylcarbocyanins such as DiR) or Fourier transform infrared spectroscopy for quantification of total lipids in EVs [2]. Although these methods may be used to discriminate between EVs with a very different protein/lipid ratio, they are not optimal for the quantification of the total amount of lipids in EVs, as their quantitative aspects are not well known. The sulfovanilin assay has been reported to detect CHOL and polyunsaturated fatty acids, but not saturated fatty acids [41], and to our knowledge there is no report describing how this reagent reacts with the many different types of lipids found in biological membranes. Furthermore, also the fluorescent dyes used to label lipid bilayers may give different signals depending on the membrane composition and lipid packing, and these dyes may also be differently distributed among cellular membranes [42].

Quantitative lipid data of vesicle preparations may also throw light on whether the lipid composition of a sample fits with the expected composition of cellular lipid bilayers, and whether they contain impurities, particles, or cellular organelles. For example, does the sample contain high amounts of lipids not expected to be found at the plasma membrane or in MVBs? In particular, lipids such as triacylglycerols (TAGs) and cholesterol esters (CEs) are mainly found in lipid droplets and in lipoproteins. Therefore, if a high content of these lipids is found in exosome preparations, it should be investigated if this is due to the presence of a subtype of vesicles/particles with a very high content of these lipids. This could possibly be tested by adding substances used

to stain lipid droplets to the preparations [43]. Also a high content of cardiolipin (CL), a phospholipid located in the inner mitochondrial membrane, in EV samples suggests a contamination by mitochondria. In this context, we and others have earlier discussed secretory autophagy as a possible mechanism for release of EVs [44–47]. We believe secretory autophagy could be a likely mechanism for the release of lipids from lipid droplets (following lipophagy) or from mitochondria (following mitophagy). The different forms of lipophagy, including macrolipophagy (autophagy of whole lipid droplets) or microlipophagy (autophagy of parts of lipid droplets) are discussed in recent reviews [48,49]. The enzyme adipose triglyceride lipase is present on lipid droplets and catalyzes the hydrolysis of TAGs to diacylglycerols (DAGs) and fatty acids. The lipase is also able to hydrolyze DAGs to monoacylglycerols (MAGs) and fatty acids, although with a lower activity compared to the hydrolysis of TAGs. Thus, secretory autophagic vesicles containing lipid droplets may contain DAGs, MAGs and fatty acids as degradation products from TAGs. To our knowledge, degradation of CEs to fatty acids and CHOL is obtained by lysosomal acid lipases and by neutral lipases in the cytoplasm [50]. Recent reviews are also available for the different forms of mitophagy, a mechanism that captures nonfunctional mitochondria in autophagosomes for their degradation [51,52]. This process is initiated following the generation of different “eat me” signals on the mitochondrial surface both by posttranslational modification of proteins on the outer mitochondrial membrane, or following externalization of CL or proteins normally present on the inner mitochondrial membrane.

There are several issues that may impact the lipid composition of the EVs released from cells in culture. We have shown that different cell densities may change the lipid composition and intracellular trafficking in cells [32]. Furthermore, fetal calf serum contains lipid particles of a size similar to EVs, and the lipid composition of the growth media and lipids added to the media have been shown to affect the lipid content of cells and their released EVs [53,54].

In terms of the phospholipid composition of EVs, it should be considered that the most common fatty acyl groups (regarding number of carbon atoms and double bonds) are those mentioned above, and that fatty acyl groups with an odd number of carbon atoms are present in very limited amounts in human cells [55]. More information about fatty acids commonly available in biological samples can be found in the supplementary information of Bowden et al. [56] and in Wikipedia (https://en.wikipedia.org/wiki/List_of_unsaturated_fatty_acids).

Although relative values of lipid classes/species can be used to demonstrate changes in the lipidome of EVs under different treatments/conditions, presentation of MS lipid data as quantitative data (e.g. as pmol/ μ g protein) is more informative. This can today be obtained using modern MS equipment and internal standards for the various lipid classes. There are several excellent reviews describing how to perform quantitative lipidomic studies and what can be expected regarding sensitivity and reproducibility, including the need for using well controlled extraction methods [22,57–60].

The availability of quantitative data will give other researchers the possibility to evaluate the purity of the samples and other issues of interest in the dataset. This is especially important in studies reporting changes in the release of EVs from cells under conditions that affect cellular metabolism or intracellular transport in such a way that compensatory mechanisms may be induced.

3. Lipid composition of EVs

In this section we discuss the lipid composition of EVs isolated from cell cultures and biofluids, including differences in the lipid composition between releasing cells and exosomes in cases where this is known. Recent advances in MS analyses have made it possible to obtain quantitative data for many lipid species, and we stress the importance of reporting as many lipid classes and species as possible. We will also discuss the distribution of the different lipid classes between the two

membrane leaflets, and some lipid classes where more information is needed to confirm their presence in EVs.

3.1. Lipid composition of EVs released by cells in vitro

The percentages of the different lipid classes found in exosomes based on 10 published exosome preparations are shown in Table 2 (reproduced from [47]), including the enrichment factors from cells to exosomes for 8 data sets. We here comment shortly on some of these data since we have discussed them extensively in earlier reviews [47,61]. Most of the studies show an enrichment from cells to exosomes of 2–3 times for CHOL, sphingomyelin (SM), glycosphingolipids and PS. Most exosome preparations also show a higher content of PC and PI in cells than in exosomes, whereas the content of PE is similar in both. The main results of the most extensive quantitative lipid data available are shown in the first column of Table 2; 22 lipid classes were quantified in PC-3 cells [33], but only 14 of them are included in the table. Moreover, addition of the ether lipid precursor hexadecylglycerol to PC-3 cells caused major changes in the lipid composition of both cells and exosomes [62]. Nevertheless, the specific enrichments of lipid classes from cells to exosomes under these conditions had many similarities to the ones previously observed [33,62]. Also another study of exosomes from PC-3 cells showed similar data for sphingolipids, but the glycerophospholipid classes were grouped together [63], such that it is not possible to compare these results with the other studies.

The data shown in parentheses in Table 2 were obtained more than 15 years ago using thin layer chromatography (TLC) analyses, where separation of all lipid classes was not obtained. Nevertheless, we find it important to mention that the study by Wubbolts et al. was the first to describe an enrichment of CHOL and sphingolipids in exosomes and highlight similarities between these vesicles and detergent resistant membranes, thus indicating that exosomes have lipid raft like properties [64]. Since PS, PC, PI and phosphatidic acid (PA) moved as one band on the TLC, it could not be stated if any of these classes were enriched in exosomes. It should also be noted that the authors did not detect Bis(Monoacylglyceryl)Phosphate (BMP; earlier called LysoBisPhosphatidicAcid, LBPA), an anionic phospholipid that is enriched in MVBs and ILVs, or the mitochondrial lipid CL in their preparations of exosomes.

The lipid composition of exosomes from Oli-neu cells [65], HepG2/C3a cells [66] and PC-3 cells [33] show several similarities. However, exosomes released from Oli-neu cells have a higher enrichment of ceramide (Cer) and a lower enrichment of SM compared to exosomes from PC-3 cells. As the studies of exosomes from Oli-neu and HepG2/C3a cells did not include measurement of CHOL, we have assumed that CHOL constitutes 43 mol% of the total lipid content in order to facilitate the comparison between data sets in the table.

The data shown for reticulocytes in Table 2 are from the very first study describing the lipid composition of exosomes and enrichments of lipids from their parent cells [67]. The enrichment factors for several of the lipid classes differ from those reported in other studies included in Table 2. This can perhaps be due to the very high levels of CHOL in reticulocytes, but it should also be noted that the internal vesicles fusing with the plasma membrane of these cells contained markers of early endosomes and not of late endosomes/MVBs [68].

The two last columns in Table 2 show data for exosomes released from platelets [69] and adipocytes [70]. These columns do not contain enrichment factors as the lipid content of the parent cells was not measured. Exosomes from adipocytes were reported to contain more SM and less PS than most of the preparations in Table 2. The study by Pienimacki-Roemer et al. contains interesting data about the lipid composition of exosomes and five other vesicle preparations (with diameters in the range 106–203 nm) released from platelets [69]. The authors concluded that the smallest vesicles were exosomes based on the high abundance of CD9, CD63 and ALIX. The authors reported a considerable variation in the lipid composition of exosomes (Table 2) and

Table 2
Lipid composition of exosomes released by individual cell types (reprinted from [47]).

Lipids	PC-3 cells [33]		PC-3 cells + HG [62]		Oli-neu cells [65]		HepG2/C3a [66]		B-lymphocytes [64]		Mast cells [96]		Dendritic cells [96]		Reticulocytes [67]		Platelets [69]	Adipocytes [70]
	%	Factor	%	Factor	% ^b	Factor	% ^b	Factor	%	Factor	% ^e	Factor	% ^{e,f}	Factor	%	Factor	%	% ^b
CHOL	43.5	2.3	59	1.7	43	2.3	43	1.9	42.1	3.0	15	1.0	NR	NR	47	1.03	42.5	43
SM	16.3	2.4	9.1	2.0	8.2	1.5	9.7	10.8	23.0	2.3 ^c	12	2.8	20	2.2	8.4 ^c	1.31	12.5	12.5
PC	15.3	0.31	10.8	0.33	26.7	0.67	20	0.67	(20.3) ^d	(0.76) ^d	28	0.66	26	0.6	23.5	1.03	15.9	33
PS	11.7	2.1	6.9	1.2	14.9	3.0	15.6	2.4	(20.3) ^d	(0.76) ^d	(16) ^d	(1.2) ^d	(19) ^d	(1.6) ^d	5.9	0.92	10.5	1.1
PE	5.8	0.55	1.1	0.21	10.9	1.0	7.4	1.2	(14.6) ^d	(0.7) ^d	24	1.08	26	1.13	12.7	0.84	3.1	4.0
PE ethers	3.3	1.2	4.7	0.81					(14.6) ^d	(0.7) ^d							3.2	
DAG	1.5	1.5	1.1	0.92														0.8
PC ethers	0.81	0.40	0.7	0.28													1.4	
PG	0.17	0.17	0.1	0.07														
PA	0.16	1.8	0.1	0.33					(20.3) ^d	(0.76) ^d								
PI	0.13	0.13	0.3	0.16			4.1	0.18	(20.3) ^d	(0.76) ^d	(16) ^d	(1.2) ^d	(19) ^d	(1.6) ^d	2.4	1.1	5.2	2.3
Cer	0.32	1.3	0.7	1.2	NR	3.3	0.63	2.0									0.40	0.2
HexCer	0.76	3.8	2.3	2.1	NR	2.0												0.02
LacCer	0.12	3.0 ^a	0.7	1.8														
Lipid analysis	MS		MS		MS		MS/GC		TLC		TLC/GLC		TLC/GLC		TLC		MS	MS
Exosome preparations ^g	SFM + SUC	SFM + SUC	SFM + SUC	SFM + SUC	SFM + SUC	SFM + SUC	uFCS	uFCS + SUC + IG	uFCS + SUC + SG	uFCS + SUC	uFCS + SUC	uFCS + SUC	uFCS + SUC	uFCS + SUC	SUC + IG	SUC + IG	SFM + SUC	SFM + SUC

%; Percent of total lipid quantified. Factor: Factor of enrichment from cells to exosomes. NR: Not reported.

GLC: gas liquid chromatography; HexCer: hexosylceramide; LacCer: lactosylceramide; PG: Phosphatidylglycerol. HG: Hexadecylglycerol.

^a Enrichments of other lipid classes are shown in Fig. 2B in reference [33].

^b Percent CHOL was not reported; CHOL set to 43% to better compare the content of other lipid classes with the other data shown.

^c Sum of SM and the glycosphingolipid GM3.

^d Sum for all classes shown in parentheses and having the same numbers.

^e Recalculated from the authors' data.

^f CHOL not reported; the sum for the other lipid classes is 100% (including LysoPC not included in this table).

^g Exosome preparations: Methods used to isolate the exosome preparations. SFM: serum free medium; uFCS: ultracentrifuged fetal calf serum; SUC: sequential centrifugation; SG: sucrose gradient; IG: iodixanol gradient.

the other EV populations. This is in our opinion the best publication comparing the lipid compositions of exosomes and other EVs excreted from the same cells. The percentages of lipids found in exosomes and the range observed in the other vesicles (shown in parentheses) are: CHOL 42.5% (30.8–37.1%), SM 12.5% (6.0–8.0%), PS 10.5% (13.9–18.9%) and PI 5.2% (2.5–3.9%). Thus, the highest content of CHOL and SM were observed in exosomes. These data fit well with a report showing that exosomes released from BV-2 cells had a much higher lipid order in the bilayer than microvesicles and apoptotic bodies from these cells [71], and that exosomes from several cell lines were less sensitive to detergents than apoptotic bodies and microvesicles [72].

Zhang et al. reported the lipid composition of large exosome vesicles (90–129 nm), small exosome vesicles (60–80 nm) and exomers (non-membranous particles with a diameter of approx. 35 nm) isolated from AsPC-1, MDA-MB-231 and B16-F10 cells using asymmetric flow field-flow fractionation [73]. The results show some peculiarities, for example, exosomes contain very high levels of PC, i.e. 80–90% of the lipids in exosomes from MDA-MB-231 and B16-F10 cells (CHOL not measured) and approx. 60% PC and 30% SM in exosomes from AsPC-1 cells. Furthermore, the authors found very high levels of TAGs, DAGs, MAGs and CL, i.e. lipids normally present in lipid droplets and mitochondria, and the lipid composition of the non-membranous particles (exomers) and the small and large exosomes was very similar. As we recently have discussed the importance of PS 18:0/18:1 in biological membranes, we checked the composition of PS species in these preparations [34]. In agreement with other studies, PS 36:1 was the dominant species in AsPC-1 cells but, surprisingly, the second most abundant species were PS 39:1 and PS 37:0. In the two other cell lines PS 36:1 was not among the 52 PS species reported. In these cell lines PS 35:0 was reported to be the dominant PS species, and these exosomes also contained PS 39:1, but not PS 37:0. To our knowledge, such large variations have not previously been reported for different cell lines. Moreover, it is surprising that the levels of phospholipids containing an odd number of carbon atoms are so high in light of the very small amounts of such fatty acids expected to be present in cells.

Additional studies of the lipidome of exosomes released by cells *in vitro* have been published, but the results were not presented in a way that showed the percentage of different lipid classes in EVs as shown in Table 2. For example, a total of 500 lipid species were identified in a lipidomic study of LIM1215 colorectal cancer cells, but the data were reported as relative abundance of lipid species only [74]. The remarkable high enrichment from cells to exosomes of TAG (24 times) and CE (5.7 times) should have been further investigated to exclude the possible presence of lipid droplets or other lipoparticles in the preparations. Haraszti et al. reported the enrichments of lipid classes from cells to exosomes in U87 glioblastoma cells, Huh7 hepatocellular carcinoma cells and human bone marrow-derived mesenchymal cells [75]. The results show a remarkable high enrichment of CL and no enrichment of SM from cells to exosomes for Huh7 and mesenchymal cells, whereas the opposite pattern was reported for U87 cells. PS was only slightly enriched from cells to exosomes in all three cell lines, whereas the content of PC and PI were higher in cells than in exosomes, more in agreement with the data shown in Table 2.

Brozowski et al. quantified 187 lipid species in EVs (pelleted by centrifugation at 18,000 rpm for 3 h, g value not provided) from three prostate cell lines, i.e. RWPE1 (non-tumorigenic), NB26 (tumorigenic) and PC-3 (metastatic) [76]. The authors showed that fatty acids, glycerolipids and prenol lipids were more abundant in EVs from non-tumorigenic cells, whereas sterol lipids, sphingolipids and glycerophospholipids were more abundant in EVs from tumorigenic and metastatic cells. It is difficult to compare these data with other published results because they are presented as relative abundance of lipid species within each lipid class. Also the presence of many lipid species with an odd number of carbon atoms and the absence of some of the most common SM species reported by other authors complicates the evaluation of these data.

Holopainen et al. recently reported significant changes in the lipid content of EVs released from mesenchymal stromal cells treated with polyunsaturated fatty acids, i.e. arachidonic acid (20:4), eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) [54].

Quantification of the lipid classes was not shown and CHOL was not measured, but the authors observed an enrichment in EVs of PS 36:1 and PE 36:1, similarly to what has been shown for exosomes released from PC-3 cells [33]. In another publication, the same group studied the lipidome of platelets and found that it was very different from the lipidome of mesenchymal stromal cells, especially higher amounts of PC, PE and PS with polyunsaturated fatty acyl groups (especially 38:4) were observed; the authors also reported changes in platelets and EVs during storage for up to 8 days [77]. Since studies from another group also have shown high amounts of polyunsaturated fatty acyl groups in blood cells [78,79], we wonder if a high content of polyunsaturated lipids in these cells contributes to giving them the flexibility required for passing through thin capillaries.

By using highly sensitive MS methods also lipids present in very low amounts in EVs can be measured. Thus, several hydroxylated metabolites of CHOL were measured in exosomes from MCF-7, MDA-MB-231 and HEK293 and serum samples. The highest levels were found for 27-OH CHOL in MCF-7, approx. 45 fmol/ μ g protein, which is approximately 0.01% of that reported for CHOL in exosomes [80].

Recently, Royo et al. investigated the lipidome of EVs released from a primary rat hepatocytes cells and a mouse hepatocyte progenitor cell line, MLP29 [81]. Although in these EV preparations the majority of EVs were around 150 nm in diameter (based on nanoparticle tracking analysis), the data show that the preparations also contained larger EVs (range 200–500 nm) than other preparations discussed in this review. Moreover, the EVs isolated from the rat hepatocytes showed a larger portion of vesicles of above 200 nm in diameter than the EVs excreted from MLP29 cells. The authors reported quantitative data for approximately 250 lipid species (CHOL and PS not reported). For MLP29 cells, each class of SM, PC, lysophosphatidylcholine and PC-ethers constituted approx. 12–13% of total lipids, and each class of PE, lysophosphatidylethanolamine, PE-ethers, PI and lysophosphatidylinositol constituted approx. 5% of the total lipids. Moreover, EVs released from the hepatocytes had a very high content of TAG (approx. 33% of total lipids) compared to 4% of TAG released from MLP29 cells. The authors also compared the mechanical stiffness of the two EV preparations by atomic force microscopy, and showed that EV from hepatocytes were softer (less stiff) and showed less resistance to mechanical distortion than EVs excreted from MLP29 cells.

Comparison of exosomes released from the apical and basolateral side of polarized cells has to our knowledge only been reported once [82]. The exosomes released from the apical side of polarized murine cortical collecting duct principal cells were larger (148 nm) than those excreted from the basolateral side (100 nm), and three times more exosomes were reported to be excreted from the apical side. However, it is difficult to compare the lipid compositions of the two exosome preparations because the MS data obtained using either the negative or positive mode do not fit with each other. Also the presence of a very high content of CL (35% of the lipids in the exosomes excreted from the basolateral side), which is normally found in the inner membrane of mitochondria, can be an indication of the presence of other vesicles or particles in the exosome preparations.

3.2. Lipidomic studies of EVs released *in vivo* or from tissues

Flaherty et al. reported that adipose tissue release “exosome-sized, lipid-filled vesicles” (diameter mean size 115–120 nm) that serve as a source of lipids for local macrophages [83]. The authors commented that “Standard purification strategies for exosomes proved inefficient, possibly because of the high lipid content; however using size fractionation columns and filtration, we were able to isolate EVs from adipose tissue”. The results show that approx. 1% of the lipid content of adipose tissue from lean mice was released every day, whereas approx. 2% of the lipid was released per day from the adipose tissue of obese animals. The authors presented a model where a lipid droplet (surrounded by a phospholipid monolayer) fills the internal space of a vesicle with a

lipid bilayer. It is difficult to support this model in light of the lipid composition shown for the vesicle preparations, i.e. approx. 25% CHOL, 25% PC, 10% SM, 10% TAG, 10% MAG, 6% CE and minimal amounts of the lipid classes normally present in the inner leaflet of the plasma membrane and exosomes, such as PS and PE.

There are several studies describing lipid analyses of prostasomes, i.e. EVs found in seminal fluid; only in one study the lipid content was analyzed by MS [84]. In that study, the two human prostatic preparations (50 and 100 nm) investigated were shown to contain very high levels of sphingolipids compared to other EVs discussed here. There are also older reports of the lipid composition of prostasomes purified from human [85], horse [86] and boar [87] seminal fluid where TLC analyses (PS and PI were co-migrating) were used. The lipid composition of these EVs was more similar to those reported for other EVs. These prostatic studies have been discussed earlier [61].

We have reported quantitative data for 107 lipid species in EVs excreted in urine [88]. These EVs showed a remarkable high content of CHOL (63%), which is close to the maximum amount of CHOL that can be included in model membranes [89]. Moreover, it was surprising that PS 18:0/18:1 was the most abundant lipid species after CHOL, and that all PE species detected were identified as ether lipids. Comparison of the lipid composition of these EVs with several of the other preparations shown in Table 2 has been discussed earlier [61]. In addition, Singht et al. reported recently the use of TLC coupled with MALDI-TOF-MS to describe different lipid profiles of urinary microvesicles and exosomes [90]. The authors showed that e.g. mannosyl-di-PI ceramides were detected only in urinary exosomes, whereas PI-ceramides were only found in urinary microvesicles. However, it is difficult to evaluate the results of this study because these lipids are known to be found in fungi and not in mammals [91,92].

Finally, a lipidomic characterization of EVs from human serum was recently reported [93]. The authors did not present quantitative data that could be used to evaluate the purity of their preparations, but the very high amount of TAG species probably indicates the presence of lipoproteins. Moreover, it is surprising to find several unusual species in very high amount, such as CE 30:3 and phospholipid species containing 12:0, 14:0 and 14:1 in the samples (see discussion above and [56]).

3.3. BMP and PIPs in EVs

It has been estimated that the anionic phospholipid BMP accounts for up to 15% percent of the total phospholipids in late endosomes, and it has so far not been reported to be present in other organelles [94]. BMP has been shown to be present in the membranes of ILVs, at least in some MVB populations [95]. This led to investigations of the possible presence of BMP in exosomes. Three studies have concluded that BMP is not present in exosomes, or at least not enriched in them [64,95,96] (for a discussion about this topic see [47]). Miranda et al. recently reported that BMP-enriched exosomes are released following inhibition of Vps34, a class III phosphoinositide 3-kinase (PI3K), but the absolute levels of BMP were not reported [97]. It would be interesting to quantify the amount of BMP in exosomes by MS, and to know to which extent this lipid is present in exosomes released by cells treated with drugs that impair lipid metabolism, intracellular transport or autophagy. It should also be mentioned that a gold-labeled antibody against BMP was used to detect BMP in MVBs/late endosomes many years ago [94] but, although this method is useful to visualize BMP in cells, it cannot be used for quantification.

As shown in Table 2, PI is present in exosomes, although in rather small amounts. Thus, it is an obvious question if also phosphorylated derivatives of PI (i.e. phosphoinositides, PIPs) are present in exosomes. Seven PIPs have been identified, and they can be converted into each other by phosphorylation or dephosphorylation of the 3-, 4- and 5-hydroxyl group of the inositol head group by phosphoinositide kinases and phosphatases, respectively [98,99]. PIPs play important roles both as precursors for specific second messengers and by specifying

organelle identity and regulating membrane dynamics. To our knowledge, there are no data published for PIPs in EVs. This may be due to the technological issues of using MS to measure PIPs. In fact, these lipids are often present in very small amounts, and different classes can have the same mass due to phosphorylation at different positions of the inositol head group. Thus, there are three classes of PIPs phosphorylated at one of the different OH groups (Phosphatidylinositol-3-phosphate, PI3P; Phosphatidylinositol-4-phosphate; and Phosphatidylinositol-5-phosphate) with the same mass, and also three classes of PIPs phosphorylated at two sites, i.e. phosphatidylinositol-3,4-bisphosphate, phosphatidylinositol-3,5-bisphosphate (PI(3,5)P₂) and phosphatidylinositol-4,5-bisphosphate, also having identical mass [100]. Since PIPs confer a unique molecular identity to the membranes of organelles and endocytic compartments, it is possible that exosomes and microvesicles could be identified by specific PIPs [99].

3.4. Distribution of CHOL and PS in the membrane leaflets of EVs

As mentioned above, a very high level of CHOL has been found in several exosome preparations. This is important for the discussion about the distribution of CHOL in the leaflets of cellular membranes. Some authors report that CHOL is mainly present in the outer leaflet of the plasma membrane, while others report that CHOL is mainly present in the inner leaflet or more or less equally distributed among the two leaflets (see discussion in [101]). The very high levels of CHOL (40–60% of total lipids) reported for several exosome preparations are difficult to explain without having a high content of CHOL in both leaflets.

We have earlier discussed that we assume that most PS in newly excreted exosomes is located in the inner leaflet [47,61]. PS is known to be located in the outer leaflet of activated blood cells, apoptotic bodies and microvesicles released from the plasma membrane, where it functions as an “eat me” signal for macrophages [102]. As there is an increasing amount of evidence that exosomes can transfer signals to other cells, we think it is unlikely that exosomes contain PS in the outer leaflet, because they will then be rapidly removed from circulation. In our opinion, it is important to investigate if PS is present in the outer leaflet of various types of newly excreted EVs and, if this is the case, to estimate the percentage of vesicles that may contain PS on the surface. One should also test if more PS is exposed at the surface during storage as reported for platelets [103]. PS binding proteins e.g. Annexin A5 or lactadherin could be useful in these studies [103,104].

There are several recent publications of importance for this discussion. Wei et al. studied EVs (assumed to be a mixture of exosomes and microvesicles) released from hypoxic and nonhypoxic cells, and reported that PS was only exposed on the surface of EVs released from the hypoxic cells [105]. In addition, Lai et al. analyzed three different types of EVs (diameters in the range 50–100 nm), and the only preparation shown to bind Annexin A5 carried low or undetectable levels of the exosomal markers CD9, CD81, TSG101 and ALIX [106]. Gong et al. discussed different ways in which PS could be externalized [107]. Interestingly, the authors showed that cells having PS on the outer leaflet were able to remove it by releasing PS-containing plasma membrane “bubbles” from the surface of otherwise intact cells. Moreover, these structures seemed to be largely devoid of cytosolic content (unlike apoptotic bodies), as evidenced by the absence of mCherry expressed in the cytosol. Budding from the plasma membrane has also been reported following PE exposure in the external leaflet due to loss of a flippase that translocates phospholipids from the outer to the inner leaflet [108]. For more information about enzymes being important for keeping the symmetry or making an asymmetric distribution of PS in membranes, i.e. flippases, floppases and scramblases, see [109,110].

In a very recent article several subtypes of tumor cell-derived EVs were shown to have different amounts of externalized PS [111]. The authors reported a PS-enriched subtype which, compared with exosomes,

was characterized by a larger size, a more negative zeta potential and lower abundance of canonical exosomal markers. By looking carefully into the images where PS was detected with gold labeling of the PS-binding protein lactadherin, gold particles are clearly present in this larger subtype of EVs, but only aggregates of gold particles not associated with vesicles were observed in these exosome preparations. Finally, in another very recent article, the authors used a new lipid-protein affinity based microfluidic device with Annexin A5 on the surface to pick up cancer-associated EVs from human plasma, as such EVs (or at least part of such EVs) are reported to have PS expressed on the surface [112].

Based on the results presented above, we conclude that more studies are needed to establish if PS is present in the outer leaflet of various EVs when they are secreted, and whether PS is translocated to the outer leaflet during storage.

3.5. Lipid species in EVs

We have discussed earlier in much detail the enrichment of lipid species from PC-3 cells to exosomes excreted from these cells [61]. We will here just comment on the remarkable enrichment in exosomes of PS 18:0/18:1, and also the enrichment of PI, PE and DAG with the same fatty acyl groups. The lipid data published for exosomes excreted from PC-3 cells resulted in a molecular dynamic simulation study demonstrating strong interleaflet digitations between PS 18:0/18:1 and SM 24:0 in the presence of CHOL [33,113]. Recently we extended that discussion further in a perspective article about the importance of PS18:0/18:1 in biological membranes [34]. Interestingly, there are major similarities between the lipid composition of exosomes, HIV virus particles released from HeLa and MT4 cells and detergent resistant membranes, which are often used as models for lipid rafts. Those similarities include both the composition of lipid classes and lipid species [61]. To increase our knowledge about the lipid composition of cell membranes and the interactions between the two membrane leaflets, it will be helpful in future studies to include quantitative data for as many lipid species and classes as possible. This is important both in studies describing exosomes released from cells, and for studies of other EVs, virus particles, and detergent resistant membranes.

4. Role of lipids in EV formation and function

4.1. Role of lipids and lipid modifying enzymes in the formation and release of EVs

As shown in Fig. 1, the two main cellular sites for the generation of EVs are the plasma membrane and the endosomal membrane. In both cases, the formation of vesicles requires membrane budding out from the cytosol by processes that involve interplay between lipids and proteins [114]. Moreover, there are indications for several mechanisms of EV formation both at the plasma membrane and at the endosomal membrane [6,12,44,115,116]. It also seems clear that cells can simultaneously secrete both types of EVs, but it can be expected that the percentage of each EV population will depend on the cellular physiological and pathological conditions. It has also to be considered that EV preparations may include material from lipid droplets and/or mitochondria, possibly due to the autophagy pathway, i.e. secretory autophagy.

In many of the studies where the mechanisms of biogenesis and release of EVs have been investigated, the focus has been on exosomes. The release of these vesicles implies more steps than the budding of microvesicles from the plasma membrane. In particular, exosome release requires the formation of ILVs in endosomes, the transport of MVBs to the plasma membrane and the fusion of the limiting membrane of MVBs with the plasma membrane. These steps probably require different molecular machineries than are not easy to differentiate. Thus, the role of specific molecules on the amount of

released exosomes, without specifying the specific step that is affected, is commonly reported. Additional methods are therefore required, and recently a method to visualize the fusion of MVBs with the plasma membrane in real-time was developed [117]. When looking at the role of lipids in EV release one should consider not only their structural role in cellular membranes and how their metabolism can affect membrane fluidity/curvature, but also their possible role as signaling molecules. Interestingly, several of the studies presented below indicate that the levels or formation of the membrane lipids with the smallest head groups, Cer, DAG and PA, are important for the formation or release of EVs. Depending on their fatty acyl compositions these lipids may have a conical structure, with the head group being at the thinnest end of the cone, whereas the opposite will be true for most lysolipids [26,118].

Finally, in terms of EV trafficking, it should be mentioned that the lipid composition of EV membranes may play a role in conferring stability to these vesicles in different extracellular environments and/or to facilitate binding to or uptake into recipient cells.

4.1.1. Phospholipids

Enzymes involved in the metabolism of glycerophospholipids or glycerolipids have been shown to play a role in the formation and release of exosomes. For example, phospholipase D2 (PLD2), an enzyme that removes part of the head group of phospholipids (mainly PC) producing PA, is required in MCF-7 cells for the formation of ILVs within a fraction of MVBs containing syntenin, and it has also been shown that its activity correlates with the amounts of exosomes released from RBL-2H3 cells [119–121]. In addition, Ghossoub et al. showed that the levels of PA in PLD2-depleted cells were reduced, and that these cells release exosomes with similar levels of SM, PC and PA compared to control cells, whereas the levels of PS, PE and PI were higher [119]. It is not clear how PLD2 controls the budding of ILVs into MVBs, but it should be noted that this enzyme, similarly to sphingomyelinase (SMase), reduces the head group size of membrane lipids. Another enzyme that generates PA from DAG, DAG kinase α , has also been shown to inhibit the secretion of exosomes in T-lymphocytes, probably by reducing the formation of mature MVBs [121,122]. In a later study, the authors found that protein kinase D was the effector involved in the effect of DAG in MVB formation and release [123]. In terms of microvesicles, an old study showed that calcium-dependent increased formation of microvesicles in erythrocytes depends on the production of DAG [124].

PIPs play important functions in the endocytic pathway [99,125]. In particular, MVB morphogenesis requires PI3K activity, as suggested some years ago by the generation of vacuoles with a reduced number of ILVs in cells treated with wortmannin [126], an inhibitor of the enzyme [127]. Additional results showed that PI3K affected the recruitment of endosomal sorting complex required for transport (ESCRT) proteins since wortmannin caused the dissociation of the ESCRT-0 protein Hrs from endosomes [128]. It should be noted that wortmannin has later been shown to target other PI3K than Vps34, the endosomal PI3K, and PI3K-dependent mechanisms such as autophagy [129,130]. However, other approaches to alter the levels of PI3P, such as inhibition of PI3K with the highly specific inhibitor SAR405, also support a role of the lipid in ESCRT recruitment and ILV formation [131]. It is possible that PI3P plays a role in exosome release by recruiting ESCRT proteins such as HRS. In fact, it has been shown that silencing of HRS reduced the secretion of EV-associated CD63 and major histocompatibility complex class II [132]. Exosome release has also been investigated in cells treated with wortmannin, but with different results [133,134]. This may be explained by nonselective effects of the drug, by the different cell types used in the studies and/or the methods used to measure exosome release. Furthermore, a recent study showed that VPS34-IN1, a newly developed and highly specific PI3K inhibitor [135], increased secretion of three EV markers (ALIX, Flotillin-1, and Flotillin-2) in primary cortical neurons, and the authors also observed that autophagy

was impaired [97]. Further studies are then needed to investigate the role of PI3P in exosome release.

Concerning other PIPs, it has recently been shown that inhibition of the formation of PI(3,5)P₂, a PIP produced from PI3P by the action of the PIP kinase PIKfyve [136,137], increases exosome secretion [16]. Moreover, in cells with reduced PIKfyve activity the fusion of MVBs with lysosomes seems to be inhibited, and cells contain more MVBs with an increased number of ILVs [16]. The molecular mechanism is unclear, but it has been shown that PI(3,5)P₂ can act as an agonist for the lysosomal Ca²⁺-channel transient receptor potential mucolipin 1, and therefore the observed effect may be related to the impaired fusion of MVBs with lysosomes observed after reduction of the PI(3,5)P₂ levels [138,139]. Interestingly, it has recently been shown that acid ceramidase, an enzyme that transforms Cer into sphingosine and fatty acids in lysosomes, also regulates this lysosomal Ca²⁺-channel activity, the interaction of MVBs and lysosomes and exosome release in mouse podocytes [140].

It has also been shown that exosome release can be regulated by addition of the ether lipid precursor sn-1-O-hexadecylglycerol to PC-3 cells [62]. Interestingly, addition of the precursor changed the protein and lipid composition of the exosomes released by these cells [62]. The mechanism of this effect is not clear, but it could be related to alterations of membrane fusion events since ether lipids have been reported to be involved in membrane fusion [141].

4.1.2. Sphingolipids

The sphingolipid Cer has often been associated to EV biogenesis and release. Cer is generated by de novo biosynthesis in the endoplasmic reticulum, by hydrolysis of SM via SMases and endosomes/lysosomes or through the so-called salvage pathway for sphingolipids [142]. Due to its complex generation and metabolism, it is difficult to discern if the effects observed by altering the levels of Cer are due to Cer itself or to the formation of other lipids. Lipidomic analyses would be very useful to interpret these results. Other important concern is that addition of Cer to cells is known to have toxic effects and induce necrosis or apoptosis in many cell types, and it may be difficult to know how these effects affect other cellular processes [143].

One of the first indications that lipids are important for exosome release was provided by Trajokovic et al. [65]. The authors reported that inhibition or siRNA-mediated depletion of neutral SMase resulted in reduced secretion of exosomes from Oli-neu cells, and suggested that the formation of cone-shaped Cer formed by removal of the large head group (phosphocholine) from SM could be the driver of the neutral SMase effect [65]. The effect of neutral SMase has been reproduced in several cell lines, but there are cell lines where exosome release is not affected by alteration of this enzyme, including our study in PC-3 cells [144–147]. It is not clear why this is the case. Interestingly, another type of SMase, the acidic SMase, seems to be required for the ATP receptor P2X₇-dependent release of microvesicles from glial cells and from osteoblasts [148,149].

Other studies have investigated EV release in other situations that are expected to affect the levels of Cer. Increasing the levels of Cer either by addition of Cer 6:0 in multiple myeloma cells [150] or C16:0 in hepatocytes [151], or blocking the conversion of Cer to SM by addition of an inhibitor of sphingomyelin synthase in N2a cells and cortical neurons, promoted exosome secretion [152]. Moreover, it has recently been shown that addition of the saturated fatty acid palmitate (C16:0) induced lipotoxic ER stress leading to EV release in hepatocytes and that this process was dependent of the ER stress sensor IRE1 α and the Cer transport protein STARD11 [151,153]. Hirsova et al. also have investigated the regulation of exosome release by palmitate, and found that incubation of hepatocytes (primary and Huh7 cells) with palmitate increased the release of EVs compared with control cells [154]. We also have investigated the release of exosomes from PC-3 cells in conditions that are expected to affect the levels of Cer. However, in these cells exosome release was not affected by inhibition of de novo Cer synthesis

formation by fumonisin B, or by inhibition of glucosylceramide synthase, i.e. the first enzyme in the synthesis of glycosphingolipids [146]. However, in the latter case the protein composition of the secreted exosomes was shown to be modified [146].

Cer can be metabolized in different ways, including its transformation into sphingosine and sphingosine 1-phosphate (S1P) by sequential activity of ceramidase and sphingosine kinase (SphK). S1P is, similarly to Cer, recognized for their function in signaling [142]. S1P receptors belong to the family of G protein-coupled receptors. Interestingly, Kajimoto *et al.* have shown that inhibitory G protein (Gi)-coupled S1P receptors on MVBs are constitutively activated through a constant supply of S1P in HeLa cells, and that this activation is required for cargo sorting into ILVs in MVBs destined to the plasma membrane to release their content [155]. In particular, the authors observed that exosomes released from cells depleted of S1P receptor or SphK isoform 2 contained less amount of CD63, CD81 and flotillin 2 than control cells, whereas the total number or size of exosomes was not changed. It is not clear yet how SphK is recruited to the MVB membrane or to which extent the sorting of other molecules is affected. In a follow up study, the authors investigated the mechanism required after S1P receptor activation, and found that it involved the G $\beta\gamma$ -mediated regulation of the Rho family GTPases and F-actin formation [156].

Recently, Yuan *et al.* investigated the effect in exosome release of acid ceramidase, the hydrolase responsible for the degradation of Cer into sphingosine and free fatty acids within lysosomes [157,158]. The authors found by nanoparticle tracking analysis that acid ceramidase gene deletion in primary cultures of coronary arterial endothelial cells augmented the secretion of exosomes (50–150 nm vesicles). The authors suggested that this could be related to a reduction of lysosome-MVB interactions because in these cells there was a decreased colocalization of a MVB marker (VPS16) with a lysosomal marker (LAMP-1) compared to control cells [158].

In conclusion, these studies support a role of sphingolipids and several enzymes involved in their metabolism in EV release, although their role may be cell dependent and several mechanisms seem to be involved. For recent reviews about Cer and other sphingolipids in exosomes see [159,160].

4.1.3. Cholesterol

EV membranes contain a high percentage of CHOL (Table 2), a lipid that increases membrane lipid order, prevents ion leakage and affects membrane fusion [161,162]. Several groups have investigated the potential role of this lipid in EV formation and release [163]. Several treatments and/or conditions that alter the CHOL levels in cells were used in these studies, such as methyl- β -cyclodextrin (m β CD), a cholesterol-sequestering agent often used to reduce the CHOL levels of cellular membranes. However, high concentrations of this compound may also induce ion leakage and extract other lipids. In some studies, lack of leakage of cytosolic proteins such as lactate dehydrogenase has been used to conclude that there is no leakage across the plasma membrane. However, leakage of ions such as K⁺ and Ca²⁺ could still occur and cause large intracellular changes [164], and this should be evaluated. CHOL complexed with m β CD can also be used to increase the cellular levels of CHOL [165]. Thus, restoring CHOL levels with m β CD-CHOL after depletion with m β CD can be used as a control. Alternative methods to reduce the CHOL levels, i.e. inhibition of the *de novo* CHOL synthesis, can also be useful [165]. It should be noted that conditions that lead to increased CHOL levels may perturb a number of cellular functions [166,167].

One of the first attempts to investigate the role of CHOL in exosome release was done with PC-3 cells [168]. A limited number of methods to quantify exosome release were available at that time, and changes in the levels of molecules expected to be in exosomes were often used as a readout of exosome release. The results of the study showed that the levels of caveolin-1 and LAMP-1 were increased in cells where the CHOL levels were reduced by approximately 40% by addition of m β CD,

or by approximately 25% after treatment with lovastatin and mevalonate to inhibit the *de novo* CHOL synthesis [168]. In another study, cells treated with m β CD released exosomes (30,000 rpm pellet, g value not provided) with higher levels of the latent membrane protein 2A of Epstein-Barr virus, a protein that regulates virus pathogenesis in infected cells [169]. In this case the involvement of CHOL in the m β CD effect was supported by addition of CHOL to the medium to restore the levels of CHOL. It is also interesting that addition of CHOL (in complex with m β CD) increased exosome release in Oli-neu cells (as measured by an increase of flotillin, ALIX and CD63) [170]. The same effect was observed when cells were treated with U18666A, a compound that prevents the action of the Niemann-Pick type C protein and in cells where this protein was depleted [170]. In both cases CHOL is trapped in late endosomes/MVBs. In addition, it has been reported that treatment with 5 mM m β CD reduced the ionophore-stimulated release of microvesicles in THP-1 cells by approximately 40% [171], but treatment of human erythrocytes with lower concentrations of m β CD had the opposite effect [172]. Further investigations to support the role(s) of CHOL in EV formation and release are needed.

As mentioned above, CHOL together with sphingolipids are the main constituents of specific membrane domains called rafts that have been related to several transport and sorting mechanisms [118,173]. Interestingly, it was reported some years ago that some molecules such as ganglioside GM1, the Src tyrosine kinase Lyn, flotillin-1, and stomatin are released to the extracellular medium via their association with lipid raft domains in exosomal membranes [174]. Other raft associated proteins as caveolin-1 and MAL have also been detected in exosomes [133]. The high content of CHOL and sphingolipids in exosomal membranes (Table 2) is in agreement with the presence of lipid rafts.

4.2. Secretion of EVs: effect on lipid composition in cells

The biological roles of EVs can be mediated both by their ability to function as carriers to remove unwanted/toxic cellular material and/or to transfer molecules between cells. Concerning their role in waste disposal, exosomes have been implicated in the maintenance of CHOL homeostasis. This is mainly based on studies of the Niemann-Pick type C1 (NPC1) lysosomal storage disorder, where free CHOL and sphingolipids accumulate in endosomal and lysosomal compartments [175]. In particular, an increased exosomal CHOL release was found under conditions causing an intracellular accumulation of free CHOL [170]. In terms of transfer of lipids and lipid-metabolizing enzymes from cell-to-cell, it has been reported that EVs contain several bioactive lipids that may affect the behavior of recipient cells, and also enzymes responsible for the synthesis of these bioactive lipids. In this way EVs could act both as carriers for lipid mediators synthesized in releasing cells as well as induce production of lipids in recipient cells. Various studies have reported effects mediated by lipid or lipid-related enzymes enriched EVs. For example astrocytes release EVs enriched in several Cer species, and this has been suggested to play a role in the spreading of Alzheimer's disease [176]. In another study, Cer 24:1 was suggested as marker of aging as it was enriched in serum EVs from aged (75–90 years) compared to young (24–40 years) women. Moreover, mouse serum EVs loaded with Cer 24:1 induced cell senescence in bone marrow mesenchymal stem cells [177]. Furthermore, several functional effects have been attributed to the presence in EVs of lipid metabolizing enzymes such neutral ceramidase and neutral SMase [178,179]. EV proteins could also affect the function of recipient cells by changing their lipid metabolism. For example, it has recently been suggested that the viral protein Nef released in exosomes from HIV-infected cells may be responsible for impairment of CHOL metabolism in recipient cells [180]. This is an emerging topic and, considering the molecular complexity of EVs, it is not easy to attribute effects in the EV-receiving cells to a specific lipid. For additional information about this topic we refer to recent reviews [181,182].

5. Clinical uses of EVs

The interest in EVs has increased in recent years due to their potential clinical applications. In particular, EVs are currently being investigated as a new type of liquid biopsies and as novel drug carriers.

5.1. The use of lipids in EVs as biomarkers

5.1.1. Lipids in urinary EVs as biomarkers

Increasing evidence shows that EVs are involved in numerous pathological processes. The protein and nucleic acid content of EVs has been thoroughly studied in an attempt to identify diagnostic and prognostic biomarkers [4]. There are also a few studies that have analyzed the EV lipid profile to get insight in the lipid signature associated with specific diseases. Early studies during the period 2009–2012 indicated that urine could be a useful biofluid to look for lipid biomarkers [183–185]. In terms of EVs, we have compared the content of 36 lipid species of urinary EVs from 15 patients with prostate cancer and 13 healthy volunteers [88]. The results showed that specific lipid species could separate the two groups with high sensitivity (93%) and specificity (100%). The largest differences were observed for lactosylceramide d18:1/16:0, which was highest in the patient group, and PS 18:1/18:1, which was highest in the control group. However, no significant differences in lipid classes between the two groups were found. Thus, these data illustrate the importance of using MS analyses for lipid species quantification in clinical laboratories.

Yang et al. recently published lipidomic data for EVs in urine obtained from four prostate cancer patients and four healthy volunteers [186]. EV preparations obtained using flow field-flow fractionation were combined into one cancer sample and one healthy control sample before analysis. The size distribution of the two preparations was not reported, but the EVs from the cancer patients seem to be considerably larger than the EVs from the healthy volunteers. The lipid data were presented as the ratio obtained between patients and healthy volunteers (i.e. absolute quantification was not shown), and the large differences reported for the content of TAG and CE in the two samples indicates that at least one sample contains different types of vesicles/particles. In another recent study, the metabolomic content of urinary EVs of patients with stage 2 and 3 prostate cancer showed several Cer species that were significantly different between the two stages, but the differences were relatively small [187].

Very recently Glover et al. reported lipidomics of urinary exosomes from hereditary α -tryptasemia patients and healthy volunteers [188]. The authors showed that both groups had EVs with a mean diameter of approximately 170 nm, and that twice as many EVs were excreted from the patients than from the controls. The results showed that 64 out of 521 lipid species from 19 lipid classes were significantly different in the vesicles obtained from the two groups; surprisingly among the 64 species there were 13 species of both TAG and CE. Remarkably, the 64 species reported to be significantly different in the two groups were found to be present in lower relative amounts in patients than in controls. The very high content of the 13 CE species and the very low amounts of the two SM species reported to be different between the two groups (low in both groups), differ considerably from data reported by others. In this study, it would have been very useful if an estimation of the amount of the various lipid classes had been provided.

5.1.2. Lipids in EVs from other biofluids as biomarkers

During recent years several lipidomic studies of EVs obtained from other biological fluids than urine have been published. Thus, Tao et al. compared the lipid composition of serum EVs from 20 pancreatic cancer patients and healthy controls [189]. Importantly, PE 16:0/18:1 was shown to be associated with the tumor stage, and this lipid was also found to be significantly correlated with patient overall survival. A long list of other lipids was also stated to be present in different amounts in the two groups. It is difficult to evaluate these data because

they are presented as relative signal intensities for the m/z signals detected, and many of these species are unusual, i.e. containing combinations of fatty acyl groups not assumed to be present in cellular membranes.

In addition, a recent publication reported the analysis of lipids in EVs isolated from plasma of multiple sclerosis patients and healthy volunteers. It has shown that sulfatides (a class of glycosphingolipids, sulfated galactosylceramide, highly expressed in brain) were found in these EVs, and a slightly higher level of sulfatide C16:0 was found in the EVs isolated from patient samples [190]. Finally, Hough et al. isolated EVs with a mean size diameter of approx. 150 nm from bronchoalveolar lavage fluid of asthmatics and healthy subjects, and reported that SM 34:1 (most likely SM d18:1/16:0) was more abundant in EVs from second-hand smoke exposure asthmatics compared to healthy controls [191]. The authors also listed nine other species to be present in different amounts in the two groups. Several of these species are surprising considering the fatty acyl groups expected to be present in human cells, and this list also includes mannosyl-di-PI-ceramide, i.e. the fungi specific glycosphingolipid mentioned above.

5.2. EVs in drug delivery

It has been a huge interest in using EVs for drug delivery in recent years. Much attention has been put on how to solve technical issues like obtaining large-scale preparations and making standardized protocols for EV purification and analyses. Although the study of EVs for drug delivery is in an early phase, it is important to consider how to document such complex products as EVs for market approval as drugs. We expect that it will be challenging to describe products containing thousands of different molecules and to demonstrate batch reproducibility.

In order to benefit from the learning in the EV field and to make products with a more simple composition than EVs, several groups have started to make liposomes, sometimes referred to as exosome- or EV-mimetics, based on specific molecular characteristics of EVs [192,193]. This can be an interesting approach, although it is challenging as long as we do not know which proteins and lipids on the surface of EVs are essential for a successful drug delivery. The importance of complex glycosphingolipids at the EV membranes for drug delivery is also unclear. For groups trying this approach, it is probably important that the lipid surface of the liposomes looks as similar as possible to the surface of EVs. When making symmetric liposomes this implies that lipid classes normally present in the inner leaflet (such as PS and PE) are not included. The best mimic of the lipids in the outer leaflet of EVs should probably be high levels of CHOL (30–40%) and more or less similar amounts of PC and SM. It should also be kept in mind that the most common PC species in cells contain one saturated and one monounsaturated fatty acyl group with 16 or 18C atoms, and that the most common SM and glycosphingolipid species contain C16:0, C24:0 and C24:1 as the N-amidated fatty acyl chains.

Several groups also try to make cell-derived vesicles by applying different forms of stress to cells, as larger amounts of such vesicles are easier to produce than exosomes (reviewed in [193]). We would like to mention that applying stress methods to cells or to load vesicles with drugs using stress methods such as sonication or extrusion, include the risk that the final preparation contains vesicles where the membrane is turned inside out.

6. Conclusion and future perspectives

This review demonstrates the added value of performing quantitative lipid analyses in EV research. We stress the importance of describing all methodological details used to purify, isolate and analyze EVs such that the studies can be reproduced. Importantly, as a final quality control, it should also be checked that the lipids reported are likely to exist in the investigated species. In conclusion, reliable MS data are important for quantification of lipids both in cells and their EVs, as well as

in recipient cells treated with EVs. Furthermore, MS analyses will allow us to further explore the potential use of EV lipids as biomarkers, and could also be helpful for the design of better carriers for drug delivery.

Declaration of Competing Interest

The authors have no competing interests to declare.

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