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EVs IN HUMAN REPRODUCTION.

EXTRACELLULAR VESICLES IN HUMAN REPRODUCTION IN HEALTH AND DISEASE.

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Extensive evidence suggests that the release of membrane enclosed compartments, more commonly known as extracellular vesicles (EVs), is a potent newly identified mechanism of cell-to-cell communication both in normal physiology and in pathological conditions. This article specifically reviews evidence about the formation and release of different EVs, their definitive markers and cargo content in reproductive physiological processes, and their capacity to convey information between cells through the transfer of functional protein and genetic information to alter phenotype and function of recipient cells associated with reproductive biology. In the male reproductive tract, epididymosomes and prostasomes participate in regulating sperm motility activation, capacitation and acrosome reaction. In the female reproductive tract, follicular fluid, oviduct/tube and uterine cavity EVs are considered as vehicles to carry information during oocyte maturation, fertilization and embryo-maternal cross talk. EVs via their cargo might be also involved in the triggering, maintenance and progression of reproductive and obstetric related pathologies such as endometriosis, polycystic ovarian syndrome, pre-eclampsia, gestational diabetes, and erectile disfunction. We provide here, the current knowledge on the present and future use of EVs not only as biomarkers, but also as therapeutic targeting agents, mainly as vectors for drug/compounds delivery into target cells/tissues.

Essential Points of the review "Extracellular Vesicles in Human Reproduction in Health and Disease".

- Extracellular vesicles are a newly identified mechanism of cell-to-cell communication, recently discovered as a communication between the mother and the embryo.
- Extracellular vesicles play and important role in normal physiology and in pathological conditions in human reproduction.
- Prostasomes participate in regulating sperm motility activation.
- Different extracellular vesicles and their cargo are implicated in promoting oocyte development and maturation.
- Exosomes and their cargo in miRNAs play and important role in embryo implantation.

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Extracellular vesicles are involved in the triggering, maintenance and progression of reproductive and obstetric pathologies.

The participation of this EVs in the human reproduction health has made them appealing players as biomarkers and to carry therapeutic agents.

Part I. Introduction

Intercellular communication is an essential process both for multicellular organisms and for the relationship of unicellular organisms with the environment and hosts (1). Classically, communication has been identified as indirect as endocrine, paracrine and autocrine or direct via cell-to-cell contact, secretion, release, and uptake of chemical moieties such as hormones, growth factors or neurotransmitters (2,3). According to The Human Protein Atlas, nearly 39% of the human protein-coding genes are annotated to give rise to membrane (28%) and secreted (15%) forms of signalling protein variants, some producing both isoforms and post-translational modifications that can alter function. These molecules which constitute potential therapeutic targets include cytokines, growth factors and coagulation factors, among others, playing physiological and pathological roles in processes such as immune defence, blood coagulation, or matrix remodelling. Of note, more than 500 of these proteins are currently known as pharmacological targets with already approved druggable targets available commercially.

A new mechanism has recently been in the spotlight for cellular communication: the release of membrane-enclosed compartments, most commonly regarded as extracellular vesicles (EVs). EVs can act to convey molecules from one cell or tissue to another. Importantly, their contents (cargo) are protected from extracellular degradation or modification. They exert their biological roles by either direct interaction with cell surface receptors or by transmission of their contents by endocytosis, phagocytosis or fusion with the membrane of the target cells. Recipient cell specificity appears to be driven by specific receptors between the target cells and EVs (4-6). EVs have been described in different body fluids including semen (7), saliva (8), plasma (9), breast milk (10), urine (11) and amniotic fluid (12), among others (4).

EVs can be classified in different populations based on their biogenetic pathway, composition and physical characteristics, such as size or density, giving rise to three major categories: apoptotic bodies, microvesicles and exosomes (5,13,14).

EV content is complex as a continually progressing field with new cargo's being identified continually. Regrettably, due to technical limitations in methods of isolation and differentiation of the different populations of EVs, mixed, heterogeneous populations are often used making interpretation of their content and functionality difficult (15-17). This constitutes a salient notion in the field at present, that populations of EV subtypes must be considered when reviewing published literature. With homogeneous sample preparation and key developments in characterisation of EVs, we now hold important insights into defining these select communicators in far greater depth. With the implementation of high resolution and sensitive instrumentation for characterisation such as mass spectrometry and next generation deep sequencing, it has been possible to develop databases gathering information about protein, lipid and RNA content of EVs from different sources: ExoCarta (online source: www.exocarte.org) (18), EVpedia (online source: www.evpedia.info) (19) and Vesiclepedia (online source: www.microvesicles.org) (20).

In the last years, EVs have been shown to participate in different processes committed to the maintenance of the normal physiology of the organism such as tissue repair, maintenance of the stem cell status of progenitor cells, platelet and immune function, nervous system homeostasis.

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EVs potential role in the pathogenesis of different diseases has also been studied, being cancer, autoimmunity, neurodegeneration, HIV-1 infection and prion diseases the widest studied areas (1,6,21). In all these cases, EVs are unique as they became small indicators of organism's homeostasis that can stably travel over the body fluids. The fact that their content reflects cell of origin and pathophysiological states highlights their usefulness as biomarkers. Importantly EVs are attributed with potential to cross tissue barriers, such as blood brain barrier, possibly by transcytosis. This fact makes them appealing targets for therapeutics development (22). EVs can be released in response to cell activation, pH changes, hypoxia, irradiation, injury, exposure to complement proteins, and cellular stress (23-25). Interestingly, EVs are also secreted by plant cells (26,27), and pathogens (28,29), including bacteria, mycobacteria, archaea, and fungi (30,31), suggesting an important evolutionary conserved mechanism of intercellular signalling.

In the field of reproductive biology there is a growing interest in understanding the role of EVs within the male and female reproductive tracts, as they may constitute a new mechanism of communication between the reproductive tract and the immature germ cells, or between the mother and the developing embryo. Such developments offer great potential implications in the establishment of a successful pregnancy or implications with understanding associated pathological conditions (32). In the present review, we will address current knowledge on the existence and functionality of EVs as cell-to-cell messengers in normal human reproductive physiology, as well as their contribution in the triggering, maintenance and/or progression of pathological conditions in the functionality of the reproductive tract. Further, we discuss their usefulness as biomarkers of altered reproductive conditions such as pre-eclampsia, spontaneous premature birth, or polycystic ovaries syndrome. We will end up gathering the current knowledge on the present and future of the use of EVs as therapeutic agents, mainly as vectors for drug/compounds delivery into target cells/tissues.

Part II. Types, Isolation and Characterization of EVs and Cargo

I. EV heterogeneity

EVs can be classified into select subtypes according to different criteria, i.e.: cellular origin, biophysical (density and size) and biochemical (biological markers) characteristics, biological function, biogenetic pathway. According to their biogenetic mechanism of formation and release, three main classes of EVs are defined: apoptotic bodies, microvesicles and exosomes, are now known (Figure 1).

i. Apoptotic bodies

Apoptotic bodies (ABs) are EVs produced by plasma membrane blebbing in cells undergoing programmed cell death. This term was coined by Kerr and colleagues (33) who defined them as 'small, roughly spherical or ovoid cytoplasmatic fragments, some of which contain pyknotic remnants of nuclei'. Indeed, one of the events that characterize apoptotic bodies is the fragmentation and packaging of cellular organelles such as the nucleus, endoplasmic reticulum or Golgi apparatus into these vesicles (34,35).

ABs have widely been described as 1 - 5 μm in diameter, thus overlapping with the size range of platelets (36,37) although some groups extend this range to 50 nm (16,38,39). Their buoyant density in a sucrose gradient is in the range of 1.16 to 1.28 g/mL (40,41).

This vesicle population is characterized by cytoskeletal and membrane alterations, including the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the lipid bilayer (42). In this way, PS serves as an 'eat me' signal for phagocytes to target and clear apoptotic debris (43,44). Moreover, PS can naturally be recognized by annexin V, which is a useful marker

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of apoptotic bodies (45). Nevertheless, care should be taken when using annexin V for this purpose as PS flipping can also be triggered by other stimuli such as mechanical disaggregation of tissues, enzymatic treatments for detachment of cells, electroporation, chemical transfections or retroviral infections, and PS exposure has also been described in healthy cells (46). PS flipping also induces microvesicle (MVs) formation, so these can also be recognized by annexin V detection (47,48). Another specific feature of ABs is the oxidation of surface molecules, creating sites for recognition of specific molecules such as thrombospondin (49) or C3b complement protein (50), which are also useful as markers of ABs.

Included in newly identified potential molecular markers of ABs, VDAC1 is a protein that forms ionic channels in the mitochondrial membrane and has a role in the triggering of apoptosis. It proves to be a useful AB marker as its biological function and subcellular localization are characteristic of this vesicular fraction (39). Calreticulin is an endoplasmic reticulum (ER) protein that could also work as an AB marker due to its subcellular localization (15), although it has also been observed in the smaller sized MVs fraction (39). It is possible that, during the apoptosis process, the ER membrane is fragmented and forms vesicles smaller in size than ABs, which would contain calreticulin and would sediment at higher centrifugal forces (51,52). Indeed, proteomic studies have related calreticulin with vesicular fractions across the full size range of MVs (53) and ABs (54).

Different functions have been attributed to ABs although most are also features of other EVs. DNA can be horizontally transmitted between somatic cells, with possible integration of this DNA within the receptor cell where can be functional (55). These vesicles are also a vehicle for the horizontal transfer of oncogenes, which are internalized by target cells and consequently increase their tumorigenic potential in vivo

(56,57). ABs have also been related to the immune response where they are associated with an under-activation of the immune system (58), and with antigen presentation with special regard to the self-tolerance (59-61).

ii. Microvesicles

Microvesicles (MVs) were reported for the first time by the group of Chargaff (62) as being sedimented at high-speed centrifugation (31,000 x g) (not specifically at lower speeds such as 5,000 x g). MVs are a population of EVs that are formed and released directly from the cell plasma membrane by outward budding and fission from viable cells (63,64). Plasma membrane blebbing is triggered by different mechanisms that are accompanied by the remodelling of the membrane proteins and lipid redistribution, which modulate membrane rigidity and curvature (65). Such changes within the periphery of the plasma membrane have been associated with cargo sorting in MVs (66).

The size range of MVs has been classically established between 100 - 1000 nm (67), thus overlapping with that of bacteria (13). Some groups extend this range up to 1500 nm (68) or even 2000 nm (69-71). The buoyant density of MVs is not as clear as that of other vesicle populations: around 1.16 g/mL in sucrose gradient (71), or 1.04-1.07 g/mL (72). The flotation density in iodixanol gradient is between 1.18-1.19 g/mL (73).

As a proposed marker for MVs population, ARF6 is a GTP-binding protein that is implicated in the regulation of cargo sorting and promotion of the budding and release of MVs through the activation of phospholipase D metabolic pathway (65,74). Additionally, data coming from our current knowledge on proteomic studies suggest numerous proteins (e.g: KIF23, RACGAP1, exportin-2, chromosome segregation 1-like protein) as unique/enriched for MVs and potentially discriminatory markers (75). Nevertheless, care should be taken with these results, as different

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EV cell sources and techniques to selectively enrich may lead to differences within EVs populations.

Among the functions described for MVs, are pivotal roles in cancer cell invasiveness (76,77), transformation potential (78), progression (63,79,80) and drug resistance (81). MVs have also been implicated in autoimmune diseases (82-84), immune system modulation and coagulation (67,85,86), embryo-maternal cross-talk (87), and embryo self-regulation (88).

iii. Exosomes

The first description of exosomes in 1981, described them as a second population of vesicles that appeared in the preparations of MVs and the term exosome was coined (89). Two years later, their biogenetic pathway was formally described by transmission electron microscopy (TEM), trying to follow the pathway of uptake and trafficking of transferrin molecules within reticulocytes in an anemic mice model (90). Exosomes (EXOs) constitute a population of nano-sized EVs that arise and are trafficked through the endosomal pathway. Endosomal sorting complexes required for transport (ESCRTs) are important for multivesicular body (MVB, which include exosomes) biogenesis. During MVBs inward budding of the limiting membrane of late endosomes facilitates formation of intraluminal vesicles (ILVs) that remain enclosed inside the greater membrane compartment of MVBs. ESCRT-independent mechanisms including neutral sphingomyelinase (N-SMase)/ ceramide formation and ARF6/PLD2 have been reported may also occur (73,91). The formed MVBs can then be targeted to plasma membrane to release ILVs, now known as EXOs or otherwise fuse with lysosomes to degrade their content (92). Members of the Rab GTPase family have been shown to modulate EXOS secretion and are thought to act on different MVBs along ESCRT-dependent and -independent endocytic pathways. It is likely that ESCRT-dependent and ESCRT-independent MVB/exosome biogenesis machineries vary from tissue to tissue (or even cell type) depending on specific metabolic needs. There are several molecular mechanisms, both canonical and alternative, implicated in the formation, release and extracellular fate of EXOs (review: (5,75).

Most studies place EXOs in a size range of 30 to 150 nm (5,93) or even 200 nm (94), thus establishing an overlap with viruses in terms of size (14). The buoyant density of EXOs in sucrose gradients has been set in a wide range of 1.10 to 1.21 g/mL (38,95), and 1.10 to 1.12 g/mL in iodixanol gradients (96).

The classically-associated markers of EXOS are molecules mainly implicated in the biogenesis of this population, which are incorporated during this process: tetraspanins (CD63, CD9, CD81), Alix, TSG101, flotillin-1, among others (5,95). Nonetheless, with the emerging interest in studying different EV populations as isolated entities, many of these classical markers have been identified as widespread between populations, although with different relative abundances. This is the case for at least CD9, CD63, HSC70 and flotillin-1. Other molecules such as TSG101 and syntenin-1 have been ratified as markers of only this vesicle population (39). Phosphatidylserine, while being described as a broad marker of EVs, has also been reported as exposed on the surface of exosomes produced by different cell types (92,97). Accumulating evidence from *in vitro* studies using cell grown in culture and ex vivo body fluids indicates the existence of more than one exosome subtype (98-105). For example, EXOs contains subpopulations, including the study of EXOS derived from apical (EpCAM-Exos) or basolateral (A33-Exos) surfaces of highly polarized cancer cells, indicated the presence of two distinct subtypes with distinct protein (98) and RNA cargo (99,106). The biological significance of these findings awaits further investigation.

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Due to the high expectations and efforts dedicated to the study of the role of EXOs in different biological processes, both in physiological or pathological conditions, the field of EXO biology has experienced an exponential growth in recent years, with a wide range of functions identified (1,107). EXOs are implicated in cancer physiology, participating in tumour progression and maintenance, resistance, immune modulation and angiogenesis (108). Their function in immune regulation has also been well studied in antigen presentation modulation, immune activation and suppression (109,110). Importantly knowledge of the seminal role of EXOs in reproductive biology is expanding rapidly. Such studies and the molecular markers and mechanisms identified have the potential for use as markers to discriminate between EV subtypes, as well as various applications of EXOs in clinical diagnosis.

II. Methods of isolation and purification of EVs

The main experimental problem when studying EVs is to achieve a homogeneous separation with appropriate yield of the EV population of interest. Different methods of isolation and purification have been developed, although to a varying extent, all carry the bias of providing completely homogenous EV populations of any one vesicle type (summarized in Table 1). In the field, there is a pressing need to define EV surface-exposed proteins for the purpose of generating mAbs that would allow – discrimination of EV class/subtype (i.e., stereotypical markers). The majority of rapid/one-step approaches for isolating EVs do not take consideration of the fact they are dealing with a possible mixture of vesicle classes/subtypes and co-isolated contaminants such as high-M_r protein oligomer and protein-RNA complexes (e.g., HDL/ LDL/AGO2) complexes.

i. Serial differential centrifugation

Differential centrifugation is the most common and well-known method for the isolation of EVs. Although each group adapts the times and centrifugal speeds depending on their samples, the basic protocol is the following. (i). centrifugation at low speed for the elimination of cells (300 x g, 10 minutes), (ii) centrifugation at up to 2,000 x g for 10 minutes to pellet membrane debris and ABs. (iii) centrifugation at 10,000-20,000 x g for 30 minutes to pellet MVs. (iv) a crude EXO preparation is pelleted by ultracentrifugation at 100,000-200,000 x g for 70 minutes. After steps (i)-(iii) of centrifugation, supernatants are transferred to new tubes for the isolation of the subsequent EV type. Pellets (ii-iv) containing different cell populations, are washed by resuspension in PBS and re-centrifugation under the same conditions. The washing step removes some impurities, but also reduces EV yield.

Apart from vesicle size, centrifugation alone cannot achieve the separation of pure populations for various reasons: sedimentation of other particles in the supernatant depending on density; distance of the particles from the bottom of the tube; and vesicle/particle aggregation (111).

To improve EV population purity, a gradient step can be added to the centrifugation protocol. This system aims to avoid as far as possible the contamination of EV pellets with large protein/protein-RNA aggregates and proteins non-specifically bound to EVs (4). The essentials of the technique are resuspension of the pellet from the previous serial differential centrifugation in a suitable buffer (i.e., PBS), then loading on either the top or base of a prepared sucrose cushion (112,113) or a sucrose gradient (114,115). Following ultracentrifugation, vesicles are recovered either from the bottom of the tube (for cushions), or from a specific fraction of the gradient, depending on their buoyant density. Moreover, substitution of sucrose by a non-ionic density gradient medium, called iodixanol (116) offers many advantages: better separation of viral particles from EVs; low toxicity towards biological material; is clinically applicable; and it

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forms isosmotic solutions compatible with the size and shape of EVs in a wide range of densities (117-119).

ii. Size exclusion methods: filtration and chromatography

Filtration for isolation of EVs is often used in combination with ultracentrifugation protocols to improve separation efficiency based on size. Filtration steps using 0.8, 0.2 or even 0.1 µm filters can be inserted between the centrifugation steps depending on the size of the desired population (112,120,121). Alternatively, ultrafiltration utilizes filtration units of different molecular weight cut-off membranes which are centrifuged at moderate centrifugal forces. They allow concentration of vesicles in the interface of the filters, from which they can be recovered by washing (122-125). All these methods face several drawbacks. The pressure of the supernatant can cause the EVs to deform or break into smaller vesicles while the filter membrane may decrease the yield. Gravity filtration has been proposed to cope with the problems associated with elevated pressures (120), but this can be time consuming and filters can become saturated.

Another option for EVs isolation in conjunction with ultracentrifugation, is based on size exclusion chromatography. In brief, the medium containing the vesicles is loaded into the chromatography device, generally a gel size exclusion column, equilibrated into the column and eluted with PBS (126-128). The technique is usually coupled with previous low-speed centrifugation to remove larger debris and subsequent ultracentrifugation to wash and concentrate the vesicles from the different chromatography fractions (129,130). Its advantages are enhanced separation of EVs from proteins and high density lipoproteins (HDL), avoidance of protein and vesicle aggregate formation, reduced sensitivity to the viscosity of the vesicle media, compatibility with the biological properties and functionalities of the isolated vesicles and preservation of the vesicular structure and conformation (126). Moreover, it offers shorter isolation times and relatively low cost. As a disadvantage, this technique offers reduced EVs recovery yields in comparison to others such as ultracentrifugation or polymeric precipitation, although it is susceptible for scale-up (131,132). Nevertheless, some studies indicate that a combination of size-exclusion chromatography and ultrafiltration may produce a yield surpassing that of classical ultracentrifugation (133,134).

iii. Other approaches

Immunoaffinity uses microbeads coated with specific antibodies for the recognition of specific surface markers of EV populations. In brief, beads are incubated with the sample containing EVs, then beads linked to their epitopes on the EV surface are recovered by magnetism or low-speed centrifugation, depending on the nature of the beads (135,136). The technique can follow centrifugation and/or filtration to clear large cellular products (96,99,137). This method differentiates EVs populations based on surface markers regardless of their size. Nevertheless, care should be taken as population specific markers are not necessarily available, and the working surface of the beads is limiting, so the EVs may not have access if they are large or present at high concentrations (116).

Aiming for a quicker and simpler method to isolate EVs, a polymeric precipitation system (ExoQuick$^{TM)}$ was commercially developed. The experimental procedure is as simple as incubating the kit reagents with the exosome-containing media and recovering the resulting polymeric complex by low-speed centrifugation. A study with human ascites samples showed that ExoQuick could provide high concentrations and purity of exosomal RNA and that the high exosomal protein concentrations from the same samples compared to other isolation methods such as ultracentrifugation, immunoaffinity isolation and chromatography (138). Even though ultracentrifugation-based protocols are preferable for exosomal protein recovery and purity,

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ExoQuick obtains better results in terms of exosomal mRNA and miRNA yield and quality (139). The method has a series of limitations. Impurities such as lipoproteins are possibly co-isolated along with EVs and the method is unable to provide isolation of different EVs subpopulations. It works ideally with small vesicles in the size range of 60 to 180 nm (111).

A new technology based on microfluidic devices has recently been developed for the isolation of EVs. It allows the reduction of sample volumes, processing times and costs, while maintaining high sensitivity. The chip technology can be based on different principles. The first developed systems relied on the recognition of EVs by specific antibodies on the surface of the device (140). The surface of the flow system was coated with anti-CD63 antibodies. When EV-containing media was pumped through the system, EXOs were restrained. The system allows SEM imaging and lysis of EVs for RNA isolation directly on the chip. However, it does not provide sufficient material for protein or functional analyses. Subsequently, the system was expanded with lipophilic staining of EXOs to allow simultaneous quantitation (141). A third microfluidic scheme used physical properties as the principle for EV isolation, separating microparticles based on their size within the micrometric size range (142). Clearly this method is not applicable to EV population analysis. This technology has also been combined with porous polymers, allowing purification of vesicles in the nanometric size range: the pore size can be modulated so that only EVs under a certain size can be filtered (143). A recent publication introduces the concept of using a combination of acoustics and microfluidics for a high-purity degree exosomes isolation. The platform is composed of two sequential modules that remove larger components and other EVs groups (microvesicles and apoptotic bodies), respectively, allowing the direct use of undiluted body fluid samples (tested in whole blood) or conditioned media from cell cultures in a single step. The system is based in the combination of microfluidics channels conformation and adjusted acoustic pressure, that make it possible to set the cutoff particle diameter (144).

The demands of clinical applications involving diagnostics and therapeutics such as low cost, reliability, and speed can eventually be met with modifications to existing technologies for improved scalability. Isolation of EVs from blood and urine is a challenge due to the presence of abundant and complex proteins and lipoproteins networks, which undoubtedly will attenuate intrinsic EV protein/RNA signatures. Distinct clinically-relevant strategies to isolate EVs are currently being investigated (75,145,146).

III. Methods for characterization of EVs

Characterization of EVs is fundamental to enable differentiation among the different subpopulations within the same biological sample, between vesicles of distinct cellular origin or even of the same origin in pathological vs physiologically normal conditions (summarized in Table 2).

i. Microscopy: morphology and size analysis

Electron microscopy (EM) techniques are the only method available to provide the appearance of EVs related to their size. Different variants offer different data to the user. Transmission EM (TEM) was initially used by Raposo and colleagues, who described EXOs as cup-shaped vesicles (147). Although different protocols can be used for TEM visualization, two general schemes offer different views. EVs can be resuspended in fixative media and laid into grids for staining and visualization. Alternatively, EV pellets from centrifugation/ultracentrifugation steps can be fixed, resin embedded, cut onto ultrathin slides, which are then stained and laid in grids. The first method is simpler and less time consuming, and offers a view of the exterior of the EVs. The second method is more informative, shows the interior of the EVs and allows immunogold

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staining of specific markers that are seen as electron-dense spots (148,149). Cryo-EM allows direct visualization of frozen EVs without previous fixation and contrast steps. The structures are seen as close as possible to their native states (not dehydrated or fixed) and demonstrate variable EV morphology (150). Indeed, such analysis showed that the classical cup shape attributed to exosomes was an artefact of fixation (2). Finally, scanning-EM (SEM) offers three-dimensional imaging of the EVs for further morphological description (151,152).

Atomic force microscopy (AFM) is an alternative for the analysis of size distribution and quantity of EVs within a sample and is based on the scanning of the sample by a mechanical probe, which physically touches the sample providing topographical information. AFM allows imaging at the sub-nanometric level. It can be adjusted to air (dry samples) or liquid modes (aqueous samples), and differences in size or number measurements are negligible between them. The possibility of measuring samples in aqueous media is advantageous as it permits the maintenance of EVs physiological properties and structure (153-155). AFM has been efficiently combined with microfluidic isolation devices to providing consecutive isolation and characterization of EVs. Mica-microfluidic chips are also of interest as they provide a non-conductive flat surface for in situ AFM analysis (155,156).

ii. Size distribution analysis techniques

Nanoparticle tracking analysis (NTA), a light-scattering technique is now widely used for the assessment of EV size distributions and concentration in the range of 50 to 1000 nm. The principle of the technique is based in the inherent Brownian motion of the particles in a solution: EVs in suspension are irradiated by a laser beam thus emitting dispersed light. This scattered light is captured by a microscope and NTA software tracks the movement of each particle in a time lapse. Silica nanospheres have been proposed for standardization, as their refraction index (RI = 1.46) is similar to that observed for most of EVs (RI around 1.39) (157).

Dynamic Light Scattering (DLS) is also used for the assessment of EV size distribution. Although the principle for size determination is also Brownian movement of particles in suspension, the way to attain this data varies from NTA technology. It has limitations when measuring polydisperse samples and those containing big EVs, since the bigger particles scatter more light, masking the smaller ones (158). It is also possible to calculate vesicle concentrations in the samples, by direct extrapolation from the distribution representations using mathematical criteria (158).

Tuneable Resistive Pulse Sensing (TRPS or qNano by its IZON commercial name) is a novel, and cheaper technique for the analysis of particle size distributions. The system is composed by a thermo-plastic polyurethane membrane containing nanopores which are selected by size requirements. Currently, the system can measure individual particles in the size range of 30 nm to 10 μm and in the concentration range of 10^5 to 10^{12} particles/mL. Since the system analyses the particles individually, multimodal populations can be studied. On the other hand, a configuration of only one pore type restricts measures to a narrow size range, which is particularly useful for analysis of a specific vesicle population. Combining pores of distinct size and geometry allows widening of this range and analysis of a greater volume of sample (159-161).

Flow cytometry has also been applied to the analysis of size distribution, concentration and qualitative characteristics of the EVs within a sample. Light scatter flow cytometry allows the analysis of vesicles with a lower size limit of usually between 300 and 500 nm (162,163), but small EVs including exosomes cannot be studied by this method. However, innovative new flow cytometry technology and the use of fluorescent labelling of EVs, has reduced the lower limit of

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detection to ~100 nm, and it is possible to discriminate between vesicles 100 to 200 nm in size (164,165). Finally, EVs can be coupled via antibodies to their surface markers, to latex beads of greater size. In this way even nano EVs can be analysed, but no quantification or differentiation between vesicle populations is possible (166,167).

iii. Molecular marker characterization

The most effective and well accepted approach to measure EV purity is the concentration of a specific EV surface-marker antigen. Approaches including western immunoblotting, ELISA using surface markers can be used with adaption for the quantitation of EVs within a sample (168,169), and ExoScreen have been employed (170).

Another approach, for the characterization and quantitation of EVs, is based on micro nuclear magnetic resonance spectrometry (μNMR) (171) EV labelling with specific EV surface molecular markers antibodies coupled to magnetic nanoparticles enables specific detection by microfluidic μNMR. The technique offers a detection sensitivity level that greatly surpasses ELISA or flow cytometry.

Finally, transmission surface plasmon resonance can provide an alternative method for the molecular characterization and quantitation of EXOs in a system called nano-plasmonic exosome assay (nPLEX). This consists of a gold film patterned with a series of nanoholes' arrays, each of which is coated with specific monoclonal antibodies for the recognition of EXO-specific proteins. Compared to previous systems, nPLEX is label-free, easy to miniaturize and scalable for higher throughput detection and improves detection sensitivity to a magnitude order lower than μNMR (172,173).

Over the past decade, recent studies and groups have employed developments in proteomic profiling to characterise specific markers for highly purified EV subtypes (EXOs and MVs). Since the emergence of the interest in studying different vesicles populations as isolated entities, many of the exosomes classical markers of EXOs have been uncovered as widespread between populations, although with different relative abundances. This is the case of CD9, CD63, HSC70, EpCAM, flotillin-1, among others (98,100). On the other hand, some new molecular markers have been identified and ratified as markers of EXOs: TSG101, syntenin-1, Alix/PDCD6IP (39,100). Numerous proteins found exclusively/enriched in MVs (e.g., KIF23, RACGAP1, chromosome segregation 1-like protein, exportin-2 [CSE1L/CAS]), warrant further study as to their potential use as discriminatory markers for MVs. Further, care should be taken care when analysing phosphatidylserine as a marker of ABs as it has also been reported to be exposed in the surface of EXOs produced by different cell types (92,97) and also MVs (47,48). An in-depth review detailing proteomic insights into EV biology and defining markers for EV subtypes and understanding their trafficking and function is provided (174).

IV. EV cargo

Membrane receptors and cargo content are the most important feature of EVs, since they define their cellular selectively, target, uptake and functionality, respectively. EV cargo includes proteins, bioactive lipids, various RNAs (including fusion gene, and splice-variant transcripts), and DNAs (described below), and other cell regulatory molecules (1,4). To date, most studies have focussed on their genetic (particularly RNA and miRNA) and protein content as sensitive methods exist for their comprehensive analysis and detection.

Protein contents in EVs has been widely studied since the application of mass spectrometry-based techniques (175). EVs have been shown as to be enriched in proteins from cytoskeleton, cytosol, plasma membrane, heat-shock proteins and proteins involved in EVs biogenesis, while proteins from cellular organelles are less abundant (1). From initial studies, EVs were shown to

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carry commonly widespread EVs proteins and a specific subset of proteins, depending on the cell, the type of vesicle and the method of isolation (5). Moreover, it has been observed that EVs number, protein content and protein concentration varies depending on the stimuli for vesiculation, even in the same subpopulation of vesicles (176).

Cytokines have also been described to be carried by EVs (1). IL-1β is among the examples of theses soluble mediators that are secreted in EVs. Indeed, secretion pathways of EVs may constitute an alternative to exocytosis for proteins that lack leader signal peptide (177). Another interesting example of cytokine cargo is IL-1α, which has been reported to be selectively carried by apoptotic bodies but no by smaller-in-size vesicles (<1μm) in endothelial cells (178), thus confirming the cargo sorting into different populations of EVs. Further examples of cytokines released into EVs are IL-18 (179), IL-32 (180), TNF-α (181) and IL-6 (182), among many others. During pregnancy, EVs cytokine cargo has been shown to be modified towards an increase in comparison to non-pregnancy, maybe contributing to the modulation of maternal immune response against the foetus. Levels of TGF-β1 and IL-10 were increased in EVs from pregnant women, along with an enhanced ability to induce caspase-3 activity in cytotoxic NK cells, thus promoting and immunosuppressive phenotype through the induction of apoptosis in these cells (183).

Lipid content of EVs has been much less studied. However, some groups have shown that EVs are enriched in certain types of lipids in comparison with their parent cells, demonstrating the sorting of these molecules. Specifically, vesicles are enriched in sphingomyelin, cholesterol, phosphatidylserine (184,185), ceramide and its derivate and, in general, saturated fatty acids (186). It is also remarkable that the ratio lipids/proteins are higher in vesicles than in parent cells. In contrast, phospatidylinositols, phospatidyglycerols, phosphatidylcholine and phosphatidylethanolamines are more present in parent cells than in vesicles (184). Recently using mass spectrometry quantitative lipidomics combinations of three lipid species were shown to distinguish cancer patients from healthy controls (187).

RNAs in EVs were first described by Valadi's group (188) in mast cells. They found that exosomes released by these cells contained mRNAs and miRNAs and were able to transfer their content to other cells, where mRNA was functional and could be translated into protein. More recent studies using high throughput sequencing techniques have shown that exosomes contain various classes of small non-coding-RNAs in addition to mRNA, i.e: miRNA, small interference RNA (siRNA), small nucleolar RNA (snoRNA), Y-RNA, vault RNA, rRNA, tRNA, long non-coding RNA (lncRNA), piwi-interacting RNA (piRNA) (189-191). Ng group (167) showed that endometrial epithelial cells cultured *in vitro* produced EVs containing a different miRNA profile from that of parent cells, thus suggesting a sorting mechanism of this miRNAs into exosomes. This could constitute a mechanism for communication between the mother and the embryo with potential implications in embryo implantation. Indeed, bioinformatic studies on the EVs miRNAs showed that some of the genes targeted by the miRNAs are involved in implantation. More recently, our investigation group deepened in the knowledge of maternal-embryo cross-talk and demonstrated that exosomes containing miR-30d were actively transferred from endometrial epithelial cells to trophoblastic cells, were the miRNA was subsequently internalized (151).

A major problem concerning RNA analysis from EVs is the variability of the results depending on the methodology used for the isolation and obtaining of the data. One of the major factors affecting this variability is the possibility that the RNA present in the medium, for example from lysed cell, could stick to the external EVs wall, thus being isolated along with internal RNA. In this sense, RNaseA treatment previous to EVs RNA isolation should be

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conducted (192). Even with this procedure, it has been stated that extravesicular RNAs associated with proteins, such as miRNAs in complex with argonaute proteins, can circumvent RNaseA degradation, thus leading to bias in result interpretation. This protective role of protein complexes has been reported either in extravesicular medium (193,194) and inside EVs (195). In order to overcome complex protection, treatment with proteinase K has been proposed for dissociation of RNA-protein complexes (196). Nevertheless, negative impact in EVs yield should be investigated as proteases may provoke vesicle lysis.

Less has been reported regarding DNA content in EVs. Some studies have currently reported the presence of double stranded DNA (dsDNA) in EVs (197,198), even distinguishing a different pattern of content among EVs subpopulations (199). A previous study conducted in a similar way in tumour cells, using DNase to cleave extravesicular DNA, showed that EVs DNA was more abundant in microvesicles from tumour cells than from normal cells and that this DNA was mainly single stranded (200). It has been shown that mitochondrial DNA (mtDNA) can also be transported between cells inside exosomes, possibly constituting a pathway to transmit altered mtDNA and associated pathologies (201). This may serve as an evidence of a trans-acting function of DNA, being able to have functional effects on the recipient cells.

Of note, both the amount and content of EV genetic cargo can be hormonally-regulated in exosomes from target cells: this is of particular relevance to reproductive tissues and is further discussed below.

V. EVs Mechanism of recognition and uptake

i. Mechanisms of EV uptake

For EVs to act in cell-cell signalling, they must recognise their specific cellular target, bind to that cell and undergo internalization (Figure 2).

Target cell recognition.

EVs may interact with recipient cells by direct signalling through ligand/receptor molecules on their respective surfaces or by direct fusion of EV and recipient cell plasma membranes (202), through lipid raft-, clathrin- and calveolae-dependent endocytosis, macropinocytosis and phagocytosis (203-208).

Cell surface and integral membrane/ adhesion proteins on distinct EVs are important in mediating associated cell recognition and adhesion. These include integrin pairs: for example, distinct EXOs integrin repertoires - specifically integrins α6β4 and α6β1 - were identified as associated with lung metastasis, whilst EXOs integrin αvβ5 associated with liver metastasis (209). The integrin profile of each EXOs subtype permits selective cellular targeting

Differences in EXOs tetraspanin complexes also appear to influence target cell interaction *in vitro* and *in vivo*, possibly by modulating the functions of associated integrin adhesion molecules (210). Exosome capture by dendritic cells was reduced by 5–30% following co-incubation with blocking antibodies specific for various integrins, adhesion molecules or tetraspanins (203). Other membrane proteins reported as important in targeting select EVs to recipient cells, include intercellular adhesion molecule 1 (ICAM-1) and milk fat globule-epidermal growth factor VIII protein (MFGE-8) (211,212). Further, the delivery efficiency of EXOs to cells is reported to be directly related to rigidity of cargo lipids including sphingomyelin and N-acetylneuraminyl-galactosylglucosylceramide (GM3) (23).

Recent new data indicates that proteoglycans and lectins can participate in EXOs binding and internalization. Proteoglycans are cell surface proteins while lectins, such as galectins 1, 3 and 5 which recognise and bind proteoglycans, are identified on EVs. Indeed proteoglycan receptors

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along the plasma membranes of cells and proteoglycans on EXO surfaces have been shown to promote docking (213).

Exosome uptake and release of cargo.

EV internalization by recipient cells is reported to occur via multiple processes such as phagocytosis (188,203,204), clathrin-mediated endocytosis (214), macropinocytosis (208), receptor-mediated (215), and direct fusion (23). However, much further understanding of the underlying mechanisms , and importantly, whether EV subtypes have distinct mechanisms of uptake their target cell specificity is required (216-220) (review(221)).

EV uptake is readily demonstrated in cell culture, using fluorescently-labelled EVs (91)). Uptake and cargo release occur very rapidly, within minutes-hours. However, such techniques do not absolutely prove release, as it is possible that the transfer and spread of fluorescence results from the culture conditions and lipid/membrane transfer.

Recent developments in modification of EVs have also facilitated monitoring and tracking their behaviour, interaction and transfer *in vivo* (222). Intracellular probes are utilized to fluorescently label mRNA within EVs to monitor EV-borne mRNA encoding luciferase. Developments in transgenic mice enable visualization of EV transfer to cells associated with tumour stroma (223) and immune cells (224,225) while EV-mediated transfer of donor genomic DNA to recipient cells supports a mechanism for genetic influence between cells (226). Such *in vivo* approaches have not specifically shown whether transfer involves a direct fusion of EVs with the recipient cells, formation of gap junctions or nanotubes, or phagocytosis of live or apoptotic cell-derived EVs by the recipient cell.

Low pH is important for EXOs uptake. There appears to be elevated stability and lipid/cholesterol content of exosomal membranes in an acidic environment (23).

Understanding recipient cell function and regulation by EXOs needs to focus on specific mechanisms of targeting and delivery, uptake and transfer, including modulation of key signalling pathways in various recipient cells both *in vitro* and *in vivo*. Processes that control target cell recognition and EV uptake are not well understood.

ii. Inhibiting EV recognition/uptake

While several uptake mechanisms have been proposed for EVs, detailed knowledge regarding the key steps in EV target cell definition and definitive mechanisms of uptake is required (221) particularly since variability is found between cell types in vesicle internalization (227). The use of inhibitors is proving useful in elucidating cell-type specific mechanisms.

As discussed, using fluorescently labelled EVs, internalization can be readily observed *in vitro* within a short period of time (91,228). Treatment with inhibitory agents such as chlorpromazine to examine clathrin-dependent uptake (205) and specific RGD inhibitory peptides (229) to target integrin-mediated EV uptake allows identification of selective processes of internalization. The efficacy of EV exchange between cells probably depends on their surface antigen repertoires since partial digestion of membrane proteins exposed on EVs with proteinase K can significantly decrease their uptake (205) and blockage of select integrins or tetraspanins with monoclonal antibodies also has suppressive effects on EV internalization (203). Further, the use of cytochalasin D, which interferes with actin polymerization and endocytosis, significantly reduces the uptake of EVs (205,206). Similarly, the inhibition or knockout of dynamin, a GTPase responsible for formation of endosomal vesicles, significantly suppresses EV uptake (207). Further research is needed to understand the precise mechanisms that underpin distinct EV entry into select target cells and importantly how to control this process.

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Part III. EVs as messengers in reproductive physiology

Normal reproductive processes are highly dynamic, with well characterized stages. The considerable intercellular interactions involved at each stage have prompted the study of the involvement of EVs in both the male and female reproductive tracts, from pre-conception to birth. EVs associated with reproductive biology have been specifically identified and studied in different fluids such as prostatic and epididymal fluid (230) seminal fluid (7,150), follicular fluid (231,232) oviductal fluid (233,234), cervical mucus (235), uterine fluid (151,167), amniotic fluid (115,236) and breast milk (237), and the originating tissues (reviewed in (238) (summarized Table 3).

There is currently increasing data pointing at EVs as key regulators of different reproduction processes such as sperm/ovum maturation, coordination of capacitation/acrosome reaction, prevention of polyspermy, endometrial embryo cross-talk and even communication between *in vitro* co-cultured embryos leading to quorum improved development (239). In these initial steps of the reproductive process (e.g.: pre-conception) EVs are widely produced by different organs and show specific functions. Once implantation has taken place, production of EVs continues throughout pregnancy being the placenta the main source of EVs. During early pregnancy, EVs are released by the extravillous trophoblast. Later on, the syncytiotrophoblast (STB) is formed and establishes contact with maternal blood-flow. From here on, STB constitutes the main site of EVs generation, and these EVs get access to the maternal systemic vasculature, where they show important roles in immune modulation, either for the innate and the adaptive response (32). Of note, EVs are also found in amniotic fluid, where they are attributed inflammatory and pro-coagulant activities (240); and in maternal breast milk. In this last case, an important influence of EVs recovery procedure has been detected on subsequent analysis (241). Among attributed roles, milk EVs have been involved in bone formation, immune modulation and gene expression regulation, with special emphasis for long non-coding RNAs (237,242).

I. EVs in the male reproductive tract: epididymis and prostate

After leaving the seminiferous tubules, spermatozoa (SPZ) are still immature cells. SPZ are stored in the epididymis where they undergo a series of morphological and biochemical modifications that provide them with motility and fertilization ability in their transit from the caput to the cauda, a process called sperm maturation (243,244). During ejaculation, SPZ mix with seminal fluid from the seminal vesicles, the prostate and the bulbourethral gland to form the ejaculated semen, which is ejected into the vaginal cavity. Seminal fluid composition is crucial in promoting sperm motility and genomic stability (243,245). Moreover, it contributes to the establishment of maternal immune tolerance (246,247). Subsequently, as SPZ travel through the female reproductive tract to the upper Fallopian tube where fertilization occurs, they interact with the endometrial and tubal milieu. Finally, to achieve successful fertilization, SPZ undergo capacitation: sperm head membranes undergo a series of biochemical modifications that enable the acrosome reaction when the spermatozoon reaches the zona pellucida of the oocyte. This leads to the release of enzymes that allow SPZ to penetrate the zona pellucida and fuse with the oocyte plasma membrane (248-250). In this context, secretions from the different components of the male and female reproductive tracts have been proposed to play a sequential role in programming sperm function (251).

i. Epididymosomes

Epididymosomes (EVs originating from the epididymis) were first described in 1967 by Piko in the Chinese hamster (252) as having diameters between 20 and 100 nm, and being associated

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with the SPZ acrosomal membrane (253). More recently, it has been shown that epididymosomes are a population of roughly spherical bilayered vesicles that display heterogeneity both in size and content that varies between the different segments of the epididymis. Their sizes range from 50 to 800 nm or even to 2-10 μm in the first segments of the caput (230). Their lipidic composition also varies: indeed, an increase in sphingomyelin and a general decrease in the other phospholipids and in the proportionof cholesterol occurs with epididymal progression from the caput to the cauda. This is in contrast to SPZ, where the proportions remain more constant. Epididymosomes also have an increased ratio of saturated/unsaturated fatty acids from the caput to the cauda, while the opposite situation is found in SPZ. Together, these data indicate that epididymosomes tend to gain membrane rigidity whilst SPZ membranes tend to become more fluid (230).

Two main classes of epididymosomes have been identified: CD9-positive epididymosomes, that preferentially bind live SPZ, and ELSPBP1-enriched epididymosomes, which present higher affinity for dead SPZ (254). CD9-positive epididymosomes are EVs of size ranging from 20 to 150 nm (255). These were recovered by ultracentrifugation from the total epididymal fluid EVs, specifically in the epididymis cauda. CD9-positive epididymal cargo transferred to SPZ includes proteins involved in sperm maturation namely P25b, GliPr1L1 and MIF (256-258), in contrast to ELSPBP1 which was widespread between all EVs. Moreover, CD9, in cooperation with CD26, plays a role in promoting this transfer (255).

ELSPBP-1-enriched epididymosomes constitute a subpopulation of vesicles obtained from the epididymal fluid by high-speed ultracentrifugation (120,000 x g) after SPZ and debris removal at 4,000 x g (259). It had been suggested that ELSPBP1 allowed distinction between dead and viable SPZ as it was only detectable in the dead SPZ population (260). Later, the same group demonstrated that epididymosomes were the only path for the transmission of molecules including ELSPBP1 to dead SPZ (255,259). Interestingly, ELSPBP and biliverdin reductase A (BLVRA) can associate and bind in tandem to dead SPZ, concurrently with the epididymal maturation of SPZ, a process during which these cells cease producing BLVRA. Therefore, BLVRA could act as a quencher of reactive oxygen species generated by dead and immature SPZ, protecting viable SPZ from oxidative stress. Moreover, BLVRA may be involved in haemic protein catabolism, changes also important in the SPZ maturation process (254,261).

Since the epididymis brings SPZ to functional maturity before they enter the vas deferens, it is not surprising that epididymosomes serve as means for protein transfer into SPZ during their transit in the epididymal duct. Some epididymosomal proteins have proven roles in sperm maturation: these include P25b, MIF or SPAM1, among others (262,263). Sperm adhesion molecule 1 (SPAM1) is a hyaluronidase with roles in both fertilization and sperm maturation. It is transferred to SPZ from epididymosomes, increasing their ability for penetrating the oocyte cumulus (264). Another protein transferred to SPZ by this mechanism is ADAM7, which is important for sperm motility, morphology and establishment of membrane correct protein composition (265,266). Of note is the transfer of the plasma membrane ATPase 4 (PMCA4), a major Ca^{2+} efflux pump, into epididymosomes: this plays a pivotal role in SPZ maturation and motility (264). Glutathione peroxidase 5 (GPX5) associates with SPZ during its transit through the epididymis, protecting them from lipid peroxidation stress and, independently, is transferred to SPZ via epididymosomes (267). Finally, components of the Notch pathway are described in epididymosomes, suggesting that these vesicles transmit Notch signalling at a distance between epididymal epithelial cells, but also between the epididymis and SPZ with important implications for sperm motility (268).

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Epididymosomes also convey miRNAs within the epididymal duct. As with proteins, distinct regions of the epididymis produce EVs with a specific set of miRNA whose profiles differ from those of parent cells, suggesting a sorting mechanism (269). Indeed, it has been proposed that epididymosomes may act as modulator of gene expression between sections of the epididymal duct (269). Recent analysis confirmed that they contain over 350 miRNA, showing a different profile from that of parent cells and dependant on the region of the epididymis from which they originate. Finally, it was demonstrated that many of these miRNAs are transported into the SPZ (270).

An emerging concept is the transfer of traits to the offspring by epigenomic modifications. In this respect, transfer (t)RNA, has been attributed a new function as a modulator of genetic expression. It was initially discovered that a respiratory syncytial virus (RSV) infection of lung and kidney cell lines, led to the production of specific tRNA fragments (tRFs) that are able to repress the expression of specific mRNAs in the cytoplasm to favour viral replication and survival (271). Subsequently, further examples of tRFs have been described with potential implications in pathological processes, such as cell proliferation in cancer (272). Mature molecules corresponding to tRNA fragments are highly enriched in mature sperm. Interestingly, these fragments are produced by sequence specific cleavage, giving place to fragments corresponding to the tRNA 5' end (273). Recently, the transfer of tRFs to maturing SPZ in epididymosomes was demonstrated in mice (274), providing an explanation for the scarcity of these molecules in testicular SPZ but with an increase with SPZ maturation. A tRF (tRF-Gly-GCC) has been identified as transferred to SPZ by epididymosomes. This tRF, represses MERVL, an endogenous retro-element, that positively regulates a set of genes that are highly expressed in pre-implantation embryos. Interestingly, male mice treated with a low-protein diet have a trend (non-significant) to increased tRF-Gly-GCC in mature SPZ and to downregulate tRF-Gly-GCC targets in embryos at 2-cell stage. This evidence supports that parental diet can affect the offspring epigenome: however, this preliminary data requires confirmation (274).

ii. Prostasomes

Prostasomes were first described as vesicles recovered from human prostatic fluid by centrifugation, that were associated with an Mg^{2+} and Ca^{2+}-dependent ATPase activity (275). They are now considered a population of EVs produced by the prostate epithelial cells that interact with SPZ, epididymal and seminal secretions at the time of ejaculation. They are EVs of size range 30 to 500 nm, surrounded by lipoprotein bilaminar or multilaminar membranes (276,277). It is likely that a population of prostasomes is exosomal, as they originate from structures resembling MVBs and exhibit classical EXO markers (278). Prostasomes' lipid composition is unusual and provides them with a characteristic highly ordered structure, rigidity and viscosity due to several factors: a high cholesterol/phospholipid ratio reaching values of 2, which greatly surpasses the values for most of biological cholesterol-rich membranes; phospholipid composition domination by sphingomyelin, which accounts for almost a half of the phospholipids found in prostasomes (279); and finally prostasomes show a strongly saturated fatty acid profile in comparison to SPZ membranes (280). It has been reported that prostasome uptake decreases the fluidity of SPZ membranes by transfer of lipids directly dependent on the prostasome/SPZ ratio (277,281). This decrement is crucial as it stands as a regulator of the acrosome reaction, preventing a premature response (282).

Different roles have been attributed to prostasomes in sperm maturation and function, either directly or indirectly. These include protection of SPZ from the female acidic environment and

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immune surveillance modulation of SPZ motility, capacitation, acrosome reaction and fertilizing ability, among others (276-278,282).

SPZ motility is vital for a successful fertilizing ability, especially for traversing the cervical mucus and zona pellucida (283). One of the first roles attributed to prostasomes was the enhancement of SPZ motility (284) in a pH-dependent manner, suggesting that prostasomes might alleviate the negative effects of vaginal acidic microenvironment on SPZ motility, thus showing a protective effect (285). Ca^{2+} has been well known as the major ion promoting SPZ motility and fertility, from initial studies carried in hamster (286). Increased SPZ Ca^{2+} levels have been linked to prostasomal delivery, directly depending on the extent of fusion/prostasome concentration and influenced by pH (287). However, it took a decade to identify a mechanism. Park and colleagues showed that a progesterone-triggered long-term sustained Ca^{2+} stimulus is involved in SPZ motility promotion, via fusion of (acidic) pH-dependent prostasomes. Specifically, prostasomes transferred progesterone receptors and different Ca^{2+} signalling cascade components to the SPZ neck region where, following progesterone stimulation, they trigger the release of Ca^{2+} from SPZ internal acidic stores to promote SPZ motility (288). Other proteins involved in intracellular Ca^{2+} homeostasis are also transported into SPZ in prostasomes, including PMCA4 (289), which along with nitric oxide synthases (NOSs) are delivered into SPZ by prostasomes. PMCA4 and NOS activity is stimulated by Ca^{2+} ions (290) and indeed, NOS spatially interacts with PMCA4 to a degree positively related to Ca^{2+} concentration levels. This supports the theory that PMCA4 expels Ca^{2+} from SPZ in the presence of NOS to reduce nitric oxide production and thus oxidative stress, which could reduce SPZ viability resulting in asthenozoospermia (289). Prostasomes also carry aminopeptidase N, a protein involved in modulating sperm motility, which acts through the regulation of endogenous opioid peptides, such as enkephalins, once in SPZ (291,292).

Interestingly, EXO-like EVs found in cervical mucus have been reported to carry sialidase activity, which reaches a maximum during the ovulatory phase in healthy women. This is likely involved in modifying the properties of the highly–glycoslyated mucus to favour SPZ access to the uterine cavity and tubes (235).

There is scarce data on the prostasomes' nucleic acid cargo and its implications for male reproductive physiology. Prostasomes contain various coding and regulatory RNAs, with potential modulatory functions (190). Interestingly, mRNA and miRNA do not represent the majority of the prostasomal RNA (278), and it has been postulated that mRNA in semen is predominantly transported inside vesicles while miRNA is mostly contained in the vesicle-free fraction of the semen, forming complexes with proteins (195). DNA inside prostasomes apparently represents random regions of the genome and is effectively transported into SPZ (293,294). Nevertheless, this DNA may be a contaminant from apoptotic bodies in the semen (295).

Capacitation is a cAMP-regulated process, whose production is in turn promoted by bicarbonate and Ca^{2+} ions and influenced by membrane dynamic changes mainly due to cholesterol composition (276,278). It has been proposed that prostasomes may act as inhibitors of the capacitation process and acrosomal reaction, mainly through transfer of cholesterol (296,297). Indeed, this might represent a mechanism to avoid premature capacitation and acrosome reaction (282,298). A switch between positive and negative regulation exerted by prostasomes may be influenced by the environment or even determined by specific prostasome subpopulations. cAMP promotes capacitation through the protein kinase A (PKA) axis, by the simultaneous tyrosine-phosphorylation of specific down-stream proteins and plasma membrane

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protein and lipid remodelling. This remodelling breaks down plasma membrane asymmetry, exposing cholesterol molecules to external acceptors to trigger the capacitation process (299). In this context, co-incubation of equine SPZ with prostasomes led to increased cAMP levels and tyrosine phosphorylation of PKA cascade proteins, in addition to the prostasome endogenous PKA activity described in previous reports. However, these changes were not correlated with increased capacitation and acrosome-reaction rates and reverted after 3 hours of co-incubation in capacitating conditions (296). Interestingly, Aalberts and colleagues observed that at least a subpopulation of prostasomes are able to bind to live SPZ only when capacitation-inducing conditions are established, probably to promote hypermotility and acrosome reaction at the precise moment it is needed. Nonetheless, care should be taken when interpolating these results into human, as they were obtained from a stallion model, a species that deposits its ejaculate directly in the uterus (299).

Following capacitation, SPZ need to undergo an acrosome reaction to enable penetration of the zona pellucida of the oocyte and fusion of plasma membranes. The zona pellucida glycoprotein ZP3 is mandatory for this process as it facilitates sperm-binding, triggering the acrosome reaction. Nevertheless, the acrosome reaction begins before the SPZ contacts the zona pellucida, probably due to the progesterone-dependent stimulus produced by cumulus cells (300). Conversely prostasomes have been proposed as inhibitors of the acrosome reaction through the transference of cholesterol to the SPZ (297,301) or as inducers by facilitating progesterone uptake by the SPZ (302), most likely by the transfer of progesterone receptors (288). Other studies also supporting the promotion of the acrosome reaction via prostasomes include delivery of molecules to the SPZ membrane ia a pig model (303), or progesterone priming, acting via the Ca^{2+} signalling axis (304). Other acrosome reaction-promoting molecules in prostasomes include hydrolases (305) and lipoxygenases (306).

In summary, the role of prostasomes in sperm-fertilizing ability in humans is most likely the result of orchestrated actions. Initially, prostasomes would attach to SPZ after mixing during ejaculation, favoured by the acidic environment of the vagina, thus transferring cholesterol to stabilize SPZ membranes and prevent premature capacitation and acrosome reaction. This would enable prostasomes to pass the barrier of cervical mucus adhered to spermatozoa with subsequent fusion and transfer of their content to the SPZ when the SPZ first contacts the oocyte. At this time, the progesterone secreted by the cumulus cells would activate Ca^{2+}-dependent pathways that promote capacitation process and acrosome reaction (276).

Finally, of note is the role of prostasomes in protecting SPZ from the potentially hostile female genital tract They appear to exert roles as protectors from female immunity, antioxidants, antibacterial, and in the process of semen liquefaction (reviews: (276,282,299)).

II. EVs in the female reproductive tract: follicular fluid, oviduct/tube and uterine cavity

Contemporarily to sperm maturation, a coordinate oocyte development must be taking place so as both gametes can meet at the appropriate location and time inside the female reproductive tract. Developing oocytes are arrested in prophase I of meiosis in primordial follicles from the fetal period until female reproductive maturity. From this moment, cohorts of these oocytes cyclically restart growth, forming the zona pellucida while granulosa cells proliferate in order to form the cumulus, which will support posterior egg fertilization. Concomitantly, meiosis is reinitiated, extruding the first polar body and arresting again at metaphase II during ovulation (307). The resumption of meiosis is stimulated by the LH peak, which in turn is initiated by a surge in estradiol-17β levels due to the secretion by the granulosa cells from the preovulatory follicle, and results in ovulation 36 hours later (308). After ovulation, the extracellular matrix of

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the cumulus cells serves as an adhesion dock for the Fallopian tubes, through which the eggs travel as far as the ampulla where they await SPZ for fertilization (309). Following fertilization embryo development to blastocyst stage, proceeds as the embryo passages through the Fallopian tubes reaching the uterine cavity of about 4 days after ovulation. The blastocyst undergoes final preparation for implantation into the maternal endometrium in the microenvironment of uterine fluid with implantation occurring 6 -10 days after ovulation (310).

The process of embryo implantation can only occur during a short period of time during the luteal phase of the menstrual cycle, which has been classically regarded as the window of implantation and that typically extends from days 5.5 and 9.5 days after ovulation in healthy normal cycling women (310,311). At this point, different factors affect and limit embryo implantation, namely: embryo quality, endometrial receptivity and embryo-endometrial cross-talk (312), where EVs stand as important potential mediators.

During all this process, EVs carry out many different supporting actions: they assist follicle and oocyte development and maturation at the initial stages, and further assist early embryo development and implantation as the conceptus reaches the uterus. Further, female tract EVs contribute in preparing endometrial vascular net, promote embryo implantation and prime the endometrium for harbouring the embryo. Moreover, these EVs also contribute to SPZ maturity, capacitation and acrosome reaction coordination, support SPZ storage while waiting for the oocyte and regulate molecule delivery into SPZ during this period. All these concepts will we discussed in the following sections.

i. Follicular fluid EVs

Oocyte maturation occurs within the micro-environment of follicular fluid (313). The easy availability of this fluid during oocyte retrieval in assisted reproductive techniques makes it attractive in the search of biomarkers for oocyte quality (314). EVs (resembling exosomes and microvesicles) were first identified in follicular fluid by da Silveira and colleagues who demonstrated follicular fluid EV uptake by granulosa cells, both in vivo and in vitro, and their protein and miRNA cargo. EV miRNAs were also present in the surrounding granulosa and cumulus cells, thus suggesting EVs as a vehicle for biomolecule transfer within the ovary. Of particular interest, the miRNA signature of follicular EVs varied with the age of the female, suggesting EVs miRNA cargo as an indicative and possible predictor of age-related decline in oocyte quality (315). Subsequently, EV miRNAs were further evaluated and a set of four differentially expressed miRNA based on age (young/old) was defined. However, these age-related miRNAs were studied in complete follicular fluid samples and as such cannot be confidentially attributed to EVs (316).

The miRNA of bovine follicular fluid is present both in exosomes and free, each with different composition (317). The exosomes were taken up by granulosa cells in vitro, resulting in increased miRNA content and variations in mRNA profiles: some of the affected genes are involved in follicle development. Moreover, some of the miRNA within exosomes may also contribute to oocyte growth as they were differentially expressed in follicles containing oocytes at different maturation stages (317). A more exhaustive characterization of the EV content of bovine follicular fluid demonstrated variation in number, protein markers and miRNA contents depending on the developmental stage of the follicles. What is more interesting, variation in miRNA signature suggested a switch in genetic programming concurrent with the follicular maturation. As such, EVs miRNAs from small follicles preferentially promoted cell proliferation pathways while those from large follicles related to inflammatory response pathways (318). A possible role of follicular fluid-derived exosomes in follicle development and growth through the

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TGFB/BMP axis ACVR1 and ID2 regulation, was demonstrated when granulosa cells were exposed to follicular fluid exosomes *in vitro*. It was proposed that these effects were triggered by the direct delivery of ACVR1 and ACRV1 regulatory miRNA within follicular exosomes to granulosa cells (319).

Cumulus-oocyte complex expansion is a critical process for ovulation. In this context, in vitro co-culture experiments using bovine follicular fluid-derived exosomes and cumulus-oocyte complexes from mouse and bovine revealed that follicular EVs are taken up by cumulus cells, promoting both cumulus expansion and related genes expansion (320).

ii. Oviduct/Tubal EVs

Fertilization of the oocyte by SPZ occurs within the Fallopian tubes/oviduct. After capacitation, SPZ must undergo an acrosome reaction and maintain hyperactivated motility in order to fuse with the oocyte, both functions being regulated by high intracellular Ca^{2+} concentration levels. In this context, the major murine Ca^{2+} efflux pump PMCA4, and particularly its splicing variant PMCA4a, is predominant in oviductal fluid, compared to uterine and vaginal fluids, and is totally associated with EVs. Moreover, these PMCA4a-carrying vesicles had exosomal characteristics and were taken up by SPZ, where the efflux pump was functionally relocated to their membranes. This was the first study describing the presence of exosomes in the oviducts and introduced the relevance of PMCA4 as a tool for the maintenance of Ca^{2+} homeostasis and SPZ viability during SPZ storage, regulating capacitation and acrosome reaction timings and SPZ motility (233,234,321,322). Subsequently, the same authors discovered that integrins (α5β1 and αvβ3), in oviductal EVs were transferred to SPZ, and were involved in EV-SPZ fusion for cargo delivery. While the oviductal EVs, include both microvesicles and exosomes, the former appeared to be more efficient in fusing with SPZ (233).

Bovine oviductal EVs produced in vitro by cell lines, have beneficial effects on the quality and development of in vitro co-cultured bovine embryos, suggesting a functional communication between the oviduct and embryo during the early stages of embryo development (323). However, these results must be treated with caution as oviductal EVs produced in vitro have been observed to carry a differential cargo compared with in vivo produced EVs. This is the case, for example of OVGP and HSPA8, oviductal proteins known to be important in the fertilization process and early pregnancy. While HSPA8 was found in both in vitro and in vivo exosomes, OVGP was absent in exosomes of in vitro origin (324).

iii. Uterine EVs

Endometrial fluid is a viscous liquid, secreted by the endometrial epithelial cells from the glands into the uterine cavity. Since the endometrium is a hormonally regulated organ, the molecular composition of the fluid varies depending on the phase of the menstrual cycle (325). Uterine fluid, a biologically and clinically relevant sample source (326) also contains contributions from the oviductal fluid and a large cohort of plasma proteins along with other factors, differentially transudated from the blood (327). It is highlighted that this uterine fluid carries information that mirrors maternal environmental exposure and possibly relays such information to the embryo, subsequently generating long-term epigenetic effects on the offspring via embryonic and placental programming.

To date, EVs have been reported throughout menstrual/estrous cycles in the endometrial fluid of different species, including humans (151,167) and sheep (328-331), and are also released by endometrial epithelial cells in culture (151,167).

Ng et al (2013) first described the production of EVs by human endometrial epithelial cells in primary culture and by the endometrial epithelial cell line ECC1. These EVs contained a specific

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subset of miRNAs, not detectable in the parent cells. Bioinformatic analysis revealed that some of the target genes of the EVs miRNAs are relevant to processes involved in embryo implantation. Importantly, they also verified the presence of EVs in human uterine fluid and the associated mucus (167).

Greening *et al*. (388) demonstrated that the proteome of highly purified EXOs derived from human endometrial epithelial cells, is regulated by steroid hormones, and thus varies with the progression of the menstrual cycle. Under follicular phase hormonal conditions, when oestrogen constitutes the main hormonal stimulus, the EXOs proteome was enriched in proteins related to cytoskeletal reorganization and signalling cascades, coinciding with the phase of endometrial restoration. Importantly, after ovulation, when progesterone is the dominant hormone driving endometrial receptivity, the proteome altered with changes indicating enrichment in extracellular matrix reorganization and embryo implantation. As in other systems, the exosomal protein profiles were shown distinct from parental cells. Importantly, this study demonstrated that endometrial EXOs were transferred and internalized by human HTR-8 trophoblast cells, enhancing their adhesive capacity, partially through focal adhesion kinase signalling (91). This was significantly higher when the exosomes were derived from cells subjected to both estrogen and progesterone to mimic the receptive phase of the menstrual cycle.

iv. Embryonic and trophectodermal EVs.

Interestingly, murine embryonic stem cells from the inner cell mass generate microvesicles that reach the trophectodermal layer and enhance the migration ability of trophoblast cells in culture, either as isolated cells or in the whole embryo. The presence of the laminin and fibronectin in the cargo of the inner cell mass EVs, enabled attachment to the integrins on the trophoblast cell surfaces and stimulated JNK and FAK kinase cascades, increasing trophoblast migration. Further, injection of these EVs inside the blastocoele cavity of 3.5 day blastocysts increased their implantation efficiency (88). It must be noted that this mechanism may be particular to the mouse and those other species in which the ICM is distal to the site of trophectoderm attachment to the endometrial surface: in women this is the reverse with the ECM tightly aligned with the attaching trophectoderm.

EVs produced by ungulate trophectoderm participate in cross-talk with the maternal endometrium (330). Bidarimath and colleagues observed that EVs from a porcine trophectodermal cell line stimulated the proliferation of endothelial cells *in vitro*, thus being potential regulators of maternal endometrial angiogenesis (332). These vesicles contained a miRNA and protein cargo likely to annotate functions in the angiogenesis process. Again, care should be taken with these data as they were retrieved from cell lines cultured *in vitro*. Further, the pig is a species with epitheliochorial placentation, and thus the *in utero* development is very different from that of the human (332). Nevertheless, study of human extravillous trophoblast cell (HTR-8/SVneo and Jeg3)-derived exosomes similarly showed that these vesicles promote vascular smooth muscle cells migration, which is important during human uterine spiral artery remodelling in successful pregnancies (333). Importantly, the two trophoblast cell lines (which are different stages along their differentiation pathway) produced differential migration results, raising the likelihood that cell origin as well as content and bioactivity of the exosomal cargo are of considerable importance, emphasising the need to keep models as close to the physiological situation as possible.

v. EVs as vehicles for embryo-maternal cross talk

The first indication that the endometrium produced EVs with unique cargo was that the human endometrial epithelial cell model ECC1 (which best represents luminal epithelium), released EVs

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containing a different miRNA profile from that of parent cells (167). These EVs could provide a mechanism for communication between the mother and the embryo with potential implications in embryo implantation. Indeed, bioinformatic analyses on the EV miRNAs showed predominance of the genes targeted by the miRNAs as involved in implantation. Furthermore, interrogation of the proteome of ECC1 EVs, cultured under conditions to represent the proliferative (estrogen-dominant) and secretory (estrogen plus progesterone) phases of the cycle, showed that the protein cargo of EVs is hormone-specific, enriched with 254 and 126 proteins respectively (91). Importantly, 35% of the endometrial EV proteome had not been previously reported, indicating the unique cargo of endometrial EVs. These findings were validated in EVs from primary endometrial epithelial cells. Functionally the EVs were internalised by human trophoblast cells, inducing increased adhesive capacity, that was at least partially mediated through active focal adhesion kinase (FAK) signalling, indicating a likely role in promoting embryo implantation (109). Interestingly, among the implantation-related proteome of these endometrial exosomes, were the cell surface metalloproteinases ADAM10 and MMP-14 (a membrane-bound MMP), for which there are abundant substrates on the trophectoderm.

Another study showed that endometrial epithelial derived EVs in the uterine fluid contain has-miR-30d during the receptive phase of the cycle. This EXOs-associated hsa-miR-30d was internalized by mouse embryos via the trophectoderm, resulting in an indirect overexpression of genes encoding for certain molecules involved in the murine embryonic adhesion phenomenon—*Itgb3*, *Itga7*, and *Cdh5*. Functionally, in vitro treatment of murine embryos with miR-30d resulted in a notable increase in embryo adhesion again indicating how maternal endometrial miRNAs might act as transcriptomic modifiers of the pre-implantation embryo (151).

Part IV. Implications of EVs in Reproductive Pathology

Given their seminal functional role and presence in various aspects of reproductive biology, a growing field of evidence is uncovering potential roles for EVs in regulating reproductive pathological conditions including endometriosis, polycystic ovaries syndrome, erectile dysfunction, early pregnancy loss, hypertension, pre-eclampsia or gestational diabetes mellitus (summarized Table 4). Given this importance in EVs during maternal environment and development, significant efforts are now focused on evaluating prognostic value and applicability of EVs as diagnostic and therapeutic agents (107,334).

I. EVs in endometriosis

Endometriosis is an estrogen-dependent inflammatory disease which is characterized by the deposition and growth of endometrial cells outside the uterine cavity, with the pelvic peritoneum and ovaries being the most common sites for ectopic growth (335). For this reason, endometriosis is considered a benign metastasizing disease (336).

Endometriosis is characterized in part by an increase in the expression of angiogenic factors and metalloproteinases. Patients with endometriosis show higher levels of these molecules in endometriotic lesions than in eutopic endometrium and eutopic endometrium of endometriosis patients shows higher levels than healthy endometrial controls (337). Indeed, by inhibiting metalloproteinases it is possible to avoid the establishment of ectopic endometriotic cysts (338). In this context, EMMPRIN, a metalloproteinase inducer, is carried in EVs produced by uterine epithelial cells and stimulates the expression of metalloproteinases in stromal fibroblasts. The secretion of both EMMPRIN and metalloproteinases, is positively regulated by IL-1β/α, whose secretion is increased in women under endometriosis conditions in whom there is a pro-

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inflammatory peritoneal environment. This would allow the increase of metalloproteinases production by fibroblasts to trigger endometriotic lesions invasion (339).

In terms of EV RNA cargo, EVs from endometrial stromal cells from women with endometriosis versus women without the disorder, showed different profiles of exosomal miRNA content between EVs derived from eutopic and ectopic endometrium from endometriosis subjects and between eutopic endometrium from women without or with disease (340). Moreover, there was a differential miRNA signature, between eutopic endometriotic and control exosomes. Among these miRNAs, miR21, is already known for a role in angiogenesis. It remains to be established whether miR-21 can promote angiogenesis following EV uptake (340).

Ectonucleotidases are enzymes involved in inflammatory processes and previously reported as expressed in the endometrium. Teixidó and colleagues investigated ectonucleotidase activity from endometriotic cysts (endometriomas) on the ovary, one of the common sites for endometriotic lesion development. Ectonucleotidases were highly enriched in endometriomas compared to simple cysts. Interestingly, the ectonucleotidase activity was also contained by exosomes derived from endometriomas and simple cyst fluids, but and was significantly higher for exosomes from endometriomas (341).

II. Polycystic Ovarian Syndrome

Polycystic ovarian syndrome (PCOS) is one of the most common hormonal disorders affecting women, characterized by androgen excess and insulin resistance, leading to androgenism, high risk of glucose intolerance, diabetes and lipid abnormalities (342). Its complex phenotypic manifestation was formally described nearly a century ago as the concurrence in women of amenorrhea, hirsutism, obesity and typical polycystic appearance of the ovaries (343).

Koiou and collaborators observed that platelet microvesicles in plasma from PCOS affected women (defined by elevated circulating androgens and insulin resistance markers) were at higher levels than in healthy controls. Moreover, there was a significant positive correlation between microvesicles and numbers of follicles in the ovaries of these women (344). Subsequent confirmation of the increase in EVs levels (mainly of exosomal size) in PCOS also demonstrated a direct correlation with insulin resistance markers. Furthermore, PCO-derived EVs showed a higher content in annexin-V along with 16 miRNA that are normally expressed at low levels, being increased with PCOS, (345).

Sang and colleagues described EVs in the human follicular fluid and identified 120 miRNAs within their cargo, 11 of which were highly expressed and with target genes in pathways involved in reproduction, endocrine and metabolic processes. Two of these miRNAs, miR-132 and -320, were significantly decreased in the follicular fluid EVs from PCOS patients compared to non-affected controls (346). Of note, miR-132 and -320 have HMGA2 and RAB5B respectively as target genes: these were associated with key roles in the etiology of PCOS in a previous genome-wide association study (347).

DENND1A is a PCOS candidate locus, characterized in a number of genome-wide association studies (347,348). DENND1A variant 2 levels, both at protein and mRNA levels, were increased in theca cells of PCOS patients compared to healthy controls. In agreement with these results, mRNA for this locus was significantly increased in exosomes extracted from urine of PCOS-affected women in comparison to normal-cycling controls. In this sense, the exosomal miRNA profile is proposed to reflect the physiological status of the source cells, providing a potential biomarker of PCOS (349). Further studies are needed to uncover the roles of EVs in the triggering and development of PCOS.

III. Erectile dysfunction

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Erectile dysfunction (ED) is the most studied sexual problem worldwide and mainly affects men over 40 years of age. It costs up to £7 million in the UK and $15 billion in the USA. The prevalence of this condition varies greatly throughout the world, highlighting the Middle East (45.1%), United States (37.7%) and specially mainland China (varying from 17.1 to 92.3%), according to a retrospective study carried on men of different ages (350).

Microparticles have been proposed as involved in endothelial dysfunction and atherogenesis, with special regard to ED. Initially, microparticles defined as membrane vesicles, apoptotic or not, smaller than 1.5 µm, were recovered from plasma after platelet depletion at 900 x g and measured by flow cytometry using specific markers (351). These circulating endothelial-derived microparticles were increased in type 2 diabetic men with ED, compared with controls and a positive correlation between microparticle counts and ED severity, determined by the International Index of Erectile Function (IIEF), was shown. However, diabetes risk factors did not influence microparticle levels and so, these were postulated to be independently linked to ED severity. Finally, microparticles were proposed as possible links between endothelial dysfunction and ED (351). Retrospectively, a molecular signature identified in microparticles enabled discrimination between diabetes and ED. The marker CD31 in microparticles was mainly related to diabetes, whereas CD62E, was directly linked to ED, without diabetes. The ratio CD31/CD62 could be used to evaluate endothelial function, with a high ratio being related to endothelial activation and a low ratio associated with apoptosis. In the study, diabetic men with ED men showed lower ratios, maybe indicating a cooperative effect of the two disorders. Finally, levels of CD31+ microparticles were directly correlated with ED aggressiveness (352).

La Vignera and colleagues, increased the centrifugal force to achieve a better clearance of platelets from serum (13.000 x g). They confirmed an increase in endothelial-derived microparticles levels in ED patients with arterial etiology, in comparison to patients with ED of psychogenic origin. Since a positive correlation was observed with typical ED metabolic parameters they proposed endothelial dysfunction as the cause underlying ED and reasserted microparticles as predictors of the condition (353). Furthermore, their levels were directly related to the aggressiveness of arterial ED (354): a combination of disorders leading to a greater vascular damage was associated with more severe ED and endothelial dysfunction, and correlated with increasing levels of endothelial microparticles (355).

ED is associated with increased endothelial apoptosis, and both can be in part, reverted by treatment with a type 5 phosphodiesterase inhibitor such as tadalafil (356). Treatment benefits were maintained for 4 weeks after the cessation of a 1-year treatment in almost half of the analysed cases (357). Subsequently, the effect of tadalafil treatment and discontinuation on the production of apoptotic endothelial-derived microparticles was examined. ED patients had increased levels of apoptotic microparticles compared to controls before the start of the treatment. 90-days of tadalafil administration improved IIEF, endothelial parameters and reduced apoptotic microparticle levels, although not to control levels. These improvements reverted by six months after treatment discontinuation (358). Interestingly, complementation of tadalafil treatment with an antioxidant, maintained the tadalafil effects at least until 6 months after treatment cessation, prolonging the duration of the antiapoptotic effect within endothelium (359). This is in accord with other studies implicating oxidative stress in endothelial dysfunction (360,361). Patients with greater severity and duration of ED, associated with the concurrence of high cardiovascular risk profiles, were non-responders to sildenafil, another type 5 phosphodiesterase inhibitor.

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Androgen deficiency has also been proposed to contribute to the development of cardiovascular disease and endothelial function impairment (362). Six months of androgen replacement therapy (Tostrex) improved endothelial and erectile dysfunction features and decreased endothelial derived microparticle levels in patients of ED and late onset hypogonadism (LOH; a new vascular risk factor) (363). Indeed, LOH worsened metabolic parameters and increased the already high endothelial microparticle levels in ED patients (364).

IV. Pregnancy complications

EVs from a variety of sources (epididymis, prostate, cervical mucus, ovarian follicle, embryo, and endometrium) have potential roles in both the establishment and development of a successful pregnancy. However, from the sixth week of gestation (365), placental-derived EVs mainly of syncytiotrophoblast origin, represent the main source of vesicles with potential implication in feto-maternal communication (32,87). Their concentrations in maternal plasma increase gradually as pregnancy progresses (366). Their release and bioactivity are favoured by both low oxygen tensions (367) and high D-glucose concentrations (368). Changes in concentration, composition and bioactivity of placental and non-placental EVs have been reported in pregnancy disorders (369). Notably, the secretion of EVs is increased in the two main EVs-related pregnancy complications: gestational diabetes (370) and preeclampsia (371).

i. EVs in early pregnancy loss

Early pregnancy loss (PL) is a common complication that affects around 15% of the gestations and shows recurrence rates of 2-3%. Importantly, up to 50% of these cases are usually of idiopathic etiology (372). Interestingly, the levels of plasma endothelial microparticles are decreased in pregnancy loss, especially in cases with recurrent miscarriage, compared to controls (373). However, these results should be viewed with caution, as in healthy pregnancy (their controls), there is also an increase in EV levels, mainly due to the contribution of placental-derived EVs (365).

In pregnancy, the haemostatic balance shifts towards upregulated pro-coagulant activity, with increased clotting factors and fibrinogen, and concurrently decreased anticoagulant factors and fibrinolytic activity (374). An excessive pro-coagulant response leading to thrombosis of the uteroplacental vasculature and subsequent hypoxia, has been proposed as a factor accounting for an important part of the fetal loss cases (375). In this regard, blood microparticles with pro-coagulant activity are increased in miscarriage cases, in parallel with the enhanced coagulation-promoting activity. These microparticles may play a role in this outcome by favouring the thrombotic phenomena (376,377). Furthermore, PL-affected women present with lower levels of platelet microparticles and higher levels of endothelial microparticles than controls, although this could not be directly related to the hypercoagulation phenotype, it was suggested to reflect endothelial dysfunction (378). In contrast, plasma platelet-derived microparticles were increased in women with recurrent miscarriage compared to controls (344): However, these results may be biased by the small size of the study population (379) and the controls may be inappropriate due to the contribution of the placenta to the total EV content.

ii. EVs in gestational vascular complications

Gestational vascular complications which include hypertension (HT) and pre-eclampsia (PE), are prevalent causes of maternal and fetal morbidity and mortality. HT may appear as a consequence of abnormal placentation into the maternal uterus, and may lead to the development of impaired liver function, progressive renal insufficiency, pulmonary edema and the new onset of cerebral or visual disturbances that might end in HELLP syndrome (hemolysis, elevated liver enzymes

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and low platelet count) and/ or eclampsia (380). PE is a complex disorder causing preterm birth, intra-uterine growth-restriction and maternal death (381). In general, different studies point towards an increase in endothelial microparticle shedding within GVC conditions, thus suggesting vascular injury (382).

1. Pre-eclampsia (PE)

PE is a pregnancy-related syndrome affecting between 2 and 8% of pregnancies and characterized by a variety of systemic symptoms. It is detected by new onset hypertension and proteinuria after the 20th week of gestation. Its etiology is not well known, but the pathogenesis of PE is conceptualized in a two-stage model with the placental defect precipitating an abnormal vascular maternal response that manifests as the signs of this pathological condition Early PE appears before 34 weeks of gestation and involves the fetus, showing reduced placental perfusion, possibly due to abnormal trophoblast invasion and/or uterine spiral arteries´ remodeling. Late PE appears after 34 weeks, and the maternal manifestations appear; a series of inflammatory, metabolic and thrombotic responses compromise vascular function up to the point of producing systemic organ damage (383).

Several published studies have attempted to elucidate the relevance of EVs of both maternal and placental syncytiotrophoblast origin, in the pathophysiology of PE. Changes in EV concentration and cargo, affect PE development via pro-inflammatory and pro-coagulatory activities enhancement. Here we summarize current knowledge of EVs in relation to PE.

a. Placental-derived EVs

The placenta plays a critical role and is undoubtedly the source of PE development. PE can develop even in the absence of a fetus, provided that trophoblast tissues are established, forming the characteristic mass known as a hydatidiform mole, a tissue abnormality formed by the distension of some or all of the chorionic villi (384).

Syncytiotrophoblast-derived exosomes and microvesicles (STMBs) are increased in PE compared to normal pregnancies (385), maybe in part due to the hypoxia resulting from abnormal placentation (333). This increase occurs specifically in early-onset PE cases but not in late-onset PE or normotensive intrauterine growth restriction (386,387). Importantly, early-onset pre-eclampsia is established in the first trimester when trophoblast invasion and vascular remodelling occurs (333), emphasising the importance of STMBs in these processes. Furthermore, variations in protein (371,388,389), lipid (390) and miRNA (371) cargo of STMBs may explain the specific roles of STMB in PE including immune response, coagulation, oxidative stress and apoptosis.

One of the main characteristics of PE is abnormal remodelling of the uterine spiral arteries, which in normal pregnancies ensures enough maternal blood flow to support fetal growth and development. Thus, a role for extravillous trophoblast (EVT)-derived EVs has been proposed in PE development. Variations in concentration, cargo and bioactivity of EVT-derived EVs as indicated above, may result from a pro-inflammatory environment, inducing these changes, impairing their physiological roles in vascular/smooth muscle tissue remodelling, and thus stimulating the emergence of PE (333,391). In PE, increased amounts of pro-inflammatory cytokines (IL-18, IL12, TNF-α) are released by monocytes and lymphocytes. PE-increased STMBs can bind monocytes to promote the production of more inflammatory cytokines, perpetuating the pro-inflammatory environment and hence stimulation of EV alterations and endothelial cell damage (385). Furthermore, villous cytotrophoblast-derived exosomes carry syncytins 1 and 2, which are involved in exosome fusion with the target cells. Importantly syncytin-2 content was reduced in exosomes derived from serum of PE patients (392).

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Antiangiogenic factors, such as sFlt1 and sEng, appear to participate in PE through a series of mechanisms that lead to the imbalance of angiogenic factors and finally to the generation of endothelial dysfunction and the maternal syndrome of PE. Increasing levels of sFLT and sEng can predict PE and directly correlate with the aggressiveness of this syndrome (393). PAI-1 and, to a lesser extent PAI-2, which is predominant in placenta, are important inhibitors of fibrinolysis. Their overactivation results in the establishment of fibrin deposits that occlude placental vasculature and spiral arteries, leading to hypertension and endothelial damage causing PE. Moreover, increasing levels of PAI-1 in plasma directly correlate with PE severity (394). Eng and PAI-2 are highly expressed and localized to the surface of STMB microvesicles and exosomes, and thus can readily influence the development of PE (395). In addition, STMB from PE patients possess increased tissue factor activity compared to normotensive patients (396) and this could increase fibrin deposition. Coagulation may be enhanced by STMB action directly by direct association with platelets leading to activation: such activity is increased in PE-derived STMBs and correlates with PE-associated thrombotic risk. Moreover, treatment with aspirin, which is usually prescribed for PE women to reduce platelet aggregation, also inhibits STMB-induced platelet aggregation (397).

Cell-free haemoglobin (HbF) is released by the placenta and increased haemoglobin (Hb) expression as well as HbF accumulation in the vascular lumen of PE placentas has been reported (398). Indeed, HbF has been proposed as an important factor marking the transition between the first and second stages of PE. HbF causes placental damage similar to that observed in PE by inducing oxidative stress, which affects the blood-placenta barrier (BPB) integrity (399). BPB disruption may lead to the release of placental factors, including HbF which leak into the maternal circulation contributing to the maternal affectations of PE. Moreover, levels of HbF correlate with PE severity symptoms (400). Placental HbF can provoke differential alterations in STBM miRNA cargo between EVs populations: three miRNAs were specifically downregulated in microvesicle populations under HbF influence. STBMs may also transport HbF itself, although these data may be an artifact of the external HbF perfusion (401). Furthermore, STBMs from PE pregnancies exacerbated the production of superoxide radicals by neutrophils in a dose-dependent manner, also correlating with PE severity. In this way, STBMs display multiple mechanisms to cause vascular damage and dysfunction in women with PE (402).

b. Maternally-derived EVs

Even before pregnancy, maternal risk factors for PE are obesity, diabetes mellitus, hypertension and Systemic Lupus Erythematous (SLE). Pro-PE EVs have altered concentrations and modified molecular contents that may alter the functioning of maternal tissues prior to pregnancy. In particular, changes in endothelial cells, leukocyte and platelet-derived EVs are associated with the risk of PE. All share the common feature of a general increase in endothelial and platelet-derived EV levels (for review see (403)).

Once pregnancy is established, maternal EVs of different cellular origin interact with embryonic tissues with potential implications in PE pathogenesis. Platelets have crucial roles in PE development and several studies report decreased platelet-derived EV levels in pregnancy compared to non-pregnancy, with further decrease in PE (403). EVs of maternal endothelial and platelet origin appear to unleash a thrombo-inflammatory response in the placenta. EVs cause activated platelet aggregation and inflammasome activation within the placental vascular and trophoblastic cells, triggering a PE-like phenotype. Further, inhibition of inflammasome or platelet activation components within the placenta abrogated the PE-like phenotype (404).

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In contrast to platelets, leukocytes and certain derived EVs populations are increased in PE in comparison to normotensive pregnancies, mainly those EVs of granulocyte and monocyte origin (405). Interestingly, low levels of NK cell-derived EVs are observed in PE, linking with PE-associated maternal immune tolerance disorders (NK cell death activity dysfunction) (406). Of interest, Holder and collaborators showed that human placenta is able to internalize exosomes from macrophages via endocytosis. Importantly, macrophage exosomes uptake induced the release of proinflammatory cytokines by the placenta (407). Previously, the same group had reported that exosomes from PE placenta can activate peripheral blood mononuclear cells (PBMCs), inducing a pro-inflammatory response to a greater extent than EVs from normal placenta, and related to their cytokine content, mainly IL1β. Moreover, PE-derived EVs stimulated an enhanced response of PMBC to external PAMPs such as LPS (407). Such outcomes may be triggered by direct stimulation by EVs of TLR, the signal subsequently internalized via NF-κB (408). Taken together, these studies indicate a potential positive feed-back loop by which an inflammatory response is overstimulated under PE conditions via EVs. Endothelial-derived EVs levels correlate with the increment of the anti-angiogenic factor sFLt1 and the ratio sFLt1:P1GF. This combined evidence suggests that apoptosis of endothelia occurs along with inhibition of angiogenesis, and correlates with PE-characteristic endothelial damage which persists between <1 week (409) to 72 hours postpartum (410).

Regarding obesity, a link between exosomes release and the progression of PE is emerging. A recent study has observed that the levels of exosomes in maternal blood are correlated with maternal BMI. A positive correlation of BMI with EXO levels was established, leading to the decrease of placental-derived exosomes proportions throughout gestation. These increased exosomes levels contributed to a further exacerbated release of IL-6, IL-8 and TFN-α from endothelial cells thus leading to worsened systemic inflammation in a BMI-dependent manner (411).

Finally, it has been observed that serum microvesicles from healthy pregnant women can reduce caspase activity and stimulate migration and tube formation in endothelial cells, while this is abrogated when the microvesicles are derived from patients with gestational vascular complications such as PE and hypertension. Further, similar opposing actions on early-stage trophoblast of these vesicles was observed (412).

iii. EVs in gestational diabetes

Gestational diabetes (GD) is defined as a carbohydrate intolerance of variable severity that appears or is first recognized during pregnancy. Along with PE, GD represents the most common metabolic complication of pregnancy, affecting between 1 and 15% of all pregnancies and increasing concurrently with obesity rates. It is characterized by pancreatic beta cell insufficient insulin production, usually due to pregnancy and characteristic insulin resistance, and is associated with maternal and fetal morbidity. Moreover, women with GD have increased risks of developing type II diabetes in the future (413,414).

To date, little is known about the contribution of EVs in this pathophysiology. Salomon and colleagues showed increased serum placenta-derived exosomes in GD pregnancies compared to control pregnancies, regardless of gestational age. In vitro, GD exosomes increased the release of proinflammatory cytokines from endothelial cells contributing to the enhanced proinflammatory state in pregnancy under GD conditions (415).

Part V. Clinical and therapeutic applications of EVs

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The involvement of EVs in a wide variety of pathophysiological processes has made them appealing players as biomarkers and to carry therapeutic agents. This may also be the case when considering reproductive disorders.

I. EVs as biomarkers

EVs have been proposed as potential biomarkers of disorders of reproductive organs. The placenta releases EVs from the sixth week of pregnancy with steady increase as pregnancy proceeds, peaking at term (397). Importantly, their release is modulated by a number of factors that arise from the placenta; hence EVs may provide mirrors of placental/foetal health and evolution (369). Since maternal blood is the primary source of placental exosomes it will contain both maternal and placenta-specific EV populations and thus placental alkaline phosphatase (PLAP) has been proposed as a marker for the placental EVs, since it is restricted to placental cell linages (365).

Alterations in both, the levels and cargo of placental-derived exosomes during pregnancy are associated with different pregnancy complications. A proteomic signature of 62 proteins in microparticles was developed from plasma samples of women at 10-12 weeks of gestation (403). This signature was able to predict and differentiate spontaneous premature births (SPB) from normal term births. Functional enrichment analyses showed processes related with preterm birth, such as inflammation, fibrinolysis, immune modulation, the coagulation cascade or steroid metabolism. Currently, the only tool for evaluation of risk of spontaneous preterm birth is measurement of cervical length by ultrasound (404). A retrospective study on plasma samples of women at early gestational age (prior to 18 weeks), demonstrated potential for exosomes in the diagnosis of PE and SPB with higher (but not significant) levels of exosomes in both pathological conditions versus normal pregnancies. More interestingly, a specific exosomal miRNA signature could differentiate between the three conditions, being more similar between normal pregnancy and SPB compared with that of PE. When these miRNA profiles were compared with those from the extravillous trophoblast HTR-8/SVneo cell line cultured under normal and low-oxygen tension (LOT) conditions there was a strong correlation between the SPB and LOT conditions, with a common variation in >45% of the SPB condition miRNA profile. Placental-exosomal miRNA cargo was related to cell migration potential and inflammatory cytokine production. Particularly, LOT-exosomes decreased endothelial cell migration potential and increased their TNF-α production, which could negatively impact spiral artery remodelling during placentation. Thus, under circumstances that favour a pro-inflammatory environment or a reduction of oxygen tension such as advanced gestational age, placental EVs may be negatively altered, impacting spiral artery remodelling and resulting in development of pathologies such as PE or SPB (405). In this sense, placental EVs may be potential early biomarkers of PE/SPB or as targets for directed therapy. Finally, both total and placental-derived EVs are increased in women delivering low-birthweight babies compared to those with normal-birthweight deliveries (416).

EVs have been further investigated as biomarkers of PE. Recent publications debate the usefulness of EVs' content for their predictive value in the diagnosis of PE. As an example, Tan and colleagues analysed three candidate biomarkers, TIMP-1, PAI-1 and P1GF, for their predictive ability in a large cohort of low-risk PE women from EVs isolated from bank plasma samples. They concluded that measurement of TIMP-1 and PAI-1 reinforced the value of the classical P1GF for PE prediction (417). Indeed, TIMP-1 and PAI-1 were analysed in specific subgroups of EVs which can be retrived thanks to their affinity to cholera toxin B and annexin V, both of which had been described previously in the search for PE biomarkers. In this study, EVs

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were purified from plasma of women at ~32 week of pregnancy, using immunoadsorption to the surface proteins, GM1 ganglioside (binds to cholera toxin B chain) and phosphatidylserine (binds to Annexin V). Using these two populations of EVs (one from each marker), a specific protein signature was identified in women with PE compared to healthy pregnant controls. It is important to highlight that such biomarker discovery is highly dependent on the selected conditions, providing a possible limitation. Indeed, in this study, large cellular debris were not removed from samples prior to the immunoadsorption step, providing a major potential source of error (418). In another study, different subtypes of microvesicles were evaluated in plasma, compared with cord blood from normal women and those with PE. Microparticles were more abundant, and had altered coagulation–related factors in cord blood in PE compared with no PE (419). Recently, Salomon and colleagues (2017) investigated whether exosomes and their miRNA cargo might provide early biomarkers of PE. Over 300miRNAs were identified in total and placenta-derived exosomes in maternal plasma across gestation with has-miR-486-1-5p and has-miR-486-2-5 being identified as candidates for further study. Functional analysis showed that these miRNAs are involved in migration, placental development and angiogenesis (420). Since PLAP is a marker of serum placental-derived exosomes, which trend upwards with gestational age, exosomal content of PLAP has been proposed as a potential biomarker of PE in saliva and gingival cervical fluid (421). Finally, reduced EV-associated endothelial nitric oxide synthase expression and activity, a common feature of PE, was elevated in EVs from PE placentas (defined by PLAP), in both serum and placental perfusates, compared with healthy controls (422). Considering the above information, it is important to note that current biomarkers of pregnancy complications, such as PE or GDM, allow us to diagnose these states only once the pathologies are established and when the clinical management is limited. In this sense, in order to progress the field, efforts should focus on discovery of new biomarkers during early gestation.

EVs have also been proposed as biomarkers of peripartum cardiomyopathy (PPCM). PPCM is an idiopathic form of cardiomyopathy characterized by left ventricular systolic dysfunction (the ejaculation fraction is reduced normally bellow 45%) and subsequent heart failure. It usually appears around the end of pregnancy and in the next few months and, it is currently only diagnosed by exclusion of other heart failure causes (423) making a search for new biomarkers of considerable importance. Initially, Walenta and collaborators (2012) reported increased levels of blood-derived activated endothelial microparticles in PPCM when compared with healthy post-partum, pregnant and non-pregnant control but also with patients of ischemic cardiomyopathy (ICM) and stable coronary arterial disease (CAD). These microparticles in PPCM were mainly platelet-derived and monocyte microparticles. Treatment with bromocriptine, a therapy proven to work in animal models and human patients, significantly reduced endothelial and platelet-derived microparticles in PPCM compared to patients treated with standard undirected heart failure therapy. Thus, specific microparticle profiles may provide biomarkers that can distinguish PPCM from normal pregnancy, vascular diseases and heart failure of different origin (424). MiR146a has also been identified as a possible exosome-associated biomarker for PPCM. The 16-kDa N-terminal prolactin fragment, the primary known trigger of PPCM, stimulates the packaging of miR-146a into exosomes from HUVECs, which then are able to reach cardiomyocytes and trigger PPCM. Thus miR-146a may provide a biomarker and therapeutic target for PPCM (425).

Placental EVs may provide indicators of infectious diseases during pregnancy. Both total and placental-derived EVs are increased in plasma from pregnant women with HIV infection compared with non-infected controls. In contrast, there were no changes in the level of plasma

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EVs due to malaria infection, neither for placental malaria nor for its peripheral variant. Nonetheless, miR-517c was found to be increased in microparticles from plasma of women with active placental malaria compared with non-infected controls (416).

II. Clinical and therapeutic aspects of EVs in reproductive biology

Intercellular transfer of genetic and protein material mediated by EVs could facilitate new diagnostic and therapeutic tools in the field of reproductive biology. As discussed, EVs are stable, versatile, cell-derived nanovesicles with target-homing specificity and the ability to transfer through *in vivo* biological barriers and hold promise for the development of new approaches in drug delivery (75). Specifically, bioengineered EVs are being successfully deployed to deliver potent drugs and the capacity for select cellular reprogramming capacity (6,41). Recently, members of the International Society for Extracellular Vesicles (ISEV) and the Society for Clinical Research and Translation of Extracellular Vesicles presented a framework for challenges associated with development of EV-based therapeutics at the preclinical and clinical levels (426). This discussion addresses development of best-practice models and current outlook for EV therapies.

Engineered or modified EVs can be designed for cell-specific targeting and delivery (427,428). A seminal study has demonstrated the selective cellular uptake of EVs surpasses that of more traditional carriers such as liposomes or nanoparticles, taking advantage of EVs' natural characteristics to deliver molecules to target cells (429). Such insights provide future possibilities for clinical applications of EVs based on their ability to circumvent the limitations of various drug delivery systems of mucosal and blood brain barrier traversal. The physicochemical configuration of EVs can also be modified to enable extended clearance compared with synthetic nanoparticles, and spatio temporal localization (ligand and cell-type specific targeting) and controlled release (229,430-432). With respect to modifying EV cargo, a recent, comprehensive study compared various passive and active drug-loading methods including electroporation, saponin treatment, extrusion and dialysis, and used porphyrins of various hydrophobicities as model drugs (433). A comprehensive overview of EV cargo loading strategies, including electroporation, sonication, direct transfection, and cellular engineering is reviewed (434,435).

The potential functional roles of EVs in human embryo development have only recently been demonstrated. Embryos may generate their own microenvironment by secreting soluble factors and membrane vesicles, which constitute a secretome with select autocrine and paracrine signaling (91,436-440). In reproductive biology, nanoparticles have been used experimentally to load sperm with exogenous genetic material that is subsequently transferred to the oocyte during fertilization (441,442). EVs have been identified in uterine fluid during the estrous/menstrual cycles including humans, sheep and mice (75,151,167,329,443). Indeed, EVs derived from the maternal endometrium contain multiple subtypes including mixtures of EVs, exosomes and packaged different proteins, miRNAs and endogenous retrovirus mRNA (91,151,329-331,339,444). In the broader context of trophectodermal preparation for implantation, EVs have been shown to mediate communication between the inner cell mass (ICM) and the trophectoderm (88). EV-encapsulated cargo is protected from degradation and are highly stable in biological fluids. Such unique properties may greatly facilitate the translation of EVs and their select bioactive cargo and surface ligands into clinical applications. The study of EVs in reproduction has the potential for expanding our current understanding of the normal physiology of reproduction and pathological conditions such as implantation failure (439). Recent studies have provided key insights into the functional capacity of maternal EVs and how the protein cargo is directly modulated by uterine hormones during implantation to subsequently modulate

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trophoblast adhesive capacity (91). This study further validated select components in primary human endometrial cells under hormonal control.

Recent studies have observed the ability of EVs to undergo cell-selective fusion (445) and tissue-specific tropism (219,446-448), as well as their capacity to transverse the blood-brain barrier (449) and penetrate dense structural tissue (450). Importantly, based on their surface composition, EVs may be directed to specific tissues and organs (219,446-448). Imaging of EVs in select targeted organs has indeed demonstrated that the interactions of EVs with target cells are highly dynamic (223,451). Such unique properties of circulating EVs make them promising applications for the delivery of therapeutic cargo. Several studies support the utility of EVs as a novel path for drug delivery and as new drug targets. Alvarez-Erviti et al., in an in vivo study, demonstrated that systemically injected neuron-targeted exosomes loaded with BACE1 siRNAs (small interfering RNA) were able to significantly reduce BACE mRNA and protein, specifically in neurons (452). Further, exosomes loaded with artificial siRNA against MAPK efficiently knocked down MAPK1 upon their delivery into monocytes and lymphocytes in vitro (453). Similarly, exosomes from iPSCs have been shown to deliver siRNA to attenuate expression of ICAM1 and neutrophils adhesion in pulmonary microvascular endothelial cells (454). Exosomes have further been applied for drug delivery to target a small-molecule, anti-inflammatory drug to select organs and immune cells (455). These studies have demonstrated the capacity for EV-mediated targeted and delivery capacity and importantly the ability for exosomes to deliver and modulate multiple pathways simultaneously in the targeted cells. All these studies are examples showing how EVs cargo can be manipulated in a way that may be useful for target-based drug development for successful in vivo drug delivery.

Recent reviews have discussed the rationale to aim for selective silencing of EVs that promote unwanted functional effects. However this is still an emerging concept in the field. Some of the strategies for specific silencing of EV subtypes (cell-specific) are likely to require careful and detailed mechanistic studies. There are inhererent difficulties in avoiding the blocking of all EV types indiscriminately, which may interfere with and perturb physiological intercellular communication. Some examples of systems for abrogating EV formation and targeting/recipient cell uptake (reviewed include (213,221,435,456): (i) inhibition of exosome formation, including treatment with dimethyl amiloride, (ii) inhibition of the endolysosomal compartment functions, including proton pump inhibitors (PPI), (iii) blocking of exosome release, (for example silencing GTPase Rab11/27A/35 using siRNA or targeting ESCRT proteins and/or GTPases involved in trafficking of exosomes), and (iv) prevention of fusion or uptake of exosomes by target cells, which can be done using a variety of reagents that block phosphatidyl serine such as diannexin, heparin to inhibit endocytosis (heparan sulphate proteoglycans), cytochalasin D to inhibit endocytosis and micropinocytosis, chlorpromazine to inhibit clathrin-dependent endocytosis, EIPA and LY294002 to block micropinocytosis, annexin-V to inhibit phagocytosis and macropinocytosis, methyl-β-cyclodextrin (MβCD), simvastatin and filipin III to target lipid raft–mediated endocytosis, nystatin to target caveolae-mediated endocytosis, dynasore to inhibit clathrin-independent endocytosis (calveolae), and nystatin to perturb lipid raft–mediated endocytosis.

Future studies are required towards investigating EVs from primary tissues and biofluids and incorporate state-of-the-art quantitative analyses, including quantitative proteomics (174,457) and sequencing technology that could be exploited to study protein and gene regulation during pregnancy. These would enable identification and monitoring of functional or low-abundant EV cargo, and cellular drivers of implantation and signaling, that hitherto, have been unreported or

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functionally masked. Unlike small molecule pharmaceutical compounds, there are no defined parameters or assays for current safety testing of EV-based therapeutics (458). Understanding biodistribution patterns and circulating timeframe of locally and systemically administered EVs is important to assessing safety, in addition to techniques which enable reproducible monitoring and safety testing of select EV marker cargo. Targeted studies using EVs (modified or engineered) will hold the potential to develop novel nanodiagnostics and nanotherapeutics to increase the success of pregnancy rates during ART or IVF. Recent work on targetable biodegradable delivery platforms for transporting biological cargo into gametes and embryos (reviewed (459)), emphasizes the need to understand how EVs enter cells. We anticipate that future investigations into the use of EVs for the intentional targeted delivery of molecular compounds will provide new horizons for reproductive science and clinical ART, ultimately leading to improvements in pregnancy success.

Part VI. Concluding remarks

Considering the body of evidence treated in the present review, there is no doubt that the field of EVs and its implication in reproduction is rapidly evolving and promises a further understanding of the processes that lead to a successful pregnancy, as well as markers of correct or compromised reproductive function. Nonetheless, there is still a difficult path to negotiate. Firstly, there is an unavoidable need to firmly define standard methods for EVs isolation, since these define the fractions considered as different EVs populations and, as such, may lead to ambiguous results that cannot be compared among studies. New challenges associated with standardization of methods for isolation, quantification and analysis of EVs from complex tissues such as blood, and the stability of EVs within such biofluid samples, need to be overcome, before the EV field can provide reliable tools for diagnosis and therapy.

It is also necessary to define the extent to which EVs are important participants in the reproductive events that lead to the delivery of healthy normal newborns, as this knowledge will lead to new therapies and clinical test to ensure good pregnancy outcomes. Sample availability is maybe one of the main limiting factors that hinders such progress. In this sense, much more is known about epididymal and prostatic EVs regulation of sperm compared with embryo maternal cross-talk through EVs. Nevertheless, EV communication may provide a cornerstone to enable better understanding of the conception and implantation processes. This is important as it paves the way to deal with those patients in which the current assisted reproductive techniques fail.

Finally, data regarding the involvement of EVs in the triggering, maintenance and progression of reproductive and obstetric related disorders is still in its infancy and further key investigations utilizing homogeneous and human-specific material is needed. The use of EVs as disease biomarkers provides the opportunity for diagnostic potential with reduced invasiveness, as they can be retrieved from body fluid instead of tissue biopsies. This is vital for embryo diagnoses, where the possibility of getting STMBs from mother blood-flow appears as an interesting alternative to invasive amniocentesis and chorionic villi sampling, further offering the possibility of an earlier diagnostic. Regarding EVs use as therapeutic agents, many different variants could be exploited. EVs could be used as vectors to deliver drugs and biological compounds in a targeted manner. Nevertheless, they could potentially be used as therapeutic targets, if they are produced by affected cells and present disease promoting characteristics. This may be achieved by inhibiting EV biosynthesis, by capturing them once produced or by blocking their uptake by target cells, and may be applicable in diseases such as pre-eclampsia. Further, they could be used as natural therapeutic agents when experimental strategies rely on their

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natural features. Understanding cell-type specificity and the long-term effects of EV remodelling, and their potential to impart transgenerational consequences on the offspring's health, ranging from metabolism to sex determination, and potential epigenetic changes affecting the mother's fertility and altering the offspring's fertility, are key factors to be addressed as Need the field moves forward. EVs derived from the immune cells including dendritic cells within the reproductive tissues also need examination, since such cells, once stimulated, may trigger detrimental immune responses. Advances in research on noncoding RNAs contained in EVs must also be considered (460). Understanding all these molecular signaling networks, utilising advances in quantitative proteomics and sequencing technology, and mediated by EVs that coordinate strategies for successful implantation, may lead to approaches to improve the outcomes of natural pregnancy and pregnancy achieved using reproductive technologies.

Abbreviations

AB: Apoptotic body

AFM: Atomic force microscopy

BLVRA: Biliverdin reductase A

BPB: Blood-placenta barrier

CAD: Coronary arterial disease

cAMP: Cyclic adenosine monophosphate

DLS: Dynamic Light Scattering

dsDNA: double stranded DNA

ED: Erectile dysfunction

ELISA: Enzyme-linked immunosorbent assay

EM: Electron microscopy

ER: Endoplasmic reticulum

ESCRT: Endosomal sorting complexes required for transport

EV: Extracellular vesicle

EVT: Extravillous trophoblast

EXO: Exosomes

FAK: focal adhesion kinase

GD: Gestational diabetes

GVC: Gestational vascular complications

Hb: Haemoglobin

HbF: Cell-free haemoglobin

HDL: High density lipoproteins

HIV: Human Immunodeficiency Virus

HT: Hypertension

ICM: Ischemic cardiomyopathy / Inner cell mass

IIEF: International Index of Erectile Function

ILV: Intraluminal vesicle

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ISEV: International Society for Extracellular Vesicles

lncRNA: long non-coding RNA

LOH: Late onset hypogonadism

LOT: Low-oxygen tension

MERVL: Endogenous retrovirus-like element

miRNA: microRNA

MMP: Matrix Metalloproteinase

mtDNA: mitochondrial DNA

MV: Microvesicle

MVB: Multivesicular body

μNMR: Micro nuclear magnetic resonance spectrometry

NK: Natural Killer cells

NOS: Nitric oxide synthase

nPLEX: nano-plasmonic exosome assay

NTA: Nanoparticle tracking analysis

PBS: Phosphate buffer solution

PBMC: Peripheral blood mononuclear cells

PCOS: Polycystic ovarian syndrome

PE: Pre-eclampsia

piRNA: piwi-interacting RNA

PKA: Protein kinase A

PL: Pregnancy loss

PLAP: Placental alkaline phosphatase

PPCM: Peripartum cardiomyopathy

PS: Phosphatidylserine

RI: Refraction index

RSV: Respiratory syncytial virus

SEM: Scanning electron microscopy

siRNA: small interference RNA

SLE: Systemic Lupus Erythematous

snoRNA: small nucleolar RNA

SPB: Spontaneous premature births

SPZ: spermatozoa

STB: syncytiotrophoblast

STMB: Syncytiotrophoblast-derived exosomes and microvesicles

TEM: Transmission electron microscopy

tRFs: tRNA fragments

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tRNA: Transfer RNA

TRPS: Tuneable Resistive Pulse Sensing

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Figure 1. Main types of extracellular vesicles present in body fluids and culture media. EVs are classified in three groups according to their biogenetic pathways. Exosomes are produced in the endosomal pathway by invagination of the membrane of late endosomes to form intraluminal vesicles (ILV) enclosed in multivesicular bodies (MVB). MVBs can then fuse with lysosomes and degrade its content, or fuse with cell plasma membrane to release ILV, now regarded as exosomes. Microvesicles are produced directly from the cell plasma membrane by outward budding. Apoptotic bodies are generated as blebs in cells undergoing programmed cell death. Abbreviations: **E.E.:** Early Endosome, **Ex.V.:** Exocytic vesicle, **L.E.:** Late Endosome, **M.V.B.:** Multivesicular Body, **I.L.V.:** Intraluminal Vesicle, **EXO:** Exosome.

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Figure 2. Pathways shown to participate in EV uptake by target cells. EVs transport signals between cells and facilitate selective reprogramming. EVs have been shown to be internalized by cells through **(1)** phagocytosis, **(2)** clathrin- and **(3)** caveolin-mediated endocytosis. There is also evidence to support their interaction with **(4)** lipid rafts resulting in EV uptake. Lipid rafts are involved in both clathrin- and caveolin-mediated endocytosis. EVs may also deliver their protein, mRNA and miRNA content by **(5)** fusion with the plasma membrane. EVs can be internalized by **(6)** macropinocytosis where membrane protrusions or blebs extend from the cell, fold backwards around the EVs and enclose them into the lumen of a macropinosome; **(7)** alternatively EVs are macropinocytosed after becoming caught in membrane ruffles. On the other hand, **(8)** intraluminal EVs may fuse with the endosomal limiting membrane following endocytosis to deliver their protein, mRNA and miRNA cargo and elicit a phenotypic response.

Table 1. Classification of the methods of isolation of extracellular vesicles based on their principle.

METHOD	TECHNIQUE	ISOLATION PRINCIPLE	GENERAL WORKFLOW	ADVANTAGES	LIMITATIONS	REFERENCES
Centrifugation	Serial differential centrifugation	Sedimentation velocity	Serial or differential centrifugation: (1) 300 x g, 10' remove cells → (2) 2.000 x g, 10' remove cell debris, apoptotic bodies → (3) 10.000/20.000 x g, 30' to isolate microvesicles → (4) 100.000/200.000 x g, 70' to isolate exosomes.	• Broad application • Standardization. • Ease of use • Reproducibility • Yield	• Sedimentation dependent on density, tube length, sample viscosity, concentration and vesicle aggregation apart from size.	(16,112,116,461)
	Density gradient	Buoyant density	Generally introduced to further purify distinct types of EVs (i.e., microvesicles or exosomes). Various different reagents including sucrose or ionidxanol. Crude EV populations loaded either on top (float down) or at bottom (float up) of gradient. Ultracentrifugation performed under pre-established conditions	• Purification - increases EVs populations purity from: protein aggregates, RNA-protein complexes, separation of EVs subpopulations within the same type.	• Yield	(4,112-115,117-119,462)
				• Soft isolation approach	• Reproducibility	
				• Clinically applicable medium (ionixodonal)	• Trained user	
				• EV homogeneisty	• Time-consuming.	
Size-exclusion	Filtration	Size/shape	Generally interspersed within centrifugation steps: prior to centrifugation, supernatants are	• Easy to use.	• Yield loss within filtering membrane.	(112,120,121)
				• Further stringency of the populations based on their canonical sized.	• Risk of vesicles deformation or	

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			challenged through syringe filters of determined pore size.		fragmentation.	
				• Reproducibility		
	Ultrafiltration	Size	Centrifugal filtration units of prefixed molecular size range that selectively retain vesicles Previous studies shown to isolate distinct subtypes of EVs using this strategy	• Easy to use.	• Yield loss within filtering membrane.	(73,122-125,463)
				• Quick technique.	• Risk of vesicles deformation or fragmentation.	
				• Reproducible		
	Chromatography	Size/charge	Purification of EVs based on surface charge or size	• High resolving power – improved purification of EVs from proteins and lipid particles.	• Usually coupled to centrifugation in order to remove cell debris and recoverEV contianingfractions.	(126-128,130)
				• Limits EVs and proteins aggregation based on buffer utilised.	• Often issues with volume or buffer associated with elution	
				• Less sensitive to the viscosity of the media.		
				• Respectful with EVs functionalities and biological properties.		
				• Shorter isolation times.		
Immunoaffinity		Presence of specific EV surface molecules	Microbeads coupled to antibodies are incubated with EVs for specific surface markers recognition (i.e., A33, EpCAM, CD63). Afterwards, beads are washed and recovered by precipitation or magnetism.	• Separation based on specific molecules further than by size.	• Sometimes coupled to centrifugation and/or filtration to initially remove larger cellular debris.	(96,98,99,135-137)
				• Selectivity	• Select surface markers of EVs are not always known/available.	
				• Resolution	• Cost	
				• Speed of isolation	• Yield	
Polymeric precipitation		Weight increase to pellet at low centrifugal force	Incubation of polymerization kit reagents with EVs solution and recovery by low-speed centrifugation-	• High speed	• Possibility of co-precipitating impurities.	(111,138,139)
				• Simple procedure	• Unable to separate EVs fractions.	
					• Ideal only for small (60 to 180 nm) EVs populations.	

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Microfluidics		Different possible principles:	(1) EVs are passed through microfluidic system and EVs specific markers are recognised by antibodies in a device surface.	• Reduced sample volume needed.	• Habitually couple to centrifugation in order to remove undesired EVs populations.	(141,142,156,464)
		(1) Presence of specific molecules.	(2) Still not applicable for EVs.	• Smaller processing times and costs, maintaining high sensitivity.	• Unable to differentiate EVs populations.	
		(2) Physical properties such as size.	(3) Combination of microfluidics and polymer filter that allow passing EVs under a certain size.	• Possibility to process, quantify and image the samples within the system itself.	• Still under development.	
		(3) Microfluidic filtration.				

Table 2. Classification of the methods of characterization of extracellular vesicles based on their principle.

METHOD	TECHNIQUE	PRINCIPLE	MAIN FEATURES	QUANTITATIVE / QUALITATIVE	REFERENCES
Microscopy	Transmission electron microscopy (TEM)	Negitive staining of EVs with electron-dense molecules (heavy metals).	• Direct imaging of EV size	• Semi-quantitative.	(147-149,462,465)
			• Size distribution	• Dehydrating (fixation)	
			• Can be couples to immunogold labelling to stain specific structures.	• Possibility to take measures within the imaging field.	
	Scanning electron microscopy (SEM)	Covering of molecules with microgold particles and electron reflexion scanning.	• Three-dimensional imaging of EVs structures.	• Semi-quantitative.	(151,152,466)
				• Possibility to take measuresmentswithin the imaging field.	
	Cryo-electron microscopy (Cryo-EM)	Plunge-frozen in liquid ethane/nitrogen.	• Avoids fixation and contrasting steps.	• Semi-quantitative.	(73,150)
			• Allows to see structures closer to their native states.	• Possibility to take measures within the imaging field.	
			• Size distribution	• Highly trained user	
	Atomic force microscopy (AFM)	Use of a cantilever with a free end that touches the surface to obtain topographical information.	• Resolution at the nanometric level.	• Quantitative.	(153-156)
			• Possibility to analyse both dry and aqueous samples.	• Size-distribution profiles determination.	
			• Can be combined with microfluidic isolation devices.	• Require homogeneous EV purification	
			• It does not provide direct imaging of EVs.		
Size distribution analysis techniques	Nanoparticle tracking analysis (NTA)	Particles are challenged with a laser beam and forward scattered light is real-time	• Size measures in the range of 50 to 1000 nm.	• Qualitative: not only size populations but also EVs markers can be analysed by fluorescent labelling.	(157,467,468)

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		captured by a microscope to calculate sizes based in particles their Brownian motion.	• Standardization is not needed but possible (interest for concentration assessments).	• Quantitative: possibility to get precise size distributions and their associated concentrations in 1 nm intervals.	
			• Size distribution	• Cost	
			• Low sample use		
			• Compatibility of fluorescence detectors		
	Dynamic Light Scattering (DLS)	Particles are challenged with a laser beam and reflected light is captured by a detector in a certain variable angle. The detector converts time dependent fluctuations in the scattered light intensity into particle size data.	• Size measurements in the range of 1 to 6000 nm for EVs concentrations from 10^6 to 10^9 particles/mL.	• Mainly qualitative.	(158,469)
			• Samples can be recovered after the analysis.	• Semi-quantitative if standards are used.	
			• Limitations with polydisperse samples and those containing big EVs.		
	Tunable resistive pulse sensing (TRPS)	A transmembrane voltage is established in a porous membrane. The crossing of EVs through the pores alters the electrophoretic flow causing a resistance that can be translated into size data.	• Size measurements in the range of 70 nm to 10 µm for EVs concentrations from 10^5 to 10^{12} particles/mL.	• Qualitative.	(159-161)
			• Single EVs measures that allow multimodal EVs populations study.	• Quantitative	
			• By modifying pores configuration the analysable EVs size and sample volume can be regulated.		
	Flow cytometry	EVs are swept along by a liquid stream to align them in single file in the centre of the stream until the interrogation point, where they are excited by a laser beam. Laser scattered light is gathered by detectors situated 180º (size data) and 90º (morphology or fluorescently stained structures data) to the laser beam.	• Analysis of EVs with a lower size limit of 250-500 nm and ability to distinguish vesicles that differ 200 nm in size.	• Qualitative: not only size populations but also EVs markers can be analysed.	(162-167)
			• New technological developments have reduced the limit of detection to ~100 nm and the discrimination power to 100-200 nm.	• Quantitative.	
			• Possibility to coupling to latex		

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			beads for easy marker analysis. • No sorting capacity • Dependent on EV surface markers or use of EV fluorescent labels		
Molecular markers characterization techniques	Western blotting / ELISA	Both techniques share the same principle: proteins are attached to support (membranes or plates, respectively) and challenge with antibodies carrying a certain label.	• Easy to perform. • Cheap and available. • Relatively quick.	• Qualitative. • Semi-quantitative in the case of Western blot and quantitative for ELISA.	(39,168,169)
	ExoScreen	ELISA sandwich-like system with modifications in the detection tandem. The method relies in that all the components of the system must stay closed (~200 nm, within the same vesicle) for a laser stimuli transfer and detection.	• Reduced time consumption. • Increased sensitivity. • EVs isolation is not mandatory. • Little sample volumes are required.	• Qualitative. • Quantitative.	(170)
	µNMR	Labelling of specific EVs surface molecular markers with antibodies coupled to magnetic nanoparticles and detection by microfluidic µNMR.	• Greatly higher sensitivity.	• Qualitative. • Quantitative.	(171)
	Nano-plasmonic exosome assay (nPLEX)	A gold film with nanoholes coated with specific antibodies for the recognition of exosomal proteins is light-excited, generating surface plasmons. Joining of EVs to the antibodies cause plasmon intensity changes that are proportional to the amount of joined EVs.	• Label-free. • Easy to miniaturize. • Scalable for higher throughput detection. • A magnitude order more detection sensitivity than µNMR.	• Qualitative. • Quantitative.	(172,173)

Table 3. Main functions of extracellular vesicles in reproductive physiology classified by their origin.

EV TYPE	MAIN FEATURES	TARGET	FUNCTIONS	REFERENCES
Epididymosomes	- First described by Piko *et al* in 1967.	SPZ	Transfer of molecules involved in sperm maturation (P25b, GliPr1L1, MIF, SPAM1, PMCA4)	(256-258,262,264)

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	- Sizes: 50 to 8000 nm or even 2-10 µm. - Two main classes: CD9-positive (affinity for live SPZ) and ELSPBP-1-enriched (affinity for dead SPZ) epididymosomes				
			Protection from oxidative stress (BLVRA)		(261)
			Protection from lipid peroxidation (GPX5)		(267)
			Morphology and membrane composition regulation (ADAM7)		(265,266)
			Sperm motility (ADAM7, PMCA4)		(264-266)
			Small RNA regulation of gene expression		(273,274)
Prostasomes	- First described by Ronquist *et al* in 1978. - Sizes: 30 to 500 nm. - Unusual lipid composition that provide them with increased ordered structure, rigidity and viscosity.	SPZ	Enhancement of sperm motility (progesterone receptors, Ca^{2+} cascade signalling components, aminopeptidase N.		(284,288,289,291,292)
			Protection from acidic female reproductive tract environment.		(285)
			Protection from oxidative stress (PMCA4).		(289)
			Prevention of premature capacitation and acrosome reaction (cholesterol)		(282,296-298)
			Posterior induction of capacitation, SPZ hypermotility and acrosome reaction at the moment of fertilization (cAMP, progesterone receptors, hydrolases, lipoxygenases).		(299,302,303,305,306)
			Protection from the hostile female reproductive tract: immunity, oxidative stress, bacteria.		(276,282,299)
Uterine microenvironment EVs	- Wide variety of origins: serum transudates, residues from womb cell apoptosis, endometrial epithelial cells and conceptus. - Variations throughout the menstrual cycle. -	Endometrium	Endometrial origin	Promotion of embryo implantation (specific miRNA cargo)	(167)
			Embryo origin	Regulation of endometrial angiogenesis (specific miRNA and protein cargo) and uterine spiral arteries remodelling.	(332,366)
		Embryo	Endometrial origin	Embryo development (enJSRV *env* gene RNA) and subsequent priming of the endometrium for embryo harbouring.	(329-331)
				Promotion of embryo implantation (miR-30d, specific protein cargo, influenced by uterine hormones – functional with trophoderm).	(91,151)
			Embryo origin	Enhancing of trophoblast cells migratory ability and implantation efficiency (laminin, fibronectin).	(88)
		SPZ	Sperm maturation (SPAM1)		(444)
			Capacitation, acrosome reaction and motility promotion (PMCA4).		(234,470)
Oviductal EVs	- First	SPZ	Regulation of SPZ storage and promotion of capacitation, acrosome reaction and hypermotility		(234,321,322,471)

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	described for their implications in SPZ final competence acquisition.		(PMCA4a).	
			Regulation of molecule delivery into SPZ (Integrins α5β1 and αvβ3).	(233)
		Embryo	Enhancement of embryo quality and early development.	(323)
Follicular EVs	- First described by da Silvera *et al*. in 2012.	Cumulus-oocyte complex	Follicle development and oocyte growth (specific miRNA cargo, ACVR1, ID2)	(317,319)
	- miRNA cargo variation with female age and reproductive aging.		Follicle maturation: proliferation of small follicles and inflammatory response of large developed follicles (specific miRNA signatures).	(318)
			Cumulus-oocyte complex expansion and related genes upregulation.	(320)

Table 4. Involvement of extracellular vesicles in reproductive-related pathologies.

DISEASE	EVs PATHOGENIC ROLE			REFERENCES
Endometriosis	Promotion of endometriotic lesions invasion and progression.			(337);(339); (341)
	Enhancement of angiogenic potential.			(337); (340)
Polycystic Ovaries Syndrome	miRNA expression regulation towards PCOS phenotype.			(346)
Erectile dysfunction	Promotion of endothelial dysfunction, vascular damage and atherogenesis.			(351); (353)
Early pregnancy loss	Induction of an excessive pro-coagulant activity.			(376); (377)
	Promotion of endothelial dysfunction.			(378)
Pre-eclampsia	Placental origin	Promotion of abnormal remodelling of uterine spiral arteries.		(420)
		Enhancement of angiogenic failure and subsequent endothelial dysfunction.		(395)
		Stimulation of pro-inflammatory and pro-coagulant activities.		(385);(395);(396);(397); (408)
		Generation of oxidative stress into the placenta and mother vasculature.		(401); (402)
	Maternal origin	General	Transportation of PE risk factors.	(403)
			Failure to ensure appropriate vascular development.	(412)
		Platelet EVs	Unleashing of thrombo-inflammatory placental response.	(404)
		Leukocytes EVs	Promotion of pro-inflammatory cytokines release by the placenta	(408)
Gestational diabetes mellitus	Promotion of pro-inflammatory cytokines production by endothelial cells.			(415)

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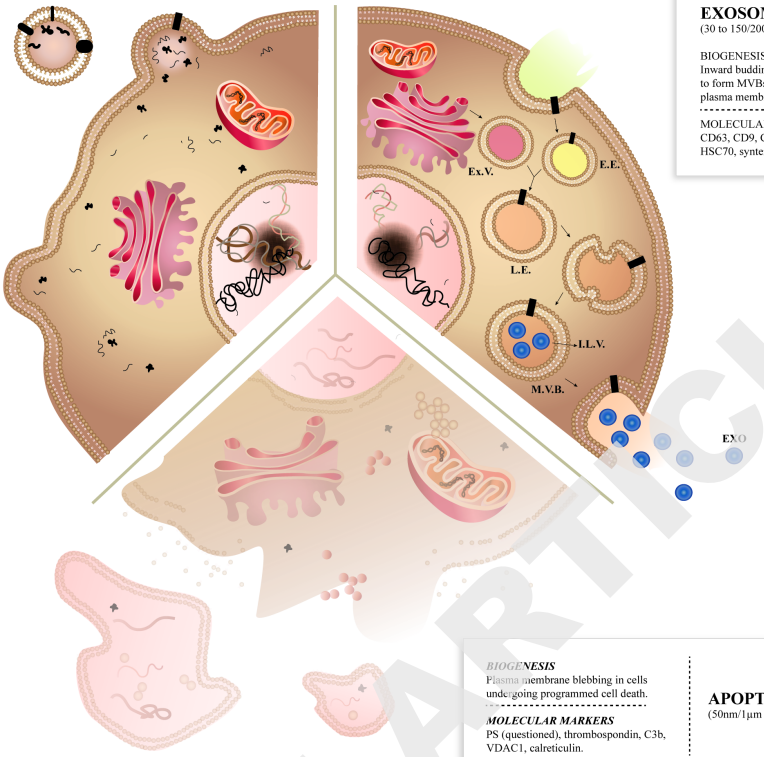
Figure 1



MICROVESICLES
(100 to 1000/2000 nm)

BIOGENESIS
Outward budding and fission directly from cell plasma membrane. Membrane budding is promoted by lipid distribution asymmetry.

MOLECULAR MARKERS
ARF6.

EXOSOMES
(30 to 150/200 nm)

BIOGENESIS
Inward budding of late endosomes membrane to form MVBs, which then fuse with cell plasma membrane to release the exosomes.

MOLECULAR MARKERS
CD63, CD9, CD81, Alix, TSG101, flotillin-1, HSC70, syntenin-1.

BIOGENESIS
Plasma membrane blebbing in cells undergoing programmed cell death.

MOLECULAR MARKERS
PS (questioned), thrombospondin, C3b, VDAC1, calreticulin.

APOPTOTIC BODIES
(50nm/1µm to 5µm)

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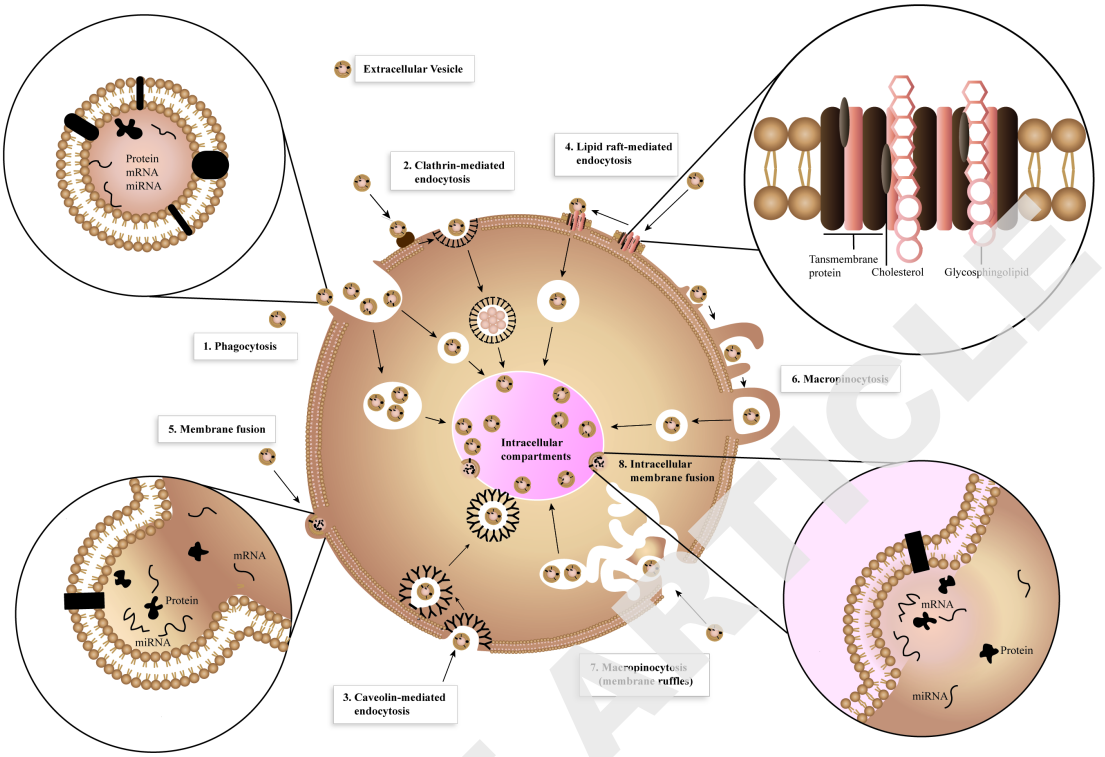
Figure 2



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