Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles

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Abstract Release of membrane vesicles, a process conserved in both prokaryotes and eukaryotes, represents an evolutionary link, and suggests essential functions of a dynamic extracellular vesicular compartment (including exosomes, microparticles or microvesicles and apoptotic bodies). Compelling evidence supports the significance of this compartment in a broad range of physiological and pathological processes. However, classification of membrane vesicles, protocols of their isolation and detection, molecular details of vesicular release, clearance and biological functions are still under intense investigation. Here, we give a comprehensive overview of extracellular vesicles. After discussing the technical pitfalls and potential artifacts of the rapidly emerging field, we compare results from meta-analyses of published proteomic studies on membrane vesicles. We also summarize clinical implications of membrane vesicles. Lessons from this compartment challenge current paradigms concerning the mechanisms of intercellular communication and immune regulation. Furthermore, its clinical implementation may open new perspectives in translational medicine both in diagnostics and therapy.

Keywords Exosome · Microvesicle · Microparticle · Apoptotic body · Cancer · Platelet · Biomarker · Autoimmune disease

Abbreviations
ADAM17 ADAM metallopeptidase domain 17
AFM Atomic force microscopy
ARF6-G ADP-ribosylation factor 6
ATP Adenosine triphosphate
CBS Center of Biological Sequence Analysis
CEA Carcinoembryonic antigen
CLL Chronic lymphocytic leukemia
DLS Dynamic light scattering analysis
DVT Deep vein thrombosis
EGFRvIII Epidermal growth factor receptor variant III
eMV Endothelial cell derived microvesicle
ERK Extracellular signal-regulated kinase
EV Extracellular vesicle
FC Flow cytometry
GPIIb/IIIa Glycoprotein IIb/IIIa
HER-2 (or Her2/Neu) Human epidermal growth factor receptor 2
Introduction

The extracellular space of multicellular organisms contains solutions of metabolites, ions, proteins and polysaccharides. However, it is clear that this extracellular environment also contains a large number of mobile membrane-limited vesicles for which we suggest the term “extracellular vesicles” (EVs). EVs include exosomes, activation- or apoptosis-induced microvesicles (MVs)/microparticles and apoptotic bodies (Fig. 1). In conventional histological sections, recognition of the secreted membrane vesicles is substantially limited by the resolving power of the light microscope, as their diameter usually falls below the limit of resolution. Furthermore, not only histological assessment but also conventional cell biology techniques including laser confocal microscopy or flow cytometry (FC) have substantial limitations when used for analysis of EVs [1]. Thus, it was not until recently that emerging evidence started to support the notion that vesicle release may be a universal adaptive cellular response (Fig. 2) [2–4].

Although initially met with skepticism, the existence of secreted membrane vesicles is now well established, and their diverse biological functions have been documented extensively. Current research interest in the field focuses primarily on two major types of EVs (exosomes and MVs), whose release may represent a universal and evolutionarily conserved process. Of note, EVs also include other vesicular structures such as large apoptotic bodies as well as exosome-like vesicles and membrane particles [2]. Exosome-like vesicles have common origin with exosomes; however, they lack lipid raft microdomains, and their size and sedimentation properties distinguish them from exosomes [2, 5]. Membrane particles are 50–80 nm in diameter, and they originate from the plasma membrane [6]. Extracellular membraneous structures also include linear or folded membrane fragments (e.g., from necrotic death) as well as membranous structures from other cellular sources including secreted lysosomes [7] and nanotubes [8].

In fact, in the extracellular environment of tissues, different types of vesicles are present simultaneously. Therefore, instead of focusing on a single type of vesicle, in this review article we summarize data on membrane vesicles collectively, from a systems biology perspective. Without claiming completeness, we discuss recent developments and some burning questions in the field.

As a step towards standardization of the terminology, we use the terms suggested recently by Théry et al. [2]. The only exception is that we use the collective term “extracellular vesicle” (EV) as a synonym of “membrane vesicle” (the designation that has been suggested for all populations of cell-derived vesicles). The phrase “exosome” refers to vesicles of 50–100 nm in diameter,
generated by exocytosis of multivesicular bodies (MVBs). Of note, biological literature also uses the term “exosome” for a macromolecular complex involved in RNA degradation, while the word “ectosome” (used by some authors to indicate neutrophil or monocyte derived MVs [9]), also refers to the outer, cortical layer of sponges [10]. “Microparticle” is a general designation used for any small particles within a given size range irrespective of origin. Even in biomedical literature the term “microparticle” is commonly used in the case of biopolymer particles (used as drug delivery systems), or dietary microparticles (food additives, pharmaceutical or toothpaste microparticles) [11], to mention just a few examples of the redundant terminology. Given that the word “particle” suggests a solid, particulate structure rather than a vesicular one, the designation “microvesicle” appears more appropriate to indicate membrane-limited structures. However, over the past decades, most studies traditionally referred to these structures as “microparticles” [e.g. endothelial cell- and platelet derived MVs were designated as endothelial microparticles (EMPs) and platelet microparticles (PMPs), respectively]. Of note, the term “microvesicle” has also been used by several authors collectively for vesicular structures released by cells instead of “membrane vesicles”.

To make the terminology unambiguous in this review, we will use the term “microvesicle” for the larger extracellular membrane vesicles (100–1,000 nm in diameter), and we will not use the term “microparticle” [2]. The abbreviation “MV” denotes “microvesicle” and not “membrane vesicle”.

Release of membrane vesicles: trogocytosis

From a cell biology perspective, vesiculation may be classified as a type of trogocytosis. The transfer of membrane components between donor and acceptor cells was first demonstrated in 1973 [12]. The phenomenon has been coined “trogocytosis” (from Greek “trogos”, meaning “gnaw” or “bite”). Some authors suggest that trogocytosis challenges classical theories of cell autonomy [13], as cells may receive membrane and cytoplasmic fragments from other cells. Two forms of membrane transfer (trogocytosis) have been described: via nanotubes or via membrane vesicles [8]. This review focuses on membrane vesicles of the extracellular environment.

The biogenesis of membrane vesicles essentially distinguishes exosomes from MVs and apoptotic bodies.

In the following section, we provide a concise overview of hallmarks of paramount membrane vesicle populations: exosomes, MVs and apoptotic bodies.

Key features of major vesicle populations

Key features of exosomes

- Exosomes were first described by Trams et al. [14] as exfoliated vesicles with ectoenzyme activity. This work was followed by the results of Harding and Stahl describing release of small vesicles and tubules from rat reticulocytes [15], and an electron microscopic study describing the exocytosis of the approximately 50-nm bodies [16].
- They are vesicles surrounded by a phospholipid bilayer (approximately 50–100 nm in diameter), their size range roughly overlaps that of the viruses (Fig. 3).
- They are released both constitutively and upon induction [2] by exocytosis of multivesicular bodies (MVBs), important intermediates in endolysosomal transport formed by the invagination and scission of buds from the endosomal limiting membrane into the lumen [17].
- Exosomes have been predominantly characterized in the case of immune cells (dendritic cells, T cell, B cells, macrophages) and tumors.
- Key mechanisms by which exosomes may exert their biological functions on cells include (1) direct contact between surface molecules of vesicles and cells, (2) endocytosis of vesicles, and (3) vesicle-cell membrane fusion [2]. Exosomes may horizontally transfer mRNA and miRNA [18]. Horizontal transfer of oncoprotein receptor [19] and transfer of HIV particles [20] have been demonstrated in the case of exosomes.
Examples for key functions of exosomes include antigen presentation [2, 21] and immunostimulatory and inhibitory activities [2].

They feature phosphatidylserine on the outer membrane leaflet [22], and markers include CD63, CD81, CD9, LAMP1 and TSG101 [23, 24].

Isolation and analytical methods include differential centrifugation and subsequent sucrose gradient ultracentrifugation [25], transmission electron microscopy (TEM), western blot and mass spectroscopy.

Basic features of exosomes have been recently covered by numerous review articles [2, 22, 23, 26–35] (Table 1).

## Key features of microvesicles (MVs)

- MVs were first described by Chargaff and West in 1946 as a precipitable factor in platelet free plasma with the potential to generate thrombin [36]. In 1967, Peter Wolf 

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### Table 1 Key features of membrane vesicle populations

|                        | Exosomes                        | Microvesicles                       | Apoptotic bodies                  |
|------------------------|---------------------------------|-------------------------------------|-----------------------------------|
| **Size range**         | Approximately 50–100 nm         | 100–1,000 nm (~100–400 nm in blood plasma) [2, 22, 38] | 1–5 μm [61]                      |
| **Mechanism of generation** | By exocytosis of MVBs           | By budding/blebbing of the plasma membrane | By release from blebs of cells undergoing apoptosis |
| **Isolation**          | Differential centrifugation and sucrose gradient ultracentrifugation [25], 100,000–200,000g, vesicle density is 1.13–1.19 g/mL | Differential centrifugation [39], 18,000–20,000g | Established protocols are essentially lacking; most studies use co-culture with apoptotic cells instead of isolating apoptotic bodies |
| **Detection**          | TEM, western blotting, mass spectrometry, flow cytometry (bead coupled) | Flow cytometry, capture based assays [38, 52] | Flow cytometry                   |
| **Best characterized cellular sources** | Immune cells and tumors         | Platelets, red blood cells and endothelial cells | Cell lines                       |
| **Markers**            | Annexin V binding, CD63, CD81, CD9, LAMP1 and TSG101 [23, 24] | Annexin V binding, tissue factor and cell-specific markers | Annexin V binding, DNA content |
| **Recent review articles** | [2, 22, 23, 26–35]             | [2, 24, 35, 53–57]                  |                                   |
Examples of key functions of apoptotic bodies are

- MVs are structures surrounded by a phospholipid bilayer. They are 100–1,000 nm in diameter [2] or ~100–400 nm in blood plasma [38], but the lower cut-off remains to be established [39]. Their size range overlaps that of bacteria and insoluble immune complexes [38] (Fig. 3)
- They are formed by regulated release by budding/blebbing of the plasma membrane.
- The rate of steady state release of budding/shedding vesicles [35] is generally low (except for tumors that release them constitutively [40]).
- Regulated release of vesicles is efficiently induced upon activation of cell surface receptors or apoptosis and the subsequent increase of intracellular Ca\(^{2+}\) [41, 42]
- They have been predominantly characterized as products of platelets, red blood cells and endothelial cells.
- Examples of key functions of MVs: they have procoagulant activity [43], represent a form of secretion of IL1/β [44], contribution to the pathogenesis of rheumatoid arthritis [45–47], contribution to the proinvasive character of tumors [48], induction of oncogenic cellular transformation [49], fetomaternal communication [50]
- They feature phosphatidylserine; however, some observations also suggest the existence of MVs without phosphatidylserine externalization [51]
- Routine isolation and analytical methods include differential centrifugation [39], flow cytometry (FC) and capture-based assays [38, 52]
- Basic characteristics, molecular and functional aspects have been summarized by several recent review articles [2, 24, 35, 53–57]

Key features of apoptotic bodies

- The term “apoptotic body” was coined by Kerr in 1972 [58], and the next milestone work in apoptosis research was conducted by Robert Horvitz et al. tracing cell lineage development in the nematode Caenorhabditis elegans [59, 60]
- Apoptotic bodies are 1–5 μm in diameter (approximately the size range of platelets) [61]
- Apoptotic bodies are released as blebs of cells undergoing apoptosis
- They are characterized by phosphatidylserine externalization, and may contain fragmented DNA [62]
- Examples of key functions of apoptotic bodies are horizontal transfer of oncogenes [63], horizontal transfer of DNA [64], yielding presentation of T cell epitopes upon uptake by phagocytic cells [65] and representation of B cell autoantigens [66]. Uptake of apoptotic bodies has been shown to lead to immunosuppression [67].
- Instead of isolating apoptotic bodies, most studies use co-cultures of cells undergoing apoptosis to investigate the functions of these structures.

In spite of extensive research, the rapidly emerging field of membrane vesicle research remains technically difficult. In the next section, we aim to overview major challenges and recent methodological improvements.

Problems and pitfalls associated with membrane vesicle measurement

In this section, we aim to summarize the difficulties associated with isolation of membrane vesicles and standardization of pre-analytical and analytical factors of membrane vesicle assessments.

Isolation of membrane vesicles

There is a widely accepted protocol for exosome isolation, which includes ultracentrifugation and a subsequent sucrose density gradient ultracentrifugation or, alternatively, sucrose cushion centrifugation [25]. In contrast, standard isolation protocols for MVs are lacking. Most groups apply centrifugation conditions from 18,000g (30 min) to 100,000g (60 min) [68]. Standard isolation protocols for apoptotic bodies are absent in the literature. In biological fluids and cell supernatants, the previously described membrane vesicle populations are present simultaneously with possible size overlap. While immune affinity isolated exosomes have been shown to have a diameter <100 nm [69], it has not yet been convincingly demonstrated that all MVs are larger than the 100-nm limit. Moreover, the statement that MVs may not be <100 nm should be made with some caution. Booth et al. have shown vesicles of this size (and even smaller) budding from the plasma membrane [70].

A further substantial problem is that during differential centrifugation prior to pelleting of a given membrane vesicle population, some of the respective vesicles may be selectively depleted. For example, centrifugation of cells or platelets results in a substantial loss of MVs [71]. To analyze blood-derived MVs, platelet-free plasma is required. However, currently there is no consensus on the centrifugation times, forces or the number of cycles for the removal of platelets. For complete removal of platelets, an 800-nm filtration is required after centrifugation [38]. Of note, forced filtration of membrane vesicles holds the risk of fragmentation into smaller vesicles [72]. To avoid this problem, we recommend gravity-driven filtration [38].
In conclusion, the isolation of membrane vesicles by differential centrifugation is complicated by the possibly overlapping size distributions of platelets and different membrane vesicle populations. Furthermore, centrifugation alone may prove insufficient to separate vesicles based on their sizes. However, differential centrifugation, when combined with sucrose gradient ultracentrifugation, can separate exosomes from MVs. A further alternative method to differential centrifugation, immunoisolation of membrane vesicles, may overcome these limitations [25, 69].

Size determination of different membrane vesicle populations

Despite the existence of several methods available to determine the size of vesicles, the precise determination of the size distribution of a given vesicle preparation seems to be unexpectedly difficult. FC is a convenient technique; however, it has severe limitations in resolving structures below 200 nm [22]. The gold standard for vesicle size determination remains transmission electron microscopy (TEM) [1]. For TEM studies, vesicles need to be concentrated by centrifugation. However, there are some concerns about the reliability of analysis of pelleted vesicles: centrifugation, dehydration and fixaton for TEM may alter the size and morphology of vesicles. Newer techniques (such as cryo-EM) led to the finding that the “cup-shaped” morphology of exosomes was an artifact related to fixation for TEM [25]. Despite these concerns, TEM is the only method by which the nature of the particle, its size and structure may be determined at the same time. Surface-bound particles are studied by atomic force microscopy (AFM). AFM has some limitations in analyzing non-rigid particles: the $z$ value (the height of the particle) seems to be much smaller than the $x$, $y$ values (characteristic for the surface area) [73]. Yuana et al. hypothesized that the vesicle volume is constant; therefore, the diameter of surface-bound vesicles is much smaller than the $x$, $y$ values, if we consider the low $z$ value. However, the low $z$ value may also result from the tapping mode analysis of the AFM (the up- and downward motion of the AFM tip may cause deformation of vesicles) or from the drying step. This is supported by our AFM data showing concordant results with TEM and dynamic light scattering analysis without a correction for the $z$ value [38].

Dynamic light scattering analysis (DLS) is a useful method for membrane vesicle sizing; however, it has limitations, particularly when the analyzed system is polydisperse. Our work based on TEM, AFM and DLS analysis, supported by the work of others [72], suggested that the blood plasma-derived MVs, isolated by 20,500g centrifugation, were 100–400 nm in diameter (mean = 170 nm) [38]. Nanoparticle tracking analysis is an alternative method to DLS and, particularly in the fluorescent mode, it is capable of analyzing highly polydisperse structures between 50 and 1,000 nm [1]. Results obtained with this method are highly concordant with the TEM measurements [1]. Other methods for vesicle size determination such as stimulated emission depletion microscopy or fluorescence correlation spectroscopy have been recently reviewed by van der Pol et al. [1].

Pre-analytical challenges

There are growing concerns about pre-analytical variables, particularly in the field of the diagnostic use of MVs. These pre-analytical factors were addressed insufficiently by earlier works in the literature. However, there are data regarding the alterations of the MV count in erythrocyte concentrates and platelet-rich plasma. It has been shown that platelets vesiculate in response to shear stress [74] and storage [75] (Fig. 4). In the study of Connor et al., it has been shown that freeze–thaw cycles of platelet-rich plasma resulted in a substantial increase of annexin V+ MV count [51]. In erythrocyte concentrates, the MV count varies with storage time, temperature, the buffer used to dilute MVs, and, most interestingly, with agitation [71]. Rubin et al. have shown that vortexing the erythrocyte concentrates for 20 s doubled the MV count [71]. These data strongly suggest that cells, particularly platelets and erythrocytes, are highly sensitive to environmental factors and respond by release of MVs (Fig. 4), which may lead to confounding results. As a consequence, during the assessment of

![Transmission electron micrograph of a platelet from normal human blood plasma releasing membrane vesicles (pMVs). Original magnification ×30,000](image)
Membrane vesicles, current state-of-the-art

In routine diagnostic approaches, all steps from venipuncture to sample analysis must be standardized precisely. These steps include (1) the diameter of the needle used for venipuncture, (2) duration of placement of the tourniquet, (3) the type of anticoagulants used, (4) centrifugal speed to yield platelet-free plasma, (5) working and storage temperature of the sample, (6) freeze–thaw cycles, (7) needle-to-analysis time, and (8) analysis protocols [39, 76]. It is critical that cells and platelets from biological fluids be pelleted shortly after collection (preferably within an hour), and before frozen stage. In order to minimize the effect of pre-analytical variables, recently, a workgroup has launched a joint project on standardization of MV analysis (International Society on Thrombosis and Haemostasis, Scientific and Standardization Committee: Standardization of Pre-analytical Variables in Plasma Microparticle Determination). The preferred protocol includes collection of blood samples in citrate containing tubes. In order to deplete blood samples in platelets, centrifugation twice with 2,500g for 15 min is recommended (F. Dignat-George, personal communication). After snap-freezing in liquid nitrogen, the samples should be stored at −80°C.

On the other hand, there are only limited data on pre-analytical variables in the case of exosomes. Some pre-analytical challenges have been demonstrated with respect to urinary exosomes as new biomarkers of kidney disease. During the pre-centrifugation step, urinary exosomes may become entrapped in the polymeric Tamm–Horsfall protein leading to a loss of exosomes [77]. Furthermore, possible viral contamination with the common presence of several hosts molecules, e.g., MHC-II molecules in both virions and exosomes, also need to be considered during exosome detection. Optiprep velocity gradients have been recently suggested to efficiently separate exosomes from HIV-1 particles [78].

Flow cytometric detection of membrane vesicles

As described above, standard FC detects vesicles above approximately ~200 nm, and therefore exosomes and smaller MVs cannot be analyzed directly by this method. Thus, it has to be emphasized that MVs smaller than the detection limit of the used flow cytometer cannot be discriminated from the instrument noise, leading to an inadequate numbering of MVs (because of not detecting the vast majority of vesicles that are present in the sample). Despite these limitations, FC is a widely used method for MV detection, and using FC, many studies have reported that MV counts correlated with a variety of diseases and different physiological conditions. Recently, there have been major improvements in the standardization of FC measurements of membrane vesicles [79, 80]. This is a prerequisite for the diagnostic and prognostic use of MV measurements. FC platforms for small size EV analysis are being developed by BD, Beckmann Coulter, Apogee; however, these are not yet widely available (except for the Apogee system).

In many of the earlier FC studies, annexin V was used as a common marker for MVs. However, recent studies suggest the presence of annexin V-negative MVs. Indeed, it has been reported that annexin V-negative MVs account for more than 80% of the platelet-derived vesicles in blood plasma [51]. This study controlled for the effect of annexin V binding, demonstrating that it requires at least 1.25 mM calcium for binding. They also drew attention to the fact that phosphate-buffered saline was not suitable for annexin V staining, as calcium forms precipitates with phosphate. In line with these results, in our work, we have found that saline with 2.5 mM Ca²⁺ results in an optimal annexin V staining [38]. Given that not all vesicles are annexin V positive, alternative labelings of MVs with PKH67 [81] or bio-maleimide [82] may also prove useful to examine correlations between MV counts and human disorders. In blood, the most important sources of MVs are platelets, and most studies analyzed the alterations of levels of platelet-derived microparticles (pMVs) under several conditions and diseases. Recent studies have suggested that the majority of the CD41⁺ pMVs were derived from megakaryocytes rather than from platelets [83]. The megakaryocyte-derived vesicles did not carry CD62P, but displayed full-length filamin A [83]. Further studies are required to clarify the potential diagnostic and/or prognostic values of megakaryocyte-derived vesicles.

An additional problem is that any structure that shares size and consequently scattering properties with MVs, would also appear during FC within the gate used for MP detection. We have demonstrated recently that insoluble immune complexes may also give signals at FC, and thus interfere with MV measurements. When all events are considered as MVs within the MV gate, the presence of immune complexes in RA synovial fluid may overestimate true vesicle counts [38]. Furthermore, we showed that, when using indirect labeling, primary and secondary antibodies formed immune complexes detectable within the MV gate as fluorescent events. Important, not only immune complexes but also streptavidine-biotinylated antibodies and antibody aggregates may form supramolecular protein complexes detectable within the MV gate. The MV and protein complex-related events may be easily discriminated by using low concentrations of detergents enough to lyse MVs but insufficient to disassemble immune complexes and protein aggregates [38]. The optimal concentration of the detergent (i.e., Triton X-100), which discriminates vesicles from protein aggregates, should be titrated for individual settings. Furthermore, it is recommended to use only direct immunolabelling when
staining vesicles in order to avoid immune complex formation. However, it is important to note that some vesicle subpopulations may be particularly resistant to such lysis; therefore, detergent lysis may not be considered as a guarantee to lyse all types of membrane vesicles.

To conclude, FC is a powerful technique to analyze membrane vesicles; however, as it has serious limitations, all FC data on membrane vesicles should be interpreted cautiously.

Potential contamination of membrane vesicle preparations

To study the effect of secreted membrane vesicles, it is of high importance to examine isolated vesicle populations. Beyond membrane vesicles, biological fluids may also contain high amounts of different types of particles (lipoproteins, viruses) or molecules with the tendency to form aggregates or complexes. These complexes or particles not only disturb the detection of membrane vesicles (as discussed in the previous section), but may also co-sediment with various membrane vesicle populations. Roughly, exosomes overlap in size with viruses and lipoproteins, while MVs overlap the size range of bacteria (Fig. 3). In the case of viruses, even sucrose gradients are inefficient at separating them from exosomes [78]. This problem may be solved using iodixanol gradients, which have been shown to separate exosomes from retroviruses [78]. Furthermore, proteins may also contaminate isolated exosome preparations, as suggested by Rood et al. [84]. The authors showed that, after ultracentrifugation or nanomembrane ultrafiltration, the pellet contained very high amounts of contaminating proteins from patients with nephrotic syndrome. Our group has also shown that preparations of MVs isolated by differential centrifugation may be contaminated by protein complexes, especially by insoluble immune complexes [38]. We have demonstrated shared size and sedimentation properties of immune complexes and MVs, which may result in contamination of conventionally isolated MV preparations.

Membrane vesicle populations may also be contaminated by microsomal fractions or organelles released from necrotic cells (in particular in tumors); thus, corresponding controls (endoplasmic reticulum-related molecules) should be run in studies of isolated membrane vesicles.

In conclusion, exosomes represent the best characterized population within the family of membrane vesicles. However, the isolation, sizing and measurement of MVs and apoptotic bodies remains elusive. There have been significant advances in FC detection and standard isolation of MVs, but pre-analytical factors still remain a challenge. Additionally, quality control of EV preparations is essential prior to functional assays, if describing a specific function of exosomes or MVs.

Conceptual and theoretical issues related to membrane vesicles

The most challenging conceptual issue in the field is the lack of standard terminology and methodology which hampers efficient information flow. International meetings may offer unique opportunities to establish such a consensus.

Current studies test isolated populations of membrane vesicles in vitro. Thus, the effect of a single vesicle type rather than a complex vesicular pattern is assessed (albeit the latter probably reflects in vivo situations more realistically). Combinatorial signaling induced by different vesicles (such as exosomes and MVs) or vesicles in combination with soluble molecules (such as cytokines), has not yet been investigated. Thus, synergistic or additive effects cannot be estimated. Size distribution of vesicles released by apoptotic cells has not yet been systematically explored. Therefore, exclusion of apoptotic bodies (>1,000 nm) from studies on membrane vesicles may lead to loss of relevant information with respect to EVs. Furthermore, lack of information on local concentrations and half lives of membrane vesicles in tissues prevents drawing conclusions from in vitro functional assays with secreted membrane vesicles.

Finally, the striking structural and functional similarities between exosomes and viruses raise interesting questions. As mentioned earlier, size distributions of exosomes and viruses show a substantial overlap (Fig. 3). Furthermore, both exosomes and many viruses are essentially lipid and protein shells enclosing nucleic acids, and their nucleic acid content is released into the cytosol of the cell. As with viruses, transcription of exosomal RNA has been convincingly shown in cells after uptake of the vesicles [19]. Thus, the description of exosomes may well fit viruses, and the borderline of these two structures might be somewhat blurred. A clear difference is the ability of viruses to replicate inside the infected cells. The intriguing possibility is raised that membrane vesicles (such as exosomes) and viruses may have phylogenetic links. HIV-1 and exosomes were reported to share a common glycome arguing for a common origin [85]. It may be hypothesized that certain cell-derived vesicles (carrying enzymes and nucleic acids) might have proven evolutionarily successful to replicate inside cells following their uptake. From this aspect, enveloped viruses such as HIV or influenza virus might be of particular interest, since during their release from cells these viruses acquire an envelope, which is a modified piece of the host’s plasma- or internal membrane. It remains an exciting question as to whether viruses like HIV hijack the exosomal dissemination system as suggested [20], a concept that has been refuted in the recent past [86], or they might have taken advantage of cellular vesiculation early during their evolution. Furthermore, it was shown that exosomes from cytomegalovirus-infected (CMV) HUVEC cells contain CMV proteins and
viral DNA [87], and exosomes from nasopharyngeal carcinoma cells with latent EBV infection, also contain viral proteins as well as virus-specific miRNAs [88].

In the next section, we make an attempt to briefly overview the experimental settings that may serve to investigate the functions of EVs.

Experimental systems to study membrane vesicles

In vitro modulation of vesicle formation may be achieved by various compounds. The family of potentially targeted pathways and proteins that participate in the release of exosomes and MVs are numerous, and their number is continuously expanding.

Stimulation of certain cell-membrane receptors leads to increased secretion of membrane vesicles. These include P2X(7)R [44, 89, 90] and thrombin receptor [91] or GPVI collagen receptor on platelets [45]. P2X(7)R can be stimulated by ATP or the antimicrobial peptide LL-37 [92], or inhibited by rollin [93] and various protein tyrosine kinase antagonists [94]. Resting B cells, T cells, mast cells and reticulocytes can also secrete vesicles after activation of their cell surface receptors [2]. Exosome secretion may be induced by activation of the T cell receptor or reduced by LPS in dendritic cells [95, 96].

Downstream pathways of cell surface receptors may also be modulated to modify vesicle secretion. Calcium ionophores stimulate vesicle release by elevating intracellular calcium levels in various cells [97, 98]. Calpeptin, an inhibitor of calmodulin, decreases MV shedding in platelets [99]. Exosome release may also be blocked in melanoma cells by the pretreatment of proton-pump inhibitors, which reduce the acidic milieu [100]. In tumor cells, the GTP binding protein ARF6-GTP activates phospholipase D which recruits extracellular signal-regulated kinase (ERK) to the plasma membrane, where ERK activates myosin light-chain kinase (MLCK). The latter molecule is needed for membrane vesicle secretion; thus, inhibition of ARF6 activation blocks MV shedding [101]. The ceramid pathway can be modulated by inhibition of the synthesizing enzyme, neutral sphingomyelinase [102].

Finally, effector molecules that take part in membrane vesicle formation may also be inhibited. The compound R5421 inhibits scramblase, and reduces MV shedding in reticulocytes [103], while inhibition of the known sheddase, ADAM17, also reduces MV production in platelets [104].

Of note, most proteins involved in vesicle trafficking also have other vital functions, thus blocking them may have detrimental consequences for cells. Strikingly, blocking of Rab27a or Rab27b impairs exosome secretion, without affecting conventional protein secretion [105].

In vivo models suitable for studying vesicle production are almost completely lacking. Scott syndrome, a rare bleeding disorder, is the only known human disease caused by inheritable deficiency of MV production of human platelets as a result of impaired phosphatidyl-serine transmembrane migration and Ca^{2+}-induced phospholipid scrambling [106]. This disease has also been described in a pedigree of German sheepdogs [107], and the gene responsible was linked to canine chromosome 27 [108]. This dog disease could serve as an in vivo animal model to study impaired platelet vesicle secretion.

Human gene polymorphisms may affect membrane vesicle secretion. IL-1β has been shown to be secreted via membrane vesicles after activation of the P2X(7) receptor and Toll-like receptors in monocytes and macrophages [44, 109]. The gain of function A348T polymorphism of P2X(7)R was shown to induce increased IL-1β secretion after LPS priming in monocytes [110], although in this case the route of secretion was not analyzed. However, the G496A loss of function mutation of the same gene was reported to decrease both secretion of IL-1β and shedding of L-selectin [111]. Polymorphisms of other genes, encoding for proteins that take part in MV formation and secretion, offer possibilities for studying membrane vesicles. Thus, polymorphisms and mutations of genes that influence EV formation may serve as valuable tools to study vesicle formation processes.

On the other hand, membrane vesicle research may also benefit from lessons of in vivo experimental models of impaired vesicle secretion. Various knock-out mice may serve as attractive mammalian candidates to study deficiency of proteins that participate in membrane vesicle formation. For instance, leukocytes of P2X(7)R-deficient mice are characterized by reduced shedding of L-selectin, and decreased secretion of IL-1β in response to LPS and ATP [112]. Tumor suppressor-activated pathway 6 (TSAP6)-deficient mice show microcytic anemia, with abnormal reticulocyte maturation and deficient transferrin receptor downregulation, features which are all dependent on exosome secretion. Exosome production is also impaired in TSAP6 null cells [113].

Mice, genetically deficient in the clearance of membrane vesicles (e.g., phosphatidyl-serine knockout mice [114] or T cell immunoglobulin and mucin domain-containing protein 4 (TIM4)-deficient mice [115]) represent another type of in vivo experimental system to study membrane vesicles. The significance of such an in vivo system is supported by the observation that TIM4-deficient mice exhibit autoimmunity and T and B cell hyperactivity [115]. Recently, Tyro3/Axl/Mer (TAM) signaling has been implicated in the ‘homeostatic phagocytosis’ of apoptotic cells and membranes [116]. Similarly to TIM4-deficient mice, TAM-deficient mice develop autoimmunity [117]. The human autoimmune disease systemic lupus erythematosus (SLE) may offer another in vivo system to
investigate membrane vesicles, as SLE is characterized by an impaired clearance of apoptotic bodies [118–123].

Comprehensive meta-analysis of proteomic studies on different types of membrane vesicles

In contrast to sporadic publications on membrane vesicle lipidomics [124–127], metabolomics [128] or glycomics [85], information regarding the protein composition of EVs may be readily extracted from numerous published proteomic studies as well as from a database [129]. In order to obtain a reliable and representative set of proteins identified in exosomes, we filtered the ExoCarta database [129] for proteomic studies, and selected only data from sources in which human exosomes were purified by density gradient ultracentrifugation [126, 129–133]. Unlike in the case of exosomes, there is no comprehensive database for proteomic data regarding MVs. While the diversity of tissue types analyzed by exosome studies makes the obtained dataset representative for these structures, at present there is a clear overrepresentation of data concerning platelet-derived MVs in the literature. In the MV dataset, we included data from proteomic studies on human platelet or plasma-derived MVs, in which protein data were supplied in a clearly accessible format [128, 134–136]. Subcellular localization of proteins can be readily analyzed using the annotations of the SwissProt/UniProt database [137, 138]. Although the proteomic studies from which the protein datasets were extracted also investigated the subcellular localization of molecules, the different methods of vesicle isolation and analysis interfered with the comparison of the results from individual studies.

One may anticipate that the subcellular localization of a protein in an intact cell is in concordance with the possible localization in a membrane vesicle. Since both exosomes and MVs are considered to consist of cytoplasm enclosed by a lipid bilayer, it was interesting to find a notable portion of nuclear proteins in both vesicle populations (Fig. 5). Given that platelets have no nuclei, the presence of nuclear proteins in MVs isolated from blood plasma supports the recent observation that circulating MVs are of megakaryocyte rather than platelet origin [83]. Nuclear proteins may originate from the preparation methods, as centrifugation may also pellet apoptotic or necrotic materials (apoptotic bodies, nuclear fragments or nucleosomal complexes) together with MVs. Dean et al. demonstrated that nuclear proteins are not evenly distributed in all size fractions of platelet-derived MVs [136]. Of note, cytosolic proteins, known to undergo nuclear translocation, may have also increased the frequency of nuclear proteins, as we included proteins in all their possible subcellular localizations (according to SwissProt/UniProt annotations) when generating Fig. 5. In contrast to the unexpectedly high ratio of nuclear proteins found in MVs, exosomes contained only limited amounts of nuclear proteins. Membrane-associated proteins, on the other hand, constituted the major fraction of exosomal proteins.

One of the possible functions of membrane vesicles is contributing to non-classical leaderless secretion of proteins such as IL-1β [139]. Taking advantage of the recently available prediction tools to determine which proteins are likely to undergo leaderless secretion [140], we used the SecretomeP 2.0 software from CBS [141] on a dataset previously filtered for cytoplasmic proteins. All membrane proteins are likely to contain a signal sequence and thus avoid leaderless secretion. Using this prediction tool, we analyzed data obtained in six studies on exosomes and four studies on MVs. From all cytoplasmic proteins in each dataset, around 30% were predicted to be secreted non-classically. The remaining molecules (such as cytoskeletal proteins) could contribute to a protective shell stabilizing vesicular structure, and may carry targeting information.

![Fig. 5 Subcellular localization of proteins identified in exosomes (a) and MVs (b). The published proteomic studies are indicated by the name of the first author.](image-url)
Since IL-1β, for example, plays a crucial role in regulating immune responses, it is critical to achieve the right concentration at the right site. Although it is degraded rapidly in human plasma (its half-life is 6 min [142]), it might be preserved for a longer time inside vesicles, thus enabling it to act systematically or locally, but distant from the site of its production. For IL-1β, the majority of leaderless secretion has been reported to occur by exosomes [143].

The fact that molecules, predicted to undergo leaderless secretion, were not detected in substantial amounts in vesicles might either suggest an alternative route for non-classical secretion (other than by secretion of membrane vesicles), or may result from limitations of current prediction tools. Indeed, transporters have also been suggested to be involved in leaderless protein secretion [144], representing a minor alternative to release by exosomes.

When exosome- and MV-associated proteins were analyzed for major biological functions by Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA, USA), in the case of both vesicle populations, cellular movement, cell-to-cell signaling, tissue development and cancer were among the top associated biological functions. The function “cell death” was associated more with MVs than with exosomes (in agreement with the observation that release of MVs is induced during apoptosis). In contrast to exosomes, MV proteins were found to be associated with inflammation, and this function was ranked 10th in the case of MVs, whereas it was ranked only 30th for exosomes (data not shown).

According to the IPA software, many of the key molecules implicated in viral entry into cells are also detectable in exosomes (Fig. 6a). These molecules expressed by both exosomes and cells bind a variety of viruses, like SV40, Filo-, Coxsackie’s-, Echo-, and Arena viruses and even HIV1. The presence of numerous virus binding proteins in exosomes is in line with the fact that viral entry via endocytic pathways is a well-known attribute to exosomes, reviewed recently by Izquierdo-Useros et al. [145]. Out of all biological pathways in the IPA knowledge base, the most adequate fit of MV proteins appeared to be integrin-mediated signal transduction (Fig. 6b). The integrin pathway is involved in regulating cell shape and motility in response to changes in the extracellular environment. MV proteins implicated in this pathway include actin, talin and vinculin, molecules involved in regulating cell shape and cellular movement. The significance of the abundant virus binding proteins in exosomes and MV molecules involved in integrin signaling remains to be elucidated experimentally.

Top canonical functions, identified by IPA software in association with exosomes and MVs, are included in Table 2. While many of the identified pathways revealed known features of membrane vesicles, germ cell–Sertoli cell signaling in exosomes, however, seems to represent an unexpected pathway. During spermatogenesis, actin-based adherens junctions at the interface of Sertoli and germ cells undergo extensive restructuring. This facilitates germ cell movement across the epithelium. The dynamics of these junctions are regulated by the integrin/RhoB/ROCK/LIMK pathway [146]. The clear nature of this pathway in the case of exosomes remains to be established, and at this point we cannot exclude the possibility that it may be an issue related to the in silico analysis rather than a real biological process existing in exosomes.

Medical implications of the extracellular vesicles

In the present review, we have described the diverse constituents of the extracellular vesicular compartment. The ubiquitous formation of membrane vesicles allows the clinician to exploit their diagnostic value in various diseases (Table 3) and conditions. Exosomes are smaller, but can serve as tumor markers. MVs are investigated thoroughly as diagnostic tools due to their larger size and accessibility in several biological fluids. Most groups have focused on blood-derived MVs. Here, we discuss the most widely studied endothelial- and platelet-derived MVs as well as some recent advances of tumor vesicle-based diagnostic studies.

Endothelial MVs (eMVs)

Endothelial cells can release exosomes, endothelial MVs (in the literature often also referred to as EMPs) and apoptotic bodies [24]. eMVs are formed in vitro after stimulation with LPS, reactive oxygen species and various cytokines [24]. They are detectable in human blood plasma by FC using endothelial cell-specific markers (CD54, CD62E, CD62P, CD31, CD106, CD105, CD144, CD146) [24]. Despite the limitations of FC, eMVs are considered to be markers of inflammation, endothelial injury and endothelial dysfunction [147–149]. As endothelial dysfunction is a well-known predictor of future cardiovascular diseases, eMVs could be used as biomarkers of vascular health.

eMVs are elevated in the blood plasma of patients with acute and chronic vascular disorders, including acute coronary syndrome [150], severe hypertension [151], end-stage renal failure [149] and pulmonary arterial hypertension [152] (for a review, see [153]) (Table 3). eMVs have a controversial role in the pathogenesis of vascular diseases [18]: eMVs may contribute to vascular injury and they are capable of inducing endothelial cell activation, impairing vasorelaxation [154], and promoting arterial stiffness. Furthermore, phosphatidylserine and/or tissue factor positive eMVs promote coagulation and
thrombosis. On the other hand, it has been shown that eMVs may induce angiogenesis or promote endothelial cell survival [24].

**Platelet MVs (pMVs)** (Fig. 4)

The majority of circulating MVs in the blood plasma are derived from platelets during platelet activation or, as suggested recently, from megakaryocytes [83]. In vitro, pMVs are formed during stimulation of platelets with collagen, thrombin or ADP. Shear stress and agitation also induces pMV formation. pMVs are easily detected by FC using CD41, CD42, CD61, and CD62 markers. Similarly to eMVs in endothelial activation, pMVs are considered as platelet activation markers. Therefore, numerous diseases are characterized by elevated levels of pMVs (Table 3), including cardiovascular diseases, autoimmune diseases [62] and type II diabetes. pMVs may also expose phosphatidyl-serine and tissue factor, and they may also contribute to the pathogenesis of vascular diseases due to

![Diagram of Ingenuity Pathway Analysis (IPA) of data from meta-analyses of published proteomic studies on exosomes and microvesicles.](image)

**Fig. 6** Ingenuity Pathway Analysis (IPA) of data from meta-analyses of published proteomic studies on exosomes and microvesicles. **a** Molecules implicated in viral entry by caveola- and clathrin-mediated endocytosis as well as by macropinocytosis. *Shaded symbols* represent molecules identified in exosomes. As shown, several exosomal proteins are present in the IPA knowledgebase as molecules that facilitate the entry of different viruses. **b** Molecules involved in integrin signaling. *Shaded symbols* represent published microvesicle-associated proteins as key participants of integrin signaling.
### Table 2: Top vesicle-associated canonical pathways identified by Ingenuity Pathway Analysis

| Canonical pathway                          | MPs/MVs Exosome | P value | Ratio | Canonical pathway                          | Exosome | P value | Ratio |
|-------------------------------------------|-----------------|---------|-------|-------------------------------------------|---------|---------|-------|
| Actin cytoskeleton signaling              | 6.05E−14        | 38/238  |       | Germ cell–Sertoli cell junction signaling | 1.20E−23 | 45/168  |       |
| Integrin signaling                        | 5.18E−11        | 32/205  |       | Integrin signaling                        | 1.65E−21 | 47/205  |       |
| RhoA signaling                            | 4.53E−09        | 21/110  |       | Caveolar mediated endocytosis signaling   | 2.92E−21 | 30/85   |       |
| Caveolar mediated endocytosis signaling   | 1.12E−08        | 17/85   |       | Virus entry via endocytic pathways        | 1.58E−20 | 32/100  |       |
| Acute phase response signaling            | 2.21E−08        | 26/183  |       | Ephrin receptor signaling                 | 2.42E−20 | 43/199  |       |

The *P* value is calculated using the right-tailed Fisher Exact Test. It is a measure of the likelihood that the association between a set of analyzed molecules and a given pathway is due to random chance. The “ratio” expresses the fraction of molecules fitting a given pathway within an analyzed dataset and the total number of molecules known to be associated with that pathway in Ingenuity’s knowledge base.

### Table 3: Diagnostic or prognostic alterations of the extracellular vesicles

| Disorders                      | Type of vesicles | Alterations of the extracellular vesicular compartment                                                                 | References |
|--------------------------------|-----------------|--------------------------------------------------------------------------------------------------------------------------|------------|
| Autoimmune diseases            |                 |                                                                                                                          |            |
| Systemic lupus erythematosus   | pMVs and eMVs   | Elevated levels of pMVs and eMVs in blood plasma                                                                        | [52, 168]  |
| Anti-phospholipid syndrome     | pMVs and eMVs   | Elevated levels of pMVs and eMVs in blood plasma                                                                        | [169, 170] |
| Rheumatoid arthritis           | pMVs            | Elevated levels of pMVs in blood plasma, elevated levels of annexin V⁺ MVs and pMVs in synovial fluid                  | [38, 45, 52, 171, 172] |
| Systemic sclerosis             | pMVs, eMVs and IMVs | Elevated levels of pMVs, eMVs and IMVs in blood plasma                                                 | [173, 174] |
| Vasculitis                     | pMVs, eMVs and IMVs | Increased number of pMVs and IMVs in acute vasculitis and increased number of pMVs and eMVs in systemic vasculitis | [175–177]  |
| Type 1 diabetes mellitus       | pMVs, eMVs      | Increased number of eMVs and pMVs, increased total MV procoagulant activity                                        | [178]      |
| Multiple sclerosis             | pMVs, eMVs      | Elevated levels of pMVs and elevated levels of eMVs during exacerbation                                              | [179, 180] |
| Cardiovascular diseases        |                 |                                                                                                                          |            |
| Acute coronary syndrome        | pMVs, eMVs      | High levels of procoagulant eMVs and pMVs are present in the circulating blood of patients. High eMV level was associated with high-risk angiographic lesions in patients with acute coronary syndromes. Levels of eMVs may predict future cardiovascular events in patients at high risk for congestive heart failure | [150, 181–185] |
| Hypertension                   | eMVs, pMVs      | Levels of eMVs and pMVs correlate with blood pressure. Hypertensive patients with microalbuminuria have higher levels of eMVs compared to hypertensive patients without microalbuminuria | [151, 186] |
| Pulmonary hypertension         | pMVs, eMVs, IMVs | eMVs predict severity of pulmonary hypertension. Elevated levels of pMVs, eMVs and IMVs predict vascular inflammation and hypercoagulability | [152, 187, 188] |
| Congestive heart failure       | eMVs            | Apoptotic eMVs are elevated in patients with congestive heart failure. Furthermore, the levels of eMVs correlate with NYHA functional classes. Patients undergoing heart transplantation due to heart failure show altered phenotypes of eMVs | [189–191] |
| Deep vein thrombosis (DVT) and venous thromboembolism | eMVs, pMVs | pMVs and eMVs are elevated in patients with DVT. Total MV count may serve as novel markers for DVT. Circulating MVs and pMVs are elevated in patients with acute pulmonary embolism | [192–194] |
| Buerger’s disease              | pMVs            | pMVs are markers of exacerbation                                                                                       | [195]      |
| Atherosclerosis                | IMVs, pMVs      | CD11a positive IMVs predict subclinical atherosclerosis. pMVs are elevated in individuals with carotis atherosclerosis | [196, 197] |
Table 3 continued

| Disorders                                | Type of vesicles                     | Alterations of the extracellular vesicular compartment                                                                 | References |
|------------------------------------------|--------------------------------------|------------------------------------------------------------------------------------------------------------------------|------------|
| Cerebrovascular disorders                | pMVs, eMVs, IMVs and erythrocyte MVs | pMVs are elevated in transient ischaemic attacks, lacunar infarcts and multifacnt dementias. pMVs, eMVs, IMVs and erythrocyte MVs are elevated in patients with subarachnoid hemorrhage and are markers of vasospasm. Circulating eMV phenotypic profiles reflect distinct phenotypes of cerebrovascular disease. |
| Hematologic diseases                     |                                       |                                                                                                                          | [198–202] |
| Paroxysmal nocturnal haemoglobinuria     | eMVs                                  | Elevated levels of eMVs                                                                                                  | [203]      |
| Sickle cell disease                      | eMVs, pMVs, erythrocyte-MVs           | Elevated levels of total MVs, eMVs, pMVs and erythrocyte MVs, particularly in sickle cell crisis                         | [204]      |
| Immune thrombocytopenic purpura (ITP)    | pMVs                                  | pMVs are elevated in patients with acute ITP and decreased in chronic ITP                                                | [205]      |
| Thrombotic thrombocytopenic purpura      | eMVs                                  | Elevated levels of eMVs                                                                                                  | [206]      |
| Cancer                                   |                                       |                                                                                                                          |            |
| Lung adenocarcinoma                      | Exosomes                              | Elevated level of exosomes and miRNA in blood plasma of patients                                                        | [163]      |
| Glioblastoma                             | Tumor-derived exosomes                | Tumor-specific EGFRvIII was detected in serum exosomes from patients                                                    | [159]      |
| Ovarian cancer                           | Tumor-derived exosomes                | Exosomal miRNA from ovarian cancer patients exhibited distinct profiles compared to patients with benign disease       | [161]      |
| Prostate cancer                          | Tumor-derived exosomes in blood and urine | Detection of tumor-specific exosomes in blood could be used as a screening test. Urinary exosomes contain biomarkers for prostate cancer |           |
| Colorectal cancer                        | Tumor-derived exosomes                | Detection of tumor-specific exosomes in blood could be used as a screening test                                           | [165]      |
| Gastric cancer                           | pMVs                                  | pMVs are markedly increased in patients with stage IV disease and might be useful for identifying metastatic gastric patients. | [209]      |
| Melanoma                                 | Exosomes                              | Elevated CD63 and caveolin 1 on exosomes                                                                               | [210]      |
| Oral cancer                              | Exosomes                              | Sera of patients with active oral squamous cell carcinoma contain FasL⁺ exosomes                                        | [211]      |
| Cancer associated thrombosis             | TF⁺ MVs                               | TF⁺ MVs are elevated in patients with colorectal carcinoma, multiple myeloma, breast and pancreatic adenocarcinoma      | [212–214] |
| Other diseases                           |                                       |                                                                                                                          |            |
| Alzheimer’s disease                      | pMVs                                  | pMVs carry amyloid β on their surface                                                                                  | [215]      |
| Type 2 diabetes mellitus                 | pMVs, IMVs                            | Elevated levels of pMVs, especially in patients with clinically apparent atherosclerosis, elevated percentage of TF⁺ MVs. Patients with nephropathy have higher number of monocyte-MVs | [216–219] |
| Metabolic syndrome                       | MVs exposing TF, eMVs                 | The level of TF exposing MVs correlate with the components of metabolic syndrome. Pioglitazone has been shown to reduce eMV levels in patients with metabolic syndrome | [218, 220, 221] |
| End-stage renal disease                  | eMVs, PMVs                            | Total annexin V⁺ MVs, pMVs, eMVs are elevated in patients with end-stage renal disease and patients with hemodialysis. eMVs predict vascular dysfunction and represent a marker of endothelial dysfunction | [149, 222] |
| Obstructive sleep apnoe                  | pMVs, IMVs, eMVs                      | Total annexin V⁺ MVs, pMVs, IMVs and eMVs are elevated in patients                                                      | [223, 224] |
| Preeclampsia                             | eMVs, IMVs, pMVs, syncytiotrophoblast MVs | Elevated levels of eMVs, syncytiotrophoblast MVs pMVs and IMVs                                                       | [225–230] |
| Sepsis                                   | pMVs, eMVs, granulocyte MVs           | pMVs and eMVs are elevated in septic shock, but their elevation predicts favorable outcomes. Patients with meningococcal sepsis have elevated numbers of pMVs and granulocyte-derived MVs | [231–233] |
their highly thrombogenic potential. Most interestingly, not only blood plasma but also synovial fluid from RA patients contain high amounts of pMVs [45] and activate synovial fibroblasts via their IL-1 content. In accordance with these results, numerous publications have demonstrated the elevated levels of pMVs in the blood plasma of patients with various rheumatic diseases (Table 3) [62]. pMVs are characterized by a highly adhesive surface and may bind to endothelial cells, leukocytes and matrix molecules [155]. Furthermore, adherent MVs may also transfer GPIIb/IIIa to cells, including neutrophils [156], leading to cell activation. In summary, pMVs possibly represent novel players in the network of inflammation and autoimmunity.

**Tumor-derived membrane vesicles**

Tumors are characterized by secretion of various forms of membrane vesicles constitutively. These comprise exosomes [157], MVs [158] and apoptotic bodies (as a result of increased apoptosis in tumors). Released membrane vesicles contain tumor-specific antigens on their surface, e.g., Her2/Neu mesothelin, MelanA/Mart-1, CEA, HER-2, and EGFRvIII [19, 157, 159]. Furthermore, membrane vesicles from cancer cells contain RNA. Several reports indicate that miRNA-based identification of cancer leads to a reliable characterization of the origin and development of tumors [160, 161]. miRNAs, circulating in serum, plasma, saliva and breast milk, are resistant to degradation [160], and therefore it was suggested that miRNAs are protected by lipid or lipoprotein complexes [162]. As certain miRNAs are characteristic for tumors, their presence within tumor-derived exosomes and MVs may serve as novel biomarkers of cancer. The exosomal miRNA levels or patterns showed correlation with lung adenocarcinoma [163], glioblastoma [159] and ovarian cancer [161]. Recently, a EV-based diagnostic platform has been developed for the diagnosis of prostate cancer [164]. In this study, prostate tumor-specific vesicles in blood plasma have been identified using simultaneous detection of prostate-, tumor- and exosome-specific markers. Using this “biosignature” of prostate cancer EVs, diagnostic specificity and sensitivity of 83 and 90%, respectively, have been reached. This group also analyzed EVs from colorectal cancer patients in blood plasma, and diagnostic sensitivity and specificity of 85 and 85%, respectively, have been reported [165]. These data suggest that tumor-derived membrane vesicles could open a new era in cancer screening and diagnostics in the near future. The technology used in these studies, however, does not specifically purify exosomes or MVs [166], yet appears to be diagnostically useful. Thus, careful isolation of given EV subpopulations in a clinical monitoring assay may be less important than previously thought. Importantly, EVs (in particular exosomes) have been recently suggested to serve as novel therapeutic agents against cancer. Ongoing Phase I and Phase II trials and therapeutic strategies have been reviewed recently by Chaput and Thery [22]. Moreover, EVs may serve as novel promising vectors for future gene therapy [167].

**Conclusions**

Based on recent convergent data, we propose that there exists a previously poorly recognized, complex and dynamic extracellular vesicular compartment. Depending on the functional state of cells in the tissues, the composition of this compartment may change spatially and temporarily. The plasticity of the compartment may enable adaptation to altered conditions, and its evolutionarily conserved nature suggests efficient and vital biological functions of this compartment. At present, we are still far from fully appreciating the biological significance of EVs. Collectively, constituents of this compartment represent large membrane surface areas, and their amount in the extracellular space might be best appraised on the basis of their membrane lipid content or lipid/protein ratio. Biological systems in which vesicles are released into large volumes of extracellular fluid, such as blood plasma or tissue culture medium (where they are not taken up immediately by surrounding cells), provide some clues about the magnitude of vesicle formation and the efficacy of clearance in tissues. Their molecular composition shows striking similarities including the shared presence of cytoskeletal proteins, membrane lipid composition and externalized phosphatidylserine, just to mention a few examples. Size distribution is another area of overlap. Even though not characterized in detail, it may be hypothesized that uptake and removal by cells occurs by similar or identical molecular mechanisms.

Similarly to cytokines that constitute a network of communication, EVs may also exert their functions in a network, which is acting in a specific context with many other players. The long list of medical implications, affecting different organs, justifies systems biology approaches to study EVs.

Understanding of this compartment challenges the current paradigms concerning the mechanisms of intercellular communication and immune regulation. It may also open new perspectives in translational medicine both in diagnostics and therapy.

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