

Exosomes and exosomal non-coding RNAs throughout human gestation (Review)

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Abstract. In recent years, research on exosomes and their content has been intensive, which has revealed their important role in cell-to-cell communication, and has implicated exosomal biomolecules in a broad spectrum of physiological processes, as well as in the pathogenesis of various diseases. Pregnancy and its normal progression rely highly on the efficient communication between the mother and the fetus, mainly mediated by the placenta. Recent studies have established the placenta as an important source of circulating exosomes and have demonstrated that exosome release into the maternal circulation gradually increases during pregnancy, starting from six weeks of gestation. This orchestrates maternal-fetal crosstalk, including maternal immune tolerance and pregnancy-associated metabolic adaptations. Furthermore, an increased number of secreted exosomes, along with altered patterns of exosomal non-coding RNAs (ncRNAs), especially microRNAs and long non-coding RNAs (lncRNAs), have been observed in a number of pregnancy complications, such as gestational diabetes mellitus and preeclampsia. The early detection of exosomes and specific exosomal ncRNAs in various biological fluids during pregnancy highlights them as promising candidate biomarkers for the diagnosis, prognosis and treatment of numerous pregnancy disorders in adolescents and adults. The present review aimed to provide insight into the current knowledge regarding the potential, only partially elucidated, role of exosomes and exosomal cargo in the regulation and progression of normal pregnancy, as well as their potential dysregulation and contribution to pathological pregnancy situations.

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1. Introduction

The human pregnancy is a delicately orchestrated biological process initiated with fertilization, followed by the subsequent mitoses of the zygote, leading to the formation of the blastocyst, and subsequently the differentiated embryo. An adequately competent blastocyst is implanted in the maternal endometrium and is comprised of the embryoblast and the enclosing trophoblast (1). The embryoblast constitutes the embryonic disc, that will further differentiate into the three germ layers (ectoderm, mesoderm, endoderm), finally giving rise to the formation of all human tissues. The cells of the trophoblast differentiate into syncytiotrophoblast (ST), participating in maternal-fetal oxygen and nutrient exchange, and extravillous trophoblast (EVT), invading into the uterine wall and remodeling the uterine spiral arteries (2). Together with the decidua, form the placenta, a transitory organ that mediates the maternal-fetal exchange of oxygen, CO₂, and nutrients through an interface between the maternal and the fetal circulation (3). Apart from its respiratory, detoxifying, nurturing, and metabolic role subserving the needs of the growing fetus, the placenta has crucial immunological and endocrine properties, as it protects the fetus from rejection and the mother from graft vs. host disease, and massively produces hormones, such as human chorionic gonadotropin (hCG) and human placental lactogen (hPL) (3).

It has been previously demonstrated that the interaction between maternal and fetal cells during implantation and throughout pregnancy is mediated by the release of exosomes and other extracellular vesicles both from the embryo and

maternal tissues, especially the placenta (4-6). Extracellular vesicles (EVs) are enclosed in a lipid bi-layer membrane and can be subdivided into heterogeneous subsets of populations based on their size and derivation (1,7). These include apoptotic bodies, with a diameter ranging from 2 to 3 μm , microvesicles, sized 100 nm-1 μm , and exosomes, representing the smallest population with a diameter of 30 to 120 nm (7). The placenta also releases syncytial nuclear aggregates with a size of 20 to 100 μm and a largely unknown function, possibly resulting from dying syncytiotrophoblasts (8). After their release into the extracellular space, EVs mediate the communication between proximal and/or distant cells, by transferring their origin- and environment-dependent cargo, including proteins, lipids, coding and non-coding RNAs (such as miRNAs and lncRNAs) and DNA, thus, inducing biological alterations in the recipient cells (7). Each subpopulation of EVs is characterized by distinct surface membrane markers and cargo, based on their biogenesis and origin, which are used for their differentiation and classification. More specifically, the human placenta releases exosomes that distinctly contain placenta-specific proteins, such as placental alkaline phosphatase (PLAP) and miRNAs belonging to chromosome 19 miRNA cluster, thus, facilitating their identification (9).

The regular initiation and continuation of pregnancy entails a precise regulation of the maternal immune system, in order not to reject the embryo as a foreign immune tissue, as well and to protect the mother from the fetus. Exosomes play an important role in the establishment of maternal immunotolerance to the fetus by mediating the inter-cellular communication between the placenta and the maternal peripheral blood T-cells (10). The concentration of placenta-derived exosomes displays an incremental tendency throughout a normal pregnancy, starting as early as 6 weeks of gestation (6). Alterations in exosome release and bioactivity, by still undefined exact mechanisms, have been associated with dysregulated maternal-fetal communication, resulting in pregnancy complications, including gestational diabetes mellitus, preeclampsia, and preterm birth, thus posing a serious threat to the survival of the fetus, as well as having a long-term impact on maternal and fetal health (9).

In recent years, research on the role of exosomes and exosomal non-coding RNAs in normal and complicated pregnancy has been extensive. The prospective beneficial research outcomes include the development of novel and sensitive non-invasive biomarkers of pregnancy status and potential complications, as well as of fetal abnormalities. The aim of this review is to update current knowledge regarding the role of exosomes and exosomal cargo in normal adolescent and adult pregnancy, as well as in pregnancy disorders and provide novel perspectives on their potential applications for the early diagnosis of pregnancy complications. Although there are no exosome studies that specifically focus on adolescent pregnancy, there is adequate related information in studies where the age range of cohorts of pregnant women includes adolescent and young adult women.

2. Exosomes

Exosome biogenesis. Exosomes, apart from their small size compared to other EVs, are characterized by a distinct

buoyant density (1.13-1.19 g/ml) and pathway of biogenesis. Exosomes originate from the endosomal compartment, which participates in vesicle trafficking (Fig. 1). They are created by curvature of the membrane of the multivesicular bodies (MVBs), leading to their initial formation as intraluminal vesicles (ILVs), enclosed in a lipid bilayer (7). Because of their endosomal origin, their membrane contains late endosomal markers, such as Tsg101, CD63, CD9, and CD81, as well as origin cell-specific membrane markers, which constitute the basis for their discrimination (9).

Exosomes can be formed by either of two major biosynthetic mechanisms, which may or may not involve the endosomal sorting complex required for transport (ESCRT). The most clearly defined mechanism relies on the ESCRT complex, which consists of four subunits. These subunits are consecutively activated to incorporate ubiquitylated proteins into ILVs, induce inward budding of the endosomal membrane and dissociation of the emerging vesicle from the membrane into MVBs, ultimately leading to exosome assembly (4). The ESCRT-independent mechanism is alternatively activated to guide exosome formation when the components of the ESCRT complex are consumed, and includes among others the tetraspanin family (CD9, CD63, CD81, CD82), ceramides, phospholipase D2, -all present in the exosomes- to guide exosome wrapping and loading of exosomal cargo (11).

The formation of MVBs can be ensued by direction to lysosomes for content degradation or to the plasma membrane for exosome release; however, they can also participate in antigen presentation by major histocompatibility class II molecules (MHCII), or be recycled (12). Vesicle tethering and fusion with the plasma membrane, as well as exosome release, are regulated by numerous proteins, such as the distinctive for every cell type Rab GTPases, syntenin, and ALIX, that have been also identified in placental exosomes, and the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) (7,13). Ceramide metabolism, intracellular calcium, endoplasmic reticulum stress, all reflecting the metabolic state of the originating cell, play a role in exosome biogenesis and release. Exosome release is subsequently followed by the selective interaction with target cells, eliciting phenotypic alterations via the transfer of exosomal cargo. Exosomes contain specific surface adhesion molecules, that mediate the interaction with specific recipient cells; e.g., trophoblast exosomes express fibronectin, syncytin-1, and syncytin-2 that are involved in selective cell targeting (14). The uptake of exosomes from the target cells and the delivery of their content can occur by clathrin-mediated endocytosis or phagocytosis or by immediate fusion of the exosome membrane with the plasma membrane. Nevertheless, exosomes can interact with target cells without being internalized via binding of exosome membrane proteins, or soluble parts of them, to target cell membrane receptors (12). However, the exact mechanisms of exosome uptake remain to be elucidated.

Exosomal Non-coding RNAs. The bioactivity of exosomes is closely correlated to the cell-to-cell transfer of their unique cargo, comprised of proteins, lipids, DNA, mRNA and RNAs that do not encode proteins, i.e. non-coding RNAs (ncRNAs). Intriguingly, ncRNAs appear to prevail in the total amount of

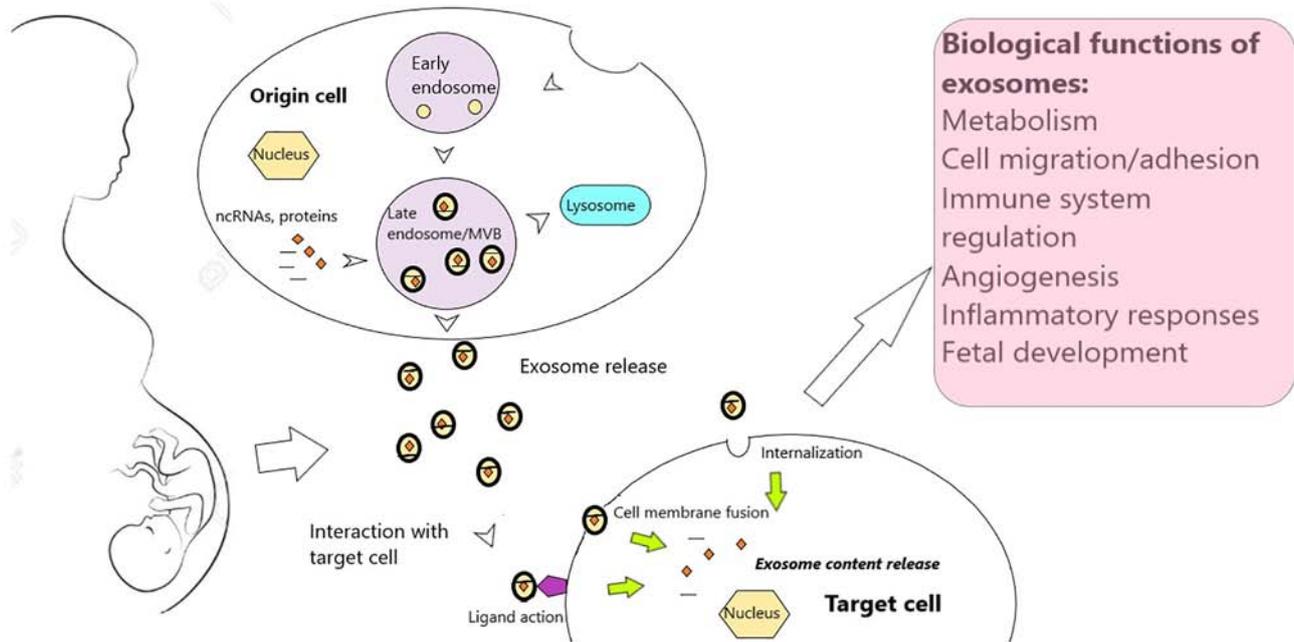


Figure 1. Exosome biogenesis and functions. Exosomes are generated within the endosomal compartment, where early endosomes mature to late endosomes and finally form MVBs. The membrane of the MVBs invaginates to form lipid-bilayer vesicles with characteristic membrane late endosomal markers, which incorporate active biomolecules, such as proteins and ncRNAs, finally leading to the formation of exosomes. Subsequently, exosomes can be directed to lysosomes for content breakdown or be released in the extracellular space, where they selectively interact with target cells via unique surface molecules, and modify their phenotype. Exosomes can enter target cells via endocytosis or phagocytosis, or by fusing with the plasma membrane. Alternatively, they can exert their biologic functions by interacting with target cell surface receptors, without being internalized. Among the important biological processes influenced by exosome signaling are metabolic adaptation, immune and inflammatory reactions, cell adhesion and migration, angiogenesis, as well as processes required for regular fetal development. MVBs, multivesicular bodies; ncRNAs, non-coding RNAs.

the human genome transcripts and may orchestrate an intricate network of interactions with other biomolecules, leading to dynamic gene regulation (7). Commonly, length is used as a criterion to separate ncRNAs into small ncRNAs, mainly microRNAs (miRNAs), with a length smaller than 200 ribonucleotides, and long ncRNAs (lncRNAs), with a length over 200 ribonucleotides (15). Lately, circular RNAs (circRNAs), have also been described. However, this review will mainly focus on the description of exosomal miRNAs and lncRNAs.

miRNAs are a subclass of ncRNAs (20-22 nt), that play a key role in physiological processes, including cell proliferation, differentiation, and migration, as well as in the onset and development of various disorders, such as immune disorders and cancer (16,17). miRNAs, after processing by a sequence of protein complexes resulting in the formation of the mature single stranded miRNA, mainly exert their biological function by inducing post-transcriptional gene silencing, as a key component of the RNA-induced silencing complex (RISC) (16,17). RISC binds principally to the 3'-untranslated region (UTR) of target mRNAs, containing specific recognition sequences, and destabilizes transcription or hampers translation (18). Furthermore, it has been lately demonstrated that miRNAs can also induce up-regulation of gene expression (7). Various cell types release miRNAs that are shielded from RNase and extreme pH and temperature alterations, either enclosed in exosomes and other EVs, or within lipoproteins or even attached to protein complexes while being in free form (19-21). Exosomal miRNAs hold great promise for the diagnosis and therapeutic targeting of various disorders because of their involvement in the regulation of key

physiological and pathological processes and their relative stability in body fluids, explaining the reason for intense scientific research in this field lately.

Another interesting subclass of ncRNAs are the lncRNAs, with a length that exceeds 200nt, that is however highly variable (7). lncRNAs result from the transcription of exonic, intergenic or distal-protein coding genomic regions, are characterized by 3'-polyadenylation, 5'-splicing and prominent thermodynamic stability (22). lncRNAs participate in various biological processes and their expression patterns are finely tuned, thus altered levels of specific lncRNAs have been associated with the onset and progression of various disorders with still undefined exact mechanisms for most of them (15,23). Importantly, the nuclear localization of lncRNAs is suggestive of a principal involvement in epigenetic gene regulation, whereas cytoplasmic lncRNAs primarily regulate genes post-transcriptionally. Subsequently, they can interact with other biomolecules (proteins, DNA, mRNA, ncRNAs) and act as recruiters, competitors, or miRNA precursors, as well as miRNA sponges, thus acting on miRNA post-transcriptional gene regulation (15,23,24). Recently, lncRNAs with still undefined exact mechanisms of loading were found within exosomes. The enclosure of specific lncRNAs in exosomes is regulated by the originating cell type and environment, while they participate in exosomal inter-cellular communication by transferring information and inducing alterations in neighboring or distant cells (25). Exosomal lncRNAs could represent useful potential biomarkers for a number of disorders, because of their protection from enzymatic degradation inside exosomes, their higher content compared to other EVs and their high tissue specificity (24).

Function of exosomes. The predominant function of exosomes that has been extensively investigated lately is their role in intercellular communication, mediated by their originating cell- and environment-dependent biologically active content. This includes proteins and coding and non-coding RNAs, that transfer important signals and reflect the originating cell state at the time of exosome generation (26). Most cells, including cancer, epithelial, immune and hematopoietic cells, produce exosomes that upon their release can act proximally in a paracrine or distally in an endocrine way, the latter by entering systemic circulation and modifying the expression and function of recipient cells (1,12). Intriguingly, exosomes can dispense cells from the need for direct contact and synchronize epigenetically distant cells by binding of their ligands to distinct recipient cell receptors concurrently. Furthermore, they could enrich the target cell membrane with surface molecules, thus widening cell targeting extent and providing new adhesion properties to them (20). In this way, exosomes participate in signal transduction, as well as in direction of harmful material to lysosomes for removal, to preserve cellular homeostasis in physiological processes, such as immune and nervous system regulation, tissue repair, sperm maturation, and, importantly, maternal immune tolerance during pregnancy (12,27). Hence, dysregulated exosomal signaling mechanisms in response to acute stress, could induce recipient cell damage and inflammation, leading to the onset and progression of pathological situations, including infections, cancer, neurodegenerative, autoimmune and cardiometabolic diseases, as well as pregnancy disorders (2,9,26). Exosomes have been isolated in a number of biological fluids, such as blood, lymph, urine, breast milk, saliva, lachrymal and mammary gland secretions, as well as in amniotic and cerebrospinal fluid (20,25). Their isolation in most biological fluids and their involvement in multifarious normal and pathological processes, renders them enticing candidate biomarkers of health and disease (28).

Placenta-derived exosomes. The establishment of human pregnancy requires a succession of maternal metabolic adaptations to fetal demands. Accordingly, the human placenta, as well as the embryo, secrete exosomes, along with other biomolecules, which ensure an intact maternal-fetal crosstalk, regulate the maternal immune and vascular system and possibly participate in placentation (6,9). All cells forming the placenta, principally syncytiotrophoblast, produce exosomes (10,29), while placental mesenchymal stem cells, *in vivo* and *in vitro*, release exosomes that promote endothelial cell migration and vascular tube formation (30,31).

Placental exosomes, similarly to exosomes of different derivation, transfer specific cargos (proteins, nucleic acids, lipids), which are indispensable for the mediation of their biological functions, encompassing the transmission of important placental information to the mother and the induction of metabolic modifications. The expression of chromosome 19 miRNA cluster (C19MC), which represents the largest miRNA gene cluster, occurs principally in the human placenta, and contains 46 miRNAs exclusively expressed in the placenta of adolescent and adult pregnancies (32). The resulting miRNAs can be subsequently selectively loaded to exosomes and transported to different cell types (33). Placental miRNAs can be secreted in a free form or enclosed and shielded from degradation inside exosomes, with mechanisms that have not

been fully elucidated; however, under normal circumstances exosomal miRNA signature highly resembles the one of the origin placental cell (33). Placental miRNAs have been detected in the peripheral blood of pregnant adolescent and adult women (9,32). In a study investigating the conditioned medium of chorionic villi of term placentas of adolescent and adult women for exosomal miRNA content, 456 distinct miRNAs were detected, most of them pertaining to C19MC cluster, but also to other non-placental specific families, such as the C14MC gene cluster and the let-7 family (34). Moreover, C19MC miRNAs expression profile could play a role in the acquisition of distinct characteristics between the more invasive EVT and villous trophoblast cells (VTs); miR-519d targets invasiveness-associated proteins, thus diminishing cell migration (35).

Microenvironmental factors, such as oxygen tension and glucose concentration impact, separately and synergistically, on the formation, secretion, and bioactivity of placental exosomes. More specifically, metabolic stress accompanying hypoxia augmented exosome release and altered placental exosome cargo and signaling to induce EVT invasion and proliferation, which occurs anyway in presence of low oxygen tension (30). Furthermore, elevated D-glucose levels correlated positively with primary trophoblast cell exosome secretion and bioactivity, possibly by enhancing exosome formation within MVBs, MVB trafficking, exosome exocytosis and altering exosomal miRNAs (2). Placental exosomes characterization and distinction can be accomplished by a set of C19MC miRNAs, and distinct membrane proteins, such as placental alkaline phosphatase (PLAP), which is derived mostly from syncytiotrophoblast (9). Lately, it has been shown that PLAP antibodies could be used to distinguish and quantify placenta-derived exosomes, possibly providing important information about the fetus and the placenta status (36). EVT-derived exosomes are uniquely characterized by the expression of human leukocyte antigen-G (HLA-G), possibly involved in maternal immune tolerance to the fetus (37). For investigating placenta-derived exosomes, cell cultures of trophoblasts, chorionic villi explants, placental perfusion, as well as maternal plasma and urine, are used (1).

3. Exosomes in normal pregnancy

Effective maternal-fetal communication via exosomes is of great importance for maternal adaptation to pregnancy, fetal survival, and normal development; however, uncovering their exact mechanisms of action remains a distant goal, due to the huge variety of bioactive molecules transported by exosomes. The peripheral blood of pregnant women contains remarkably more exosomes than in non-pregnant women (10), which could be identified as early as the sixth week of pregnancy in peripheral blood (9). Their concentration displays incremental tendency with the advancement of a normal pregnancy, peaking towards the end of pregnancy, and is maintained high until labor (1,2). Placenta-derived exosomes concentration, as indicated by the presence of PLAP, increases across gestation (6). The fraction of placenta-derived exosomes to total exosomes was raised in the peripheral blood of pregnant women in the final stages of pregnancy, starting on from mid-gestation (38). Placental exosomes concentration is normally determined

Table I. Studies evaluating miRNAs in normal pregnancy.

miRNA	Target	Source	(Refs.)
Embryo implantation miR-30d	Adhesion (Itgb3, Itga7, Cdh5)	EF exosomes	(41)
Pregnancy miR-519d	EVT invasiveness (CXCL6, NR4A2, FOXL2)	EVT-derived cell line	(35)
miR-517a-3p	Maternal immune modulation(NO/cGMP/ PRKG1 pathway blockade)	Peripheral blood NK cells	(48)
miR-210-3p miR-376c-3p miR-151a-5p miR-296-5p miR-122-5p miR-550a-5p	Endothelial cell migration	Umbilical serum exosomes	(49)
miR-517-3p miR-516b-5p miR-512-3p	Inhibition of viral replication	Human trophoblast	(50)

Itgb3, integrin beta-3; Itga7, integrin alpha-7; Cdh5, cadherin-5; EF, endometrial fluid; EVT, extravillous trophoblast; CXCL6, C-X-C motif chemokine ligand 6; NR4A2, nuclear receptor subfamily 4 group A member 2; FOXL2, forkhead box L2; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; PRKG1, protein kinase CGMP-dependent 1; NK cells, natural killer cells; miR, microRNA.

by placental mass (2). The levels of placental miRNAs can fluctuate throughout pregnancy as well; for example, placental miRNA-141 plasma levels rise as pregnancy progresses (9) and the concentration of C19MC gradually declines upon parturition (26).

Exosomes and exosomal miRNA contribute to embryo-endometrium communication, requisite for effective implantation and placentation (14,26,39). Endometrial epithelial cells liberate exosomes, which subsequently interact with trophoblasts to promote their adhesion to the uterine cavity by the activation of numerous signaling pathways, including focal adhesion kinase signaling (40). These exosomes contain miRNAs, such as miR-30d, that was demonstrated to upregulate adhesion-related genes (integrins beta-3 and alpha-7, cadherin-5) (41) (Table I). Tissue remodeling accompanying implantation occurs under subtle inflammatory conditions and placental EVs may be involved in this process by regulating cytokine secretion (42). Trophoblast-derived exosomes induce monocyte migration and differentiation to tissue macrophages and markedly increase the secretion of cytokines and chemokines, including IL-1 β , IL-6, serpin-E1, granulocyte and granulocyte/monocyte colony-stimulating factor (G-CSF and GM-CSF, respectively), and TNF α , that promote the development of the trophoblast (4). During normal pregnancy, the communication between the mother and the growing fetus is accomplished by the exchange of exosomes, produced both from the mother and the fetus. In a mouse model, maternal exosomes could translocate to the fetus, overcoming placental barriers (43).

The fetus, as well, releases exosomes that target uterine and cervical cells and could possibly activate inflammatory pathways signaling the beginning of labor (2). In pregnant mice,

the addition of exosomes, isolated from other pregnant mice, during late pregnancy could induce parturition-associated alterations, and such effect was not observed with the addition of early pregnancy exosomes (44). Commonly, the decrease of progesterone levels is essential for the onset of labor; surprisingly, exosomes promoted the initiation of parturition, regardless of progesterone levels (45).

The essential state of maternal immune tolerance to fetal tissues consists of intricate interacting mechanisms involving various biomolecules, importantly exosomes as well. Maternal immune cells internalize exosomes of embryonic origin, which induce an idiosyncratic antigen-specific immunosuppression (partially mediated by regulatory T cells), thus aiding the embryo to escape maternal immunosurveillance (1). It has been demonstrated that trophoblast derived EVs enhance T cell differentiation to regulatory T cells by transferring the heat shock protein HSPE1 (46). Furthermore, placental exosomes play a role in the attenuation of effector T-cell response involved in maternal immunoregulation, protecting the invading trophoblast from degradation (10). Upon *in vitro* interaction of mononuclear and dendritic cells with exosomal proteins, differentiation of stem cells occurs, promotion of cell migration, as well as inhibition of activation of natural killer (NK) cells and macrophages. More specifically, placenta-derived exosomes are internalized due to their specific ligands, and subsequently inhibit the expression of the activating NK cell receptor NKG2D and induce the expression of proapoptotic molecules, such as Fas ligand (FasL) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), leading to blockade of NK cell activation and cytotoxicity, and apoptosis of peripheral blood mononuclear cells, respectively (9,10,47). Moreover, exosomal miR-517b

was found to induce the expression of TNF α and other apoptosis-promoting ligands (1). It was suggested that miR-517a-3p is carried by placental exosomes to NK cells during pregnancy, blocks the NO/cGMP/PRKG1 pathway and possibly activates NF- κ B, leading to maternal immune modulation. Accordingly, miR-517a-3p is absent in the NK cells of non-pregnant women and remarkably diminishes in the cells of pregnant women following parturition (48).

Other significant roles of exosomes during normal pregnancy relate to their potential role in vascular remodeling and in resistance to viral infections. As the invasion of cytotrophoblasts progresses, the remodeling of endometrial spiral arteries ensures competent supply of oxygen and nutrients to the fetus. Predominantly in presence of low oxygen tension, placental EVs seem to detect hypoxic conditions and stimulate angiogenesis, while the embryo releases exosomal miRNAs and vascular endothelial growth factor A (VEGFA) (1). The capacity of exosomes to induce endothelial cell migration is higher in the first trimester of pregnancy compared to the second and third trimester (9). The expression of a group of miRNAs, that relate to endothelial cell migration (miR-210-3p, miR-376c-3p, miR-151a-5p, miR-296-5p, miR-122-5p, and miR-550a-5p), was found modified in umbilical serum exosomes (6,49). Interestingly, non-placental cells upon exposure to trophoblast-derived exosomes enhance their defense to viral infections, an effect mediated by the transfer of exosomal miRNAs into trophoblast cells. For instance, miR-517-3p, miR-516b-5p, and miR-512-3p, belonging to C19MC lead to inhibition of viral replication and autophagy on target cells (50).

4. Exosomes in pregnancy complications

It has been suggested that exosomal concentration, content, and function could be related to dysregulated placental activity and that it may be altered in pregnancy-related disorders (8,9). Indeed, in comparison to normal gestation, as well as in comparison to non-pregnant subjects, in women with pregnancy complications exosome release appears to be quite prominent (9,16).

Exosomal concentration during normal pregnancy was associated with trimester of pregnancy and maternal body mass index (BMI), with obese women characterized by significantly elevated exosome quantity, while in the latter group exosomes induced a more marked secretion of inflammatory cytokines (TNF- α , IL-6, IL-8) from endothelial cells, in comparison to normal weight or overweight pregnant women (38). Apart from the BMI, glucose levels and fetal body weight displayed a strong association with the concentration of placental exosomes during pregnancy, underscoring the potential role of the latter in conditioning maternal tissues for gestational metabolic alterations and their potential predictive use in pregnancy complications (51). Moreover, alterations of exosomal miRNA content and exosome-derived cell-free DNA (cfDNA) were linked to pregnancy disorders (1,52) (Table II). These observations, along with their tissue-specificity, suggest that exosomes could potentially offer a novel type of liquid biopsy, reflecting the placental status and alerting promptly to various pregnancy pathologies (26). However, there is a

great heterogeneity of isolation methodologies, and confusion arises from the lack of standardized methods for vesicle subpopulation discrimination. Furthermore, most studies use plasma from the second and third trimesters of pregnancy compared to first trimester samples, problems that need to be resolved before the utilization of exosomes in clinical applications (9). Here, we summarize current findings regarding the potential exosomal contribution to common pregnancy disorders, including preeclampsia and gestational diabetes mellitus, as well as preterm birth, and selected fetal abnormalities.

Exosomes in preeclampsia. Preeclampsia (PE) represents one of the most common and significant systemic pregnancy disorders, comprising women with new onset increases of arterial blood pressure, developing mainly in the third trimester of pregnancy, as a result of various factors (53). PE prevention and reversal would be of great importance, given the high prevalence of PE, the close correlation of PE with intrauterine growth restriction (IUGR) and premature birth, and its high maternal and neonatal morbidity and mortality; 76,000 women and 500,000 neonates die each year globally because of PE (2,54). Although PE pathogenesis has not been fully elucidated, shallow EVT invasion and deficient spiral artery remodeling, and resultant placental hypoxia are key processes involved in abnormal placentation and the development of PE (4).

There has been intense scientific interest regarding quantitative and qualitative alterations of placenta-derived exosomes in pregnancies complicated with PE. As it regards quantity, the plasma content of placenta-derived exosomes is higher in early-onset PE (PE occurring before the 33rd pregnancy week), while it is reduced in late-onset PE (PE after the 34th pregnancy week), compared to the equivalent weeks of normal pregnancy (55). Intriguingly, an increase in the number of total and placental exosomes in the circulation of women who will later on present with PE, can be observed already from the first trimester of pregnancy, supporting the attractive perspective of using placental exosomes as candidate non-invasive biomarkers of PE (2,29). Exosomes in PE possess a distinct lipidomic and proteomic profile, probably participating in immune reactions, vascular regulation, and oxidative stress (9). Pregnancies complicated with PE are characterized by a unique exosomal protein cargo in comparison with uncomplicated pregnancies (56).

Interestingly, exosomes seem to be involved in the pathogenesis of PE. Infusion of exosomes from the plasma of women with PE to pregnant mice, reduced their body weight, increased their blood pressure, and disrupted fetal development, an effect not observed when exosomes from normal pregnancy were injected (4). In PE, the release of EVs and remnants from trophoblast cells induced endothelial damage and vascular inflammation, key processes in the pathogenesis of PE (56). Endothelial nitric oxide synthase (eNOS), which is essential for the synthesis of vasoactive nitric oxide (NO), as well as neprilysin, a vasoactive-cleaving enzyme, are incorporated in EVs released from the syncytiotrophoblast; thus, EVs could serve as indicators of reduced NO bioactivity and increased risk of hypertension accompanying PE (1,57). Considering the impact of oxygen tension on the release of

Table II. Studies evaluating miRNAs and lncRNAs in diverse pregnancy complications.

miRNA/lncRNA	Target	Source	(Refs.)
Early-onset preeclampsia			
miR-210 (↑)	Trophoblast invasion	Plasma exosomes	(4)
miR-517-5p (↑), 423-5p (↑)	Unknown	Plasma	(26)
Preeclampsia			
miR-155 (↑)	eNOS expression blockade	Plasma, placenta	(59,60)
miR-141 (↑)	Trophoblast invasion	Trophoblast-derived exosomes	(4)
hsa-miR-486-1-5p, 486-2-5p	Unknown	Plasma total exosomes and placenta-derived exosomes	(58)
miR-495 (↑), 494 (↑), 136 (↑)	Cell proliferation, apoptosis	Peripheral blood and umbilical cord MSCs exosomes	(61)
lncRNA NONHSAT116812, NONHSAT145880	Unknown	Plasma, placenta	(63)
lncRNA H19	Trophoblast cell invasion, migration (↑ FOXO1)	MSCs exosomes	(64)
Gestational diabetes mellitus			
miR-518a-5p, 518b, 518c, 518e, 520c-3p, 525-5p	Unknown	Serum exosomes	(1)
miR-125a-3p (↑), 99b-5p (↑), 197-3p (↑), 22-3p (↑), 224-5p (↑)	Cell migration, carbohydrate metabolism	Placenta, skeletal muscle, plasma exosomes	(34)
miR-16-5p (↑), 17-5p (↑), 20a-5p (↑)	Unknown	Plasma	(70)
lncRNA MALAT1 (↑)	Unknown	Serum	(71)
Preterm birth			
miR-515-5p (↑), 516-5p (↑), 518b (↑), 518f-5p (↑), 519a (↑), 519e-5p (↑), 520a-5p, 520h, 526b-5p (↑)	Unknown	Placenta	(74)
miR-223 (↑), 302b (↓), 548 (↓), 1253 (↓)	Unknown	Plasma	(75)
Intrauterine growth restriction			
miR-103a-3p (↓), 126-3p (↓), 195-5p (↓), 499a-5p (↓)	Unknown	Plasma	(16)
Down syndrome			
miR-15a (↑), let-7d (↑), 23a (↑), 99a (↑), 142 (↑), 191 (↑), 199 (↑), 3156 (↑)	CNS development, congenital abnormalities, heart defects	Plasma	(82)

eNOS, endothelial nitric oxide synthase; MSCs, mesenchymal stem cells; FOXO1, forkhead box protein O1; CNS, central nervous system; ↑, upregulated; ↓, downregulated; miR, microRNA; lncRNA, long non-coding RNA.

placental exosomes, it seems alluring that the evaluation of placental exosomes might be used for the timely detection of asymptomatic women and adolescents susceptible to PE (58).

The involvement of exosomal miRNAs and proteins in the pathogenesis of PE in adolescent and adult pregnant women and their predictive potential are also under evaluation (32). More specifically, exosomal miR-155, which is upregulated in PE both in the plasma and the placenta, blocked the expression of eNOS, while the fact that aspirin largely reversed the occurrence of early onset PE in women at risk was partially attributed to exosomal miR-155 down-regulation (59). Furthermore, exosomal miR-210 was markedly upregulated in early-onset PE in comparison to normal pregnancy, and it

has been previously found to interfere with trophoblast invasion and migration (4), while C19MC miRNAs miR-517a/b and miR-517c also display distinct expression profiles in PE, possibly linked to similar biological processes (16). Trophoblast-derived exosomes in PE contain increased levels of miR-141, also shown to participate in trophoblast invasion (4). Studies aiming to identify potential future biomarkers for PE, evaluated circulating miRNAs altered in the serum of patients who subsequently presented with PE; 11 up-regulated miRNAs, including miR-155, as well as 5 down-regulated miRNAs, were detected (60). It has also been suggested that exosomal hsa-miR-486-1-5p and hsa-miR-486-2-5p, which are consistently increased in PE, could be used as biomarkers for

the prediction of PE (58). In another study, exosomal miR-495, miR-494, and miR-136, which are upregulated in PE and possibly implicated in the establishment of PE by reducing cell proliferation and apoptosis, could also be employed in the clinical setting for the early diagnosis of PE (61). Furthermore, the expression of non-exosomal hsa-miR-325 was downregulated in placentas derived from adolescent and adult women with preeclampsia, suggesting a potential pathogenetic role, by interfering with oxidative stress pathways and heat shock protein production (62).

LncRNAs have also been implicated in the modulation of trophoblast invasion and the establishment of PE (63). In a study evaluating free circulating lncRNAs profiles in PE, 163 alternatively expressed lncRNAs were found in the placenta of women with late onset PE and two lncRNAs (NONHSAT116812 and NONHSAT145880), were significantly correlated with both early and late onset PE, denoting their possible use as non-invasive biomarkers of PE (63). Strikingly, exosomes released from mesenchymal stem cells (MSCs) highly express lncRNA H19, which acts as a competing endogenous RNA (ceRNA) for miRNA let-7b, thus upregulating FOXO1 and activating signaling pathways that enhance trophoblast cell survival, invasion, and migration (64). These findings could offer entrepreneurial strategies for combating PE.

Exosomes in gestational diabetes mellitus. Gestational diabetes mellitus (GDM) represents another severe pregnancy disorder, with an alarming mounting prevalence parallel to the obesity outbreak (65). GDM can have devastating consequences, such as premature delivery, perinatal complications, macrosomia, and future risk of development of type 2 diabetes and cardiovascular disease, both for the mother and the child (16). GDM is characterized by pathological glucose metabolism presenting across gestation and/or unprecedented or unrecognized abnormal glucose tolerance, which to date is diagnosed by an oral glucose tolerance test (OGTT) at 24-28 weeks of gestation (15). Normally, along gestation, insulin sensitivity rises to a peak over the first and the second trimester to ensure the essential for the progression of pregnancy energy storage, and subsequently declines, leading to insulin resistance that shifts glucose and fatty acids from the mother to the fetus to provide it with adequate nutrients (15). In case of new onset or preexisting abnormal maternal insulin resistance, maternal pancreatic β -cells cannot secrete enough insulin to counterbalance the high glucose levels present in circulation, leading to maternal hyperglycemia, hyperinsulinemia, and hypoxia, all impacting negatively on the maternofetal interface (1). In addition, GDM is characterized by a more exacerbated proinflammatory condition than the one normally observed during pregnancy; gestational tissues, as well as adipose tissue, release proinflammatory cytokines that participate in the deterioration of insulin resistance (15,66).

Remarkably, it has been demonstrated that an increased concentration of placental exosomes at 11-14th gestational week could predict GDM (20). Moreover, GDM was correlated with an increased concentration of total and placental exosomes in the maternal blood compared to uncomplicated pregnancies of corresponding gestational ages, denoting their prospective use in identifying women at risk for GDM before disease onset (9). However, the contribution of placental

exosomes to total exosomes was reduced compared to normal pregnancies, suggesting either a dysregulated release from the placenta or a predominant release of exosomes of a different origin (2). It has been reported that placental exosomes could transfer bioactive mediators between maternal tissues and the placenta that influence immune and vascular systems, as well as the pancreas and the adipose tissue, suggesting a potential role in GDM pathogenesis (20). Upon exposure to placental exosomes derived from adolescent and adult patients with GDM, skeletal muscle cells of non-diabetic individuals displayed attenuated insulin-induced migration and glucose uptake; conversely, exosomes from healthy individuals induced insulin-stimulated glucose uptake in skeletal muscle cells from GDM patients (34). Hyperglycemia has been demonstrated to influence first-trimester trophoblast-derived exosomes, with regards to their concentration and bioactivity, and intensify proinflammatory cytokine secretion from endothelial cells, predisposing to disruption of maternal glucose metabolism and GDM (66). Nevertheless, exosomes were also found to induce the secretion of the anti-inflammatory IL-4 from endothelial cells, indicating that exosomal modulation of the inflammatory state could be multidimensional (2). Moreover, the excessive placental glycogenolysis in GDM, that triggers glucose transport to the fetus, finally leading to fetal overgrowth, seems to be affected by adipose tissue-derived exosomes (67). Another indication of the implication of placental exosomes in the pathophysiology of GDM arises from their highly elevated content of biologically active dipeptidyl peptidase-4 (DPP-4) in advanced GDM pregnancies (DPP-4 inhibits pancreatic insulin secretion by cleaving glucagon-like polypeptide-1, which enhances insulin secretion), and that the DPP-4-specific inhibitor vildagliptin can inhibit DPP-4 activity in placental EVs, paving the way for novel therapeutic approaches (68).

Exosomes in GDM have been shown to have a distinct composition profile regarding their protein and ncRNA content, driving scientific interest to their potential applications in the prediction and treatment of GDM. The differentially expressed proteins are suggested to participate in energy metabolism, insulin sensitivity and inflammation; urinary exosomes in GDM overexpressed damage associated molecular pattern (DAMP) protein S100A9, that may serve as an indicator of inflammation and immune activation (69). Existing evidence supports that exosomes in GDM contain higher levels of miRNAs belonging to the C19MC cluster (miR-518a-5p, miR-518b, miR-518c, miR-518e, miR-520c-3p, and miR-525-5p) (1). Furthermore, exosomal miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p, and miR-224-5p were increased in the placenta, in skeletal muscle and in circulation of adolescents and adults with GDM, thus contributing to the notion that the placental metabolic function could be reflected in the distinct miRNA content of placental exosomes (34). Pathway analysis has demonstrated that the altered miRNAs are involved in fatty acid metabolism, inflammatory immune reactions, insulin release and glucose transport, as well as in placental, suggesting a role of these miRNAs in peripheral insulin resistance and the development of GDM (16). Lately, the potential therapeutic application of exosomes to target GDM has been proposed (20).

Several studies evaluating free circulating miRNA profiles have identified specific miRNAs altered in GDM and

have suggested potential pathogenetic mechanisms for their involvement in insulin resistance and β -cell dysfunction (15), as well as miRNAs that could serve as predictive biomarkers for GDM development, such as miR-16-5p, miR-17-5p and miR-20a-5p (70). Studies evaluating and identifying the involvement of lncRNAs in the pathogenesis and diagnosis of GDM are scarce at the moment. For example, lncRNA MALAT1, that has been suggested to participate in the pathogenesis of diabetic microangiopathy, has been found elevated in the serum analyzed between the 36th and the 40th week of pregnancies complicated with GDM compared to normal pregnancies (71). For a more comprehensive description of the potential roles of ncRNAs in GDM, see Filardi *et al*, 2020 (15).

Exosomes in pre-term birth. Every parturition before the completion of the 37th pregnancy week is defined as a preterm birth. In accordance with the estimations of the World Health Organization, 15 million babies are delivered prematurely every year (72). Adverse outcomes of prematurity account for the most deaths among children younger than five years old. The etiology of preterm birth is multifactorial; multiple pregnancies and infections, as well as PE and GDM are among the causes leading to premature delivery (72). The sequelae of preterm labor, such as retinopathy of prematurity, respiratory distress, cerebral palsy, can be devastating for the infant, all contributing to morbidity and, in the worst-case scenario, mortality; thus, timely recognition of pregnancies at risk for prematurity is of an imperative priority (16).

It has been reported that maternal plasma exosome function could mirror the progression of pregnancy and predict preterm labor (52). The isolation of exosomes from maternal serum at the 30th pregnancy week showed a greatly reduced number of exosomes in gestations that would be preterm (9). The secretion of IL-2 by activated T-cells was attenuated upon interaction with exosomes derived from the blood of women with preterm labor, as compared to interaction with exosomes derived from the blood of women with normal labor (4). Upon pathway analysis of exosomal protein expression at normal and premature deliveries, it has been indicated that inflammatory and endocrine signaling alterations may destabilize pregnancy homeostasis, while variations in the protein content of placenta-derived EVs have been postulated to reveal a predisposal for premature delivery (73). Determination of miRNAs ferried by exosomes suggests that they display remarkable variations in women delivering prematurely and that could be possibly used to signal pre-term birth (16).

Most studies have revealed the potential application of circulating miRNAs in the plasma of pregnant women as biomarkers of prematurity; nevertheless, the exosomal transport of these miRNAs has not been verified (1). Nine miRNAs pertaining to the C19MC cluster, namely miR-515-5p, miR-516-5p, miR-518b, miR-518f-5p, miR-519a, miR-519e-5p, miR-520a-5p, miR-520h, and miR-526b-5p, were increased in pre-term gestations (74), while Gray *et al* reported an increase in miR-223, and a decrease in miR-302b, miR-548, and miR-1253, in the plasma of women delivering preterm compared to the ones delivering at term (75). Moreover, placental mRNA and miRNA expression profiles have been evaluated in association to pre-pregnancy BMI of adolescent and adult women delivering extremely prematurely (76). A low pre-pregnancy BMI

in male placentas has been correlated with a distinct mRNA profile, targeted by miR-4057 and miR-128-1-5p and involving nutrient metabolism and angiogenesis pathways (76). Another study by Payton *et al*, identified a set of placental mRNAs and miRNAs associated with the birth weight of extremely preterm infants born to adolescent and adult mothers (77).

Exosomes in fetal abnormalities. Except for maternal pathologic situations, exosomes have been associated with several fetal disorders. Importantly, the placental barrier is permeable for maternal exosomes that can subsequently target fetal tissues. Among important fetal disorders, intrauterine growth restriction (IUGR) is defined as an incapacity of a fetus to achieve its growth potential and is accompanied by considerable metabolic consequences (78). It has been demonstrated that the number of total and placental exosomes in the maternal plasma of IUGR pregnancies does not differ significantly with normal pregnancies (36). Nonetheless, the contribution of placental to total exosomes was markedly decreased in pregnancies with IUGR, implying that placental exosomes could reveal fetal developmental status, while particular placenta-characteristic non-exosomal miRNAs were downregulated in IUGR placentas, but not in maternal plasma (79). A study evaluating circulating miRNA profiles in adolescent and adult pregnant women delivering selective IUGR monozygotic twins implicates miR-199a-5p in the pathogenesis of selective IUGR, by interfering with oxidative stress and placental angiogenesis pathways (80). Furthermore, Baker *et al* linked inadequate fetal growth in pregnant, folate-deficient adolescents, to particular miRNA alterations, namely to the upregulation of miR-222-3p, miR-141-3p, and miR-34b-5p (81).

Circulating non-exosomal miRNA profiles have been evaluated for various fetal abnormalities rendering them candidate non-invasive screening biomarkers for these disorders and potential ensuing research target of exosome content. More specifically, a prenatal miRNA profile of women pregnant with fetuses with Down syndrome has been delineated and includes miR-15a, let-7d, miR-23a, miR-99a, miR-142, miR-191, miR-199 and miR-3156, which are upregulated in women pregnant with Down syndrome fetuses (82). Accordingly, a number of alternatively expressed circulating miRNAs have been identified in the blood of women pregnant with a fetus with congenital heart disease (CHD) and these miRNAs have been postulated to regulate fetal cardiac development (16). Intriguingly, exosomes derived from diabetic mice, upon injection to normal pregnant mice, could induce CHD to their fetuses (83). Furthermore, another study identified six circulating miRNAs that displayed important concentration variations in the serum of women carrying fetuses with neural tube defects compared to normal pregnancies (16). Apparently, these results could have great implications, as the capability of identifying and monitoring an increased risk of fetal abnormalities crucial for the development and survival of the offspring, could pave the way for non-invasive prenatal diagnosis.

5. Clinical applications

As already described, exosomes and bioactive exosomal cargo could critically transform current methods of

monitoring pregnancy progression and diagnosing early pregnancy disorders by representing sensitive, non-invasive biomarkers, or even future therapeutic targets. The possibility of identifying promptly increased risk of adverse pregnancy situations, and thus being capable of preventing them or monitoring them, represents an alluring perspective. Exosomes and their cargo, especially exosomal miRNAs, as well as circulating, non-exosomal ncRNAs, have been extensively investigated for alterations that could lead to the development of sensitive biomarkers for various pregnancy disorders. Specific exosomal proteins, including tissue inhibitor of metalloproteinases 1 (TIMP1), plasminogen activator inhibitor type 1 (PAI1), and placental growth factor (PIGF), have been demonstrated to reliably predict PE (84). Nevertheless, placental circulating RNA biomarkers outweigh protein biomarkers because their alterations can be identified earlier, while they have a higher correlation with placental status (62).

It has been demonstrated that therapeutic use of exosomes as drug carriers could prove to be advantageous, because of the low immunogenicity of autologous exosomes, their high stability in circulation, their capability of effective bioactive cargo transfer in target cells and their limited side effects, explained by their highly specific action on target cells (20). Remarkably, placenta-derived exosomes could also be of therapeutic use; exosomes originating from the mesenchymal stromal cells of the placenta are beneficial for individuals with Duchenne muscular dystrophy, as they promote the differentiation and fusion of myocytes and they upregulate myoblast fibrogenic genes (85). Moreover, it is expected that analyzing exosomal miRNAs in GDM-complicated pregnancies will improve our understanding of the pathogenesis of GDM and pave the way to novel therapeutic strategies for combatting GDM (20). Possibly, MSC-derived exosomes could be promising tools in regenerative medicine due to their immunoregulatory properties, with potential applications in GDM-associated myopathy and other GDM-associated maternal and fetal complications. It has been suggested that exosomes derived from adipose tissue stem cells could represent a novel therapeutic approach to stress urinary incontinence, observed often at women after parturition (86). Furthermore, it has been speculated that trophoblast-derived and placental MSC-derived exosomes could be employed in the treatment of PE, considering their angiogenic properties (4). Strikingly, genetic engineering of MSCs-derived exosomes to inhibit the expression of genes etiologically correlated with PE and modifying exosomal proteins to achieve a more targeted delivery of exosomal cargo, holds great promise for the precise treatment of PE (87).

6. Conclusions and future perspectives

Accumulated evidence supports the view that exosome-mediated communication plays a key role in an array of physiological processes required for the regular initiation and progression of pregnancy. These include maternal metabolic adaptations to gestation, immune tolerance, and inflammatory processes, as well as resistance to infections, in all of which exosomes released from the placenta have been reported to have a prominent role. Recent studies have

started to unveil the exact pathways of exosome and exosomal cargo contribution to the interface of maternal and fetal environment across normal gestation, as well as their alterations in situations of threatened pregnancy homeostasis in adolescents and adults. Variations in the concentration, the content, and the biological effects of exosomes have been implicated in the onset of pregnancy disorders, including preeclampsia, GDM, preterm birth and fetal anomalies. However, further studies are needed to improve our understanding of the exact mechanisms of exosomal participation in the pathogenesis of these diseases. Moreover, more studies investigating miRNAs and lncRNAs transferred by exosomes, mainly of placental origin, could offer a more thorough insight of the roles of exosomes and their unique cargoes in healthy and pathological states and be of clinical utility. Furthermore, although the current knowledge on exosomes and their bioactive cargo is based on studies of pregnant women with age range that includes adolescent and young adults, there seems to be a need of studies that specifically focus on exosomes in adolescent pregnancy that might reveal possibly unexpected findings.

The distinctive exosomal properties open the way for the use of exosomes as biomarkers of pregnancy disorders at the presymptomatic stage, which is a field full of promise. However, the greatest obstacle encountered in the clinical use of exosomes is the lack of standardization of pure exosome isolation techniques and the use of low-efficiency techniques, such as ultracentrifugation. Other exosome isolation techniques include polymer precipitation, size-based isolation techniques (mainly ultrafiltration and size-exclusion chromatography) and immunoaffinity chromatography (88). Factors as sample type (e.g., plasma, urine), the trimester of gestation, the status of the patient at the time of sample collection, influenced by circadian rhythms, food intake, etc., should also be considered because they could influence the accuracy of findings (26). Regarding exosomal miRNA studies, there is low reproducibility of miRNA profiles, probably because of the above-mentioned factors, as well as the possible use of different methods of analysis (miRNA-array or RNA-sequencing). Therapeutic exosome implementations have been also proposed, given the capacity of exosome signaling and cargo transfer between distant tissues, and could consist of either targeting specific exosomal cargo or loading desired drugs to exosomes for targeted cargo delivery (20). The use of exosomes as candidate non-invasive biomarkers and as therapeutic biomolecules could fundamentally upgrade the current approach of pregnancy complications diagnosis and management.

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Availability of data and materials

Data sharing is not applicable to this article, as no datasets were generated or analyzed during the current study.

Authors' contributions

IM performed the literature search, wrote the first draft of the manuscript and designed the figures and tables. CY conceived the study, and was responsible for project coordination and critical revision of the manuscript. KN performed the literature search and review of the first draft. FB performed critical revision of the manuscript. GPC performed critical revision of the manuscript and the final draft. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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