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Review

Placental Exosomes as Biomarkers for Maternal Diseases: Current Advances in Isolation, Characterization, and Detection

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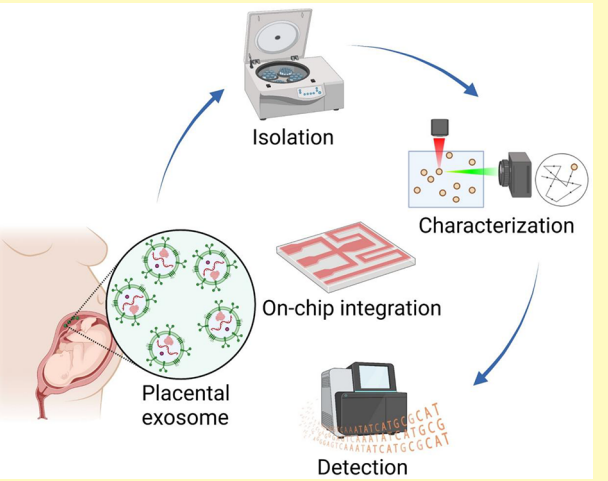
ABSTRACT: Serving as the interface between fetal and maternal circulation, the placenta plays a critical role in fetal growth and development. Placental exosomes are small membrane-bound extracellular vesicles released by the placenta during pregnancy. They contain a variety of biomolecules, including lipids, proteins, and nucleic acids, which can potentially be biomarkers of maternal diseases. An increasing number of studies have demonstrated the utility of placental exosomes for the diagnosis and monitoring of pathological conditions such as pre-eclampsia and gestational diabetes. This suggests that placental exosomes may serve as new biomarkers in liquid biopsy analysis. This review provides an overview of the current understanding of the biological function of placental exosomes and their potential as biomarkers of maternal diseases. Additionally, this review highlights current barriers and the way forward for standardization and validation of known techniques for exosome isolation, characterization, and detection. Finally, microfluidic devices for exosome research are discussed.



KEYWORDS: *placental exosome, syncytiotrophoblast extracellular vesicles, maternal disease, biomarker, liquid biopsy, exosome isolation and characterization*

A GENERAL INTRODUCTION TO MATERNAL HEALTH AND EXOSOMES

Complications during pregnancy encompass various conditions that impact both the health of the mother and the developing fetus. These complications include, but are not limited to, pre-eclampsia (PE), gestational diabetes mellitus (GDM), placental abruption, preterm birth, stillbirth, and recurrent miscarriage.[1] Among them, PE and GDM have drawn much attention in recent years, as they are two of the most serious pregnancy complications. Women with PE or GDM face a significantly increased risk of adverse pregnancy outcomes such as premature birth, fetal macrosomia, fetal growth restriction, spontaneous abortions, hypertension, and other cardiovascular diseases,[2,3] About 10 million women are affected by PE worldwide, leading to the annual mortality of approximately 76,000 women and 500,000 babies.[4] GDM is more prevalent, affecting 1 in every 6 births, resulting in nearly one million cases of pregnancy-related hyperglycaemia.[5] These disorders are becoming a burden to low- and mid-income countries due to limited access to maternal care.[6,7]

PE is typically identified through physical indications such as elevated blood pressure, proteinuria, uterine-artery Doppler velocimetry, renal damage, hemolysis, and/or platelet activation.[8] Current diagnostic and prognostic biomarkers for PE include placental markers (PAPP-A, PlGF, sFlt-1), cystatin C, RASSF1A, HTRA3, NGAL, cytokines (IL-10, TNF-α, IFN-γ), serum lipids, free fetal hemoglobin, alpha 1 macroglobulin, and possibly a low level of placental protein 13 (PP13).[8] GDM can be diagnosed following oral glucose tolerance testing, measurement of fasting and random plasma glucose concentrations, and glycated hemoglobin levels.[9] While these biomarkers may have their merits, they cannot consistently identify women who are at risk of developing these complications.[8,10−12]

Exosomes are emerging candidates for new diagnostic biomarkers and therapeutic targets. Recent studies also indicate that placental exosomes may be crucial to under-

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Table 1. Different Subtypes of Placental-Derived EVs

EV subtype	Size	Subcellular origin	Buoyant density	Sedimentation density	Enriched proteins
Exosome (small EVs)	30−150 nm	Endocytic pathway	1.13−1.19 g/mL	100,000−200,000*g*	Tetraspanins (CD81, CD63, CD9), ESCRT components (Alix, TSG101), syntenins, metalloproteinase-domain-containing protein 10
Microvesicle	150−1000 nm	plasma membrane budding	1.16−1.19 g/mL	10,000−20,000*g*	flotillin-1, KIF23, RACGAP, CSE1L, ARF6, EMMPRIN, CD40
Apoptotic bodies	1−5 μm	Dying cell fragment	1.16−1.28 g/mL	1000−2000*g*	alpha-actinin-4, GP96, lysosome-associated membrane glycoprotein 2, mitofilin, phosphatidylserine, histones, calnexin

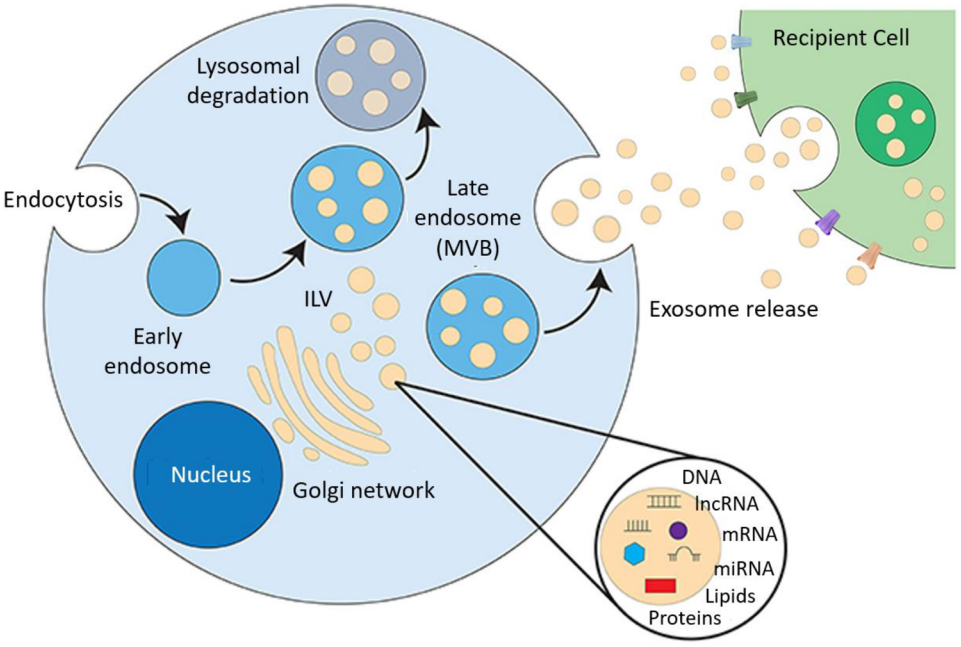

Figure 1. Biogenesis, contents, release of exosomes, and action on recipient cell. The formation of exosomes begins with the inward folding of the plasma membrane to create early endosomes. During the maturation, the endosomal membrane undergoes inward budding, which results in the formation of intraluminal vesicles (ILV) that encapsulate cytosolic components such as nucleic acids and functional proteins. These mature late endosomes, also known as multivesicular bodies (MVB), then fuse with the plasma membrane to release ILV into the extracellular space as exosomes. Along with generic markers, exosomes contain nucleic acids (DNA, lncRNA, mRNA, miRNA), lipids, and proteins. After secretion, exosomes can transfer signals to recipient cells via either endocytosis, direct membrane fusion, or receptor−ligand interactions. Adapted with permission from ref 24. Copyright 2019 Author(s).

standing pathophysiological development during pregnancy. Several available reviews have illustrated the diagnostic, prognostic, and therapeutic potential of exosomes in maternal diseases.[13,14] Although Ghafourian et al. discussed implications of exosomal miRNA as diagnostic markers for pregnancy-related conditions,[13] placental exosomes themselves and other contents should also be considered as potential biomarkers. Boriachek et al. mentioned the role of exosomes as cell-to-cell communication vehicles in modulating physiological and pathological processes in different diseases, as well as highlighting current techniques for exosome isolation and detection.[15] In an effort to standardize exosome isolation and detection techniques, Coumans et al. presented a set of methodological guidelines for the study of extracellular vesicles, including exosomes.[16] Moreover, the community in the International Society for Extracellular Vesicles (ISEV) has constantly updated recommendations to establish a standardized and evidence-based platform for extracellular vesicle research.[17−20] However, a knowledge gap remains in our understanding of the full potential of placental-derived exosomes as biomarkers for maternal diseases. This review emphasizes the role of these exosomes in pregnancy and how they can be used for diagnosis purposes. Existing methods and challenges in placental exosome isolation, characterization, and detection are also explored. Finally, we discuss the potential of microfluidic devices in exosome research.

NOMENCLATURE, BIOGENESIS, AND RELEASE OF PLACENTAL EXOSOMES

The ISEV defines "extracellular vesicle" (EV) as an umbrella term for "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate, i.e., do not contain a functional nucleus".[19] EVs are often categorized into three broad classes (namely, exosomes, microvesicles, and apoptotic bodies), based on their size, origin, and sedimentation properties (see Table 1). Note that the proper definition of the term "exosome" refers only to EVs originating from the endosomal system. However, accurately proving the subcellular origin of a particular subpopulation of EVs is challenging due to the overlap in sizes and markers of these particles as well as inconsistent expression among different cell types. As a result, the term "exosome" is sometimes used to refer to small EVs or

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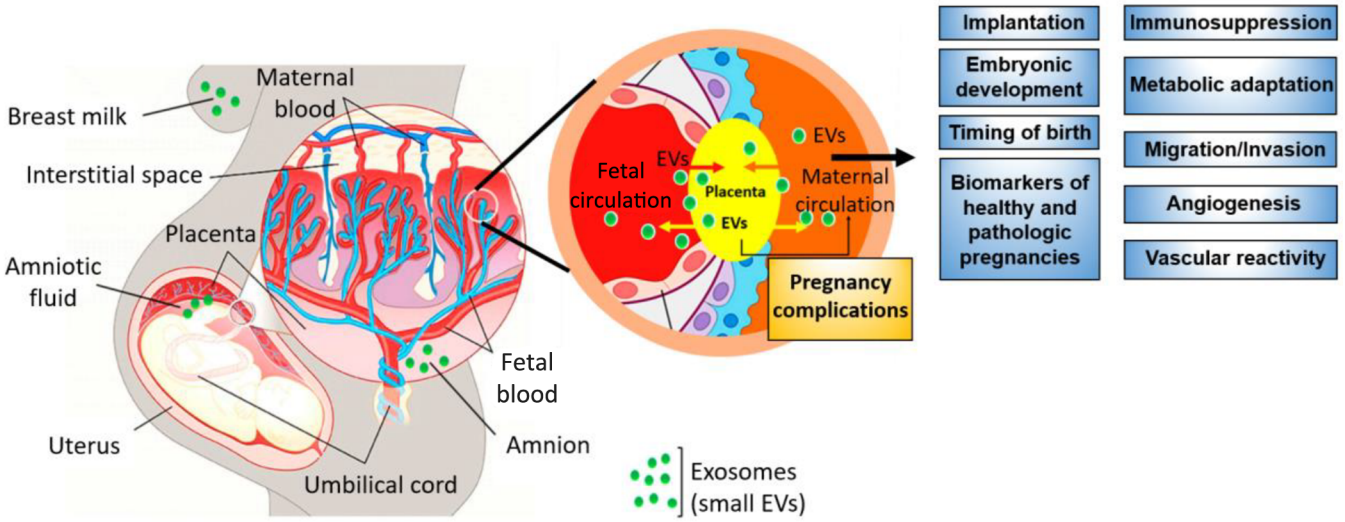

Figure 2. Illustration of exosomes acting as a fetal−mother communicating signal. Exosomes play an important role in enabling the successful implantation and development of the embryo. They are exchanged between the maternal and embryonic tissues and facilitate these processes by suppressing immune responses and ensuring a steady supply of nutrients, thereby adapting the maternal organism to pregnancy. Exosomes can also deliver pregnancy complication signals and might be used as diagnostic biomarkers. Adapted with permission from ref 51. Copyright 2020 MDPI.

extracellular nanoparticles in studies that lack biogenesis or purity information.[21]

The biogenesis of exosomes involves the endocytosis process, followed by the fusion of endocytic vesicles with early endosomes (see Figure 1). A detailed process of endocytic vesicle formation, endosome maturation, and secretion has been extensively documented and can be found in various sources such as ref 22. It is worth mentioning that exosome biogenesis can occur through either the endosomal sorting complex required for the transport (ESCRT)-dependent pathway or the ESCRT-independent (lipid-mediated) pathway. As a result, generic exosome markers often include both ESCRT components (such as Alix, TSG101, and HSP70) and tetraspanins involved in ESCRT-independent biogenesis of exosomes (such as CD9, CD63, and CD81). However, not all exosomes will necessarily possess all of these markers depending on cell type and the conditions under which the exosomes are released. Interestingly, de Menezes-Neto et al. proposed a comprehensive set of 34 exosome markers by selectively combining lists of proteins commonly found in exosomes from various publications and online databases.[23]

The human placenta consists of various cell types, with primary placental cells (trophoblasts) being the most abundant. Trophoblast cells fuse to form the syncytiotrophoblast (STB), which is responsible for sensing and regulating oxygen and nutrient exchange between the mother and fetus during pregnancy. This STB cell type is the main source of releasing placental exosomes in response to a change in gestation conditions.[25−27] While the term "placental exosome" typically refers to exosome released by STB (or STB-EVs, STB microparticles), other cell types found in the placenta, such as mesenchymal cells, mesenchymal-derived macrophages, fibroblasts, and fetal vascular cells (endothelial cells, vascular smooth muscle cells, and pericytes), may also contribute to the release of circulating exosomes during pregnancy. Placental exosome is characterized by the presence of placental alkaline phosphatase (PLAP, serving as a marker for STB origin),[28,29] and this property can be used for isolation and quantification.[30] Levels of maternal circulating placental exosomes have been reported at as early as 6 weeks of pregnancy, and they gradually increase until term before returning to nonpregnant levels within 48 h post-delivery.[31] Distinguishing the potential applications of exosomes to other types of EVs is challenging due to the overlapping size and protein markers of these particles, making it sometimes unclear which subclass of EVs researchers are precisely describing. However, it is important to note that exosomes have a specific origin from the endocytic pathway, leading to the selective packaging of molecular cargo. This selective packaging is indeed relevant to their function as biomarkers. For instance, a study demonstrated higher levels of certain cytokines (IL-10, IL-6, IFN-γ, and TNF-α) in the placental exosome fraction compared to the macrovesicle and microvesicle fractions.[32] This differential packaging of cytokines and other molecular cargos in exosomes may provide valuable insights into their functional relevance and their potential as biomarkers for maternal health conditions. Future research and advancements in EV characterization techniques will further improve our understanding of the differences and similarities among EV subtypes and their biomarker potential in maternal health.

FUNCTION AND ROLE OF EXOSOMES IN PREGNANCY

Similar to other EVs, exosomes were previously considered as a cellular mechanism for eliminating waste and metabolic byproducts.[33] However, it has been revealed that exosomes transport a diverse array of important molecules, including proteins, mRNA, non-coding RNAs (such as miRNA and lncRNA), and fragments of DNA, thereby acting as a means of transmitting genetic information and powerful biological signals from the originating cell to neighboring or distant recipient cells (Figure 1). Exosomes have specific cell surface molecules, such as differential expression of exosomal integrins, that enable them to target recipient cells.[34,35] They interact with recipient cells through multiple mechanisms, including direct binding to target receptors or release of molecular cargo into a target cell via endocytosis or direct membrane fusion. The composition of their molecular cargo is specific to each

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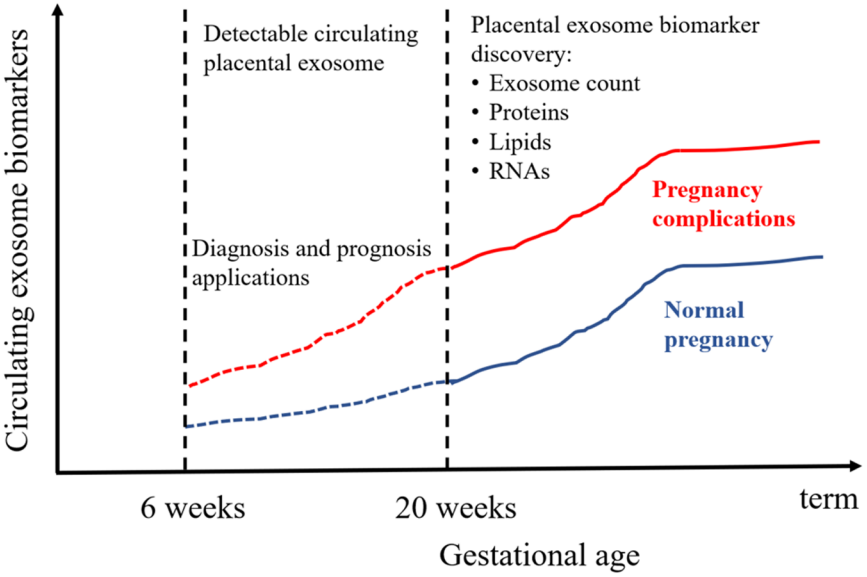

Figure 3. Representative diagram of current research on the circulating placental exosome in pregnancy complications.

cell type and is influenced by tissue physiology and cellular activities. Exosomal cargo has been shown to have the following hallmarks: regulation of gene expression, reprogramming of target cell, intracellular signaling and cell-to-cell communication, affecting cell survival and proliferation, differentiation and neoplasia, modulation of the immune system, inducing angiogenesis, activating invasion and metastasis; hence, they are valuable diagnostic and prognosis biomarkers.[36] Exosomes may be assembled and released as a result of signals from nearby cells, remote tissues, or local environmental conditions (e.g., the level of oxygen, glucose, and free fatty acid).[37] In this way, exosomes facilitate intercellular communication and serve as both autocrine and paracrine signaling vehicles.

Exosomes released by the blastocyst may affect gene expression and receptivity of endometrial cells, thereby facilitating and regulating the implantation.[38] The role of exosomes in pregnancy has been recognized as a regulator of several processes, such as maternal immune response and metabolic modulations.[39−41] During pregnancy, the release of exosomes is influenced by several cellular microenvironment factors such as hypoxia or high glucose concentration.[42−44] Placenta-derived exosomes have been found to regulate the migration and invasion of recipient cells, playing a vital role in cell-to-cell communication and potentially contributing to the placentation and development of maternal−fetal vascular exchange.[45−49]

In both physiological and pathological pregnancies, placental exosomes have been shown to be released into the maternal bloodstream, suggesting their role as messengers between the mother and fetus during pregnancy. In addition, exosomes are enriched in various biological fluids, as they can traverse physiological barriers, including the endothelial and blood−brain barrier.[50] This makes exosomes an ideal candidate for diagnostic biomarkers. In particular, it has been shown that there is a transfer of exosomes between the mother and fetus during pregnancy, as exosomes from the mother can cross the placental barrier and reach fetal tissues.[51] Czernek et al. have thoroughly summarized this aspect within their review;[51] see Figure 2. The detection of fetal exosomes in maternal plasma suggests that they could be used as biomarkers for pregnancy monitoring, as this could be done through minimally invasive methods using liquid biopsy.

It is suggested that total exosome count alone can also be used as an indication of conditions in pregnancy. The level of exosomes in maternal peripheral circulation was 20−50 times higher than that observed in nonpregnant women and progressively increases further with gestational age.[25,49] These increased concentration of maternal exosomes have been shown to provoke the migration of endothelial cell[49] and vascular smooth muscle cell.[48] Additionally, exosomes isolated from pregnancies complicated by GDM have been found to significantly enhance the release of proinflammatory cytokines from endothelial cells.[52] Existing studies have gathered compelling evidence supporting the significant involvement of exosomes derived from placental cells in intercellular communication pathways that play a potential role in the development of maternal−fetal vascular exchange and placentation.[49]

During pregnancy, exosomes play a crucial role in facilitating communication between the placenta and immune cells in peripheral blood.[37] Placental exosomes can interact with different cell types, including endothelial cells, T cells, NK cells, and monocytes/macrophages,[40] and they can suppress maternal T-cell signaling components (via exosomal FasL and/or PD-L1 expression), resulting in immunosuppression and the promotion of maternal immune tolerance toward the fetus.[25,53] Additionally, exosomes from trophoblast culture stimulate the expression of proinflammatory cytokines, such as IL-1β, in human macrophages, promoting monocyte differentiation and macrophage polarization.[54] Another report has demonstrated that placental exosomes modulate the maternal immune response during pregnancy by activating the NKG2D receptor on NK cells.[55] Interestingly, it is suggested that placental exosomes may protect endothelial cells from viral infection.[56]

Molecular analysis of placental exosomes during pregnancy provides valuable insights into their role in pregnancy complications. The altered expression of molecular cargo in placenta-derived vesicles is crucial to the pathophysiology of conditions such as PE, GDM, and preterm delivery. In PE, the

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up-regulation of tissue factor, fms-like tyrosine kinase (Flt-1), and endoglin in placental exosomes contributes to an enhanced inflammatory response, which is key to the development of this complication.[57] For GDM, proteomic analysis of exosomes obtained from GDM patients has identified significant differences compared to exosomes from normal glucose tolerant patients. The quantitative approach of Sequential Windowed Acquisition of All Theoretical Mass Spectra (SWATH) has revealed important proteins such as pappalysin-1 (PAPP-A) and calcium/calmodulin-dependent protein kinase II beta (CAMK2β) that are associated with the pathophysiology of GDM.[58] Additionally, the miRNA profile of GDM exosomes has been characterized, with miRNAs targeting glucose homeostasis and insulin signaling pathways identified.[41] Moreover, it has been shown that GDM placental exosomes can alter glucose uptake in skeletal muscles, further highlighting their role in glucose metabolism regulation.[41] In the context of preterm delivery, proteomic analysis of maternal plasma exosomes has identified proteins that exhibit differential expression compared to term-in-labor patients.[59] These proteins are involved in inflammatory and metabolic signaling pathways, suggesting their involvement in preterm labor.[59] Differences in circulating exosomal miRNA profiles have been observed between mothers delivering at term and those delivering preterm, with miRNAs targeting pathways related to p53, TGF-β signaling, and glucocorticoid receptor signaling.[60] Overall, these findings contribute to a better understanding of the underlying mechanisms and may pave the way for identifying new biomarkers and therapeutic targets in the management of these conditions.

DIAGNOSIS AND PROGNOSIS

To uncover exosome biomarkers in maternal health diseases, the changes in biomarker levels are usually analyzed at a later stage of pregnancy (typical after 20 weeks of gestation), when the disease has established within patients. Following the discovery of a novel biomarker, further validation is required at early stages of pregnancy to ascertain their utility in diagnosis and prognosis (Figure 3). To date, alterations in exosome count and exosome cargo (such as proteins, lipids, and RNAs) have been found to have clinical relevance to maternal health diseases.

Changes in Placental Exosome Count. Syncytiotrophoblast EVs play a significant role in promoting maternal tolerance to the fetus, and dysregulation of these EVs can potentially lead to the development of various pathologies.[61] Recent studies have shed light on the potential use of these specific EVs in the diagnosis of maternal diseases. Generally, total exosome count and placental-specific exosome count are both increased under most pathological conditions.[52,62,63] During pregnancy, there is a significant increase in the concentration of total exosomes and placental exosomes in maternal plasma of presymptomatic women who later develop PE.[64,65] Previous reports have demonstrated that the placenta responds to changes in tension by releasing more exosomes, affecting both content and bioactivity on cell targets.[66] In particular, exosomes from trophoblast cells cultured at 8% oxygen have been found to increase the level of endothelial cell migration. In contrast, exosomes from trophoblast cells cultured at 1% oxygen have been observed to reduce endothelial cell migration.[67]

It has been demonstrated that exosomes expressing placental alkaline phosphatase (PLAP) are circulating in the peripheral blood of pregnant women.[25,49] Immunohistochemistry analysis has shown that most chorionic trophoblastic cells exhibit positive staining for PLAP.[68] Moreover, a positive correlation has been established between the number of total exosomes and PLAP$^+$-exosomes from the first to third trimester of normal pregnancy,[49] as well as within the first trimester.[31] These findings suggest that quantifying exosomal PLAP can serve as a good method for evaluating placental exosomes in pregnancies.

Within GDM, the release of placental EVs is also significantly enhanced.[52] A longitudinal study has demonstrated that both total and placental-specific exosome counts are elevated in GDM compared to a normal pregnancy of the same gestational age.[69] Additionally, it has been shown that a high level of D-glucose stimulates the release of exosomes from primary trophoblast cells, though the exact mechanism is not fully understood.[43] It is believed that glucose enhances the release of exosomes by speeding up the process of exocytosis, promoting the trafficking of mature endosomes to the plasma membrane, facilitating the formation of exosomes within mature endosome, or by a combination of these mechanisms.[37]

Changes in Placental Exosomal Cargo: Proteins and Lipids. In a study, the proteomic content of plasma exosomes from healthy pregnant women was analyzed, revealing the identification of 340 common proteins.[31] This examination provided insights into the potential use of exosomes as biomarkers for pregnancy-related conditions and helped to advance our understanding of the role that exosomes play in maternal health. Further quantitative proteomic analysis by SWATH-MS suggested association between circulating exosome signaling pathways and pathological conditions such as GDM[58] and preterm birth.[59] In another independent proteomic analysis, about 400 proteins were identified, and 25 proteins were found to be unique to women with PE compared to healthy pregnant women.[70] Moreover, Navajas et al. identified and validated 66 exosomal proteins than can be useful biomarkers for PE.[71] Another study found 14 up-regulated and 15 down-regulated proteins in exosomes from PE compared to normal control.[72]

Further studies revealed promising candidate protein biomarkers in maternal diseases. Vargas et al. discovered the presence of syncytin-1 and syncytin-2 in placental exosomes and found that the expression of syncytin-2 protein is markedly down-regulated in PE compared to healthy pregnancies.[73] Another study observed an increase in neprilysin expression in placental microvesicles (10,000 g fraction) and exosomes (150,000 g fraction) in PE.[74] Tan et al. investigated protein biomarkers for PE from circulating extracellular vesicles and validated a prediction model using PlGF, TIMP-1, and PAI-1.[75,76] Kandzija et al. showed that small STB-EVs expressed the enzyme dipeptidyl peptidase IV (DPPIV) in an active form, and DPPIV enzymatic activity of small STB-EVs from patients with GDM was increased 8-fold in comparison with normal pregnancy.[77] While these findings are promising, further research is needed to fully understand the proteomic content of exosomes in pre-eclampsia and other maternal diseases. Additionally, it is important to establish the clinical utility of these biomarkers through large-scale validation studies, including multicenter trials, to ensure their reliability and generalizability.

The lipid content of placental exosomes has also been explored as a potential biomarker for various maternal health diseases. In a lipidomic analysis, after quantification of

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Table 2. A Summary of Studies Involving Exosomal Proteins and Lipid Biomarkers in Maternal Diseases

Complications	Biomarker	Analysis/validation method	Citation
PE	14 up-regulated and 15 down-regulated proteins	LC-MS/MS	72
PE	25 proteins	1D Gel-LC-MS/MS; IHC	70
PE	Syncytin-2	Western blot, bead-associated flow cytometry, ELISA	73
PE	66 proteins	LC-MS/MS	71
PE	Neprilysin	Immunostaining, Western blot	74
PE	CD105, IL-6, PlGF, TIMP-1, ANP, PAI-1, pro-calcitonin, S100b, TGF-b, VEGFR1, BNP	Antibody array, ELISA, LC-MS/MS	75, 76
PE and recurrent miscarriage	Phosphatidylserine, phosphatidic acid, phosphatidylglycerol, ganglioside mannoside 3	LC-MS/MS	78
GDM	DPPIV	Western blot	77
GDM	78 proteins	SWATH-MS; ELISA	58
Preterm birth	72 proteins	SWATH-MS	59

approximately 200 lipids in STB-derived exosome, up-regulation of phosphatidylserine and down-regulation of ganglioside mannoside 3, phosphatidic acid, and phosphatidylglycerol were identified in pregnancies complicated with PE and recurrent miscarriage.[78]

Table 2 shows a summary of studies involving exosomal proteins and lipid biomarkers in maternal diseases. However, it should be emphasized that, in these studies, the non-homogenous isolated EVs may have components other than placental exosomes. As such, a multiplex platform is needed for diagnostic applications, as multiple biomarker analysis may create a more reliable and predictive model.

Changes in Placental Exosomal Cargo: RNAs. Exosomes may also transfer RNAs (including miRNAs, lncRNAs, and mRNAs) and alter the transcriptome of target cells. As such, exosomes are a valuable source of circulating RNAs for diagnosis and prognosis. Table 3 summarizes studies involving exosomal RNA biomarkers in maternal diseases.

miRNAs are the most intensively studied targets in maternal diseases. In general, exosomes released by normal placental cells exhibit a miRNA profile that is similar to the parent cell.[66] On the other hand, pathologically derived exosomes show a different miRNA profile compared to that of the originating cell.[37] Findings in the pathological miRNA profile emphasize the importance of conducting more intensive screening of exosomes obtained from placental sample. Elevated exosomal number and changes in miRNA content have been associated with detrimental effects toward endothelial cells, leading to endothelial dysfunction and impaired angiogenesis.[94−97] It has been shown that the uptake of STB-EV into endothelial cells can lead to the transfer of placenta-specific miRNAs into their endoplasmic reticulum and mitochondria.[98] This transfer of miRNAs is associated with endothelial damage, as well as oxidative and endoplasmic reticulum stress.[98] However, more study is needed to further understand the specific roles and mechanisms of action of these differentially expressed miRNAs in maternal diseases.

In women with PE, there is an observed increase in total exosomal miRNA level and exosomes exhibit a distinct miRNA profile in these cases.[79] Salomon et al. discovered 12 differentially expressed exosomal miRNAs between normal and PE pregnancies, with miR-486-1-5p and miR-486-2-5p contributing the most in separation of the PE group from the normal control.[64] The identified miRNAs have been found to play a role in pathways involving migration, placental development, and angiogenesis.[64] Elevated levels of miR-210 and miR-15a-5p have been linked to the progression of PE, and even more in the severe form.[79,80] Shen et al. detected an increase in placental-derived exosomal miR-155 in serum samples of patients with PE, which is associated with the suppression of endothelial nitric oxide synthase expression in endothelial cells by targeting the 3′-untranslated region.[81] In a human ex vivo placenta perfusion model of PE, STB-EVs exhibit altered miRNA content, specifically 2 members of the chromosome 19 miRNA cluster placenta, namely, miR-517a and miR-517b.[99] Pillay et al. conducted a study to identify differentially expressed exosomal miRNAs in early and late onset of PE.[83] They discovered that miR-122-5p and miR-3605-3p were among the miRNAs that exhibited differential expression between the two groups. Devor et al. employed the TaqMan low-density array to identify 8 miRNAs differentially expressed in pre-eclamptic women; some of these miRNAs (miR-134, miR-376c, miR-486-3p, and miR-590-5p) were differentially expressed even in the first trimester.[84] Li et al. found 7 miRNAs displaying differential expression in serum exosomes from women with PE.[85] In another analysis focused on severe PE, 15 up-regulated and 14 down-regulated miRNAs were identified.[86] Exosomal miR-517-5p, miR-520a-5p, and miR-525-5p were down-regulated during the first gestational trimester in women that later developed either gestational hypertension, PE, or fetal growth restriction.[87]

In GDM, Gillet et al. identified 10 up-regulated exosomal miRNAs that are involved in trophoblast proliferation and differentiation, glucose transport, and insulin secretion/regulation.[89] Another independent research group found 5 overexpressed exosomal miRNAs (miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p, and miR-224-5p) from GDM chorionic villi that were also increased in expression in skeletal muscle tissue from GDM pregnancies, suggesting that exosomal miRNAs can affect skeletal muscle insulin sensitivity.[41] Exosomal levels of miR-518a-5p, miR-518b, miR-518c, miR-518e, miR-520c-3p, and miR-525-5p are also found to be increased in GDM.[90]

Fallen et al. conducted a study where they identified several miRNAs in EVs that can potentially be diagnostic biomarkers for preterm birth.[93] Moreover, another finding showed a total of 173 miRNAs that were differentially expressed in exosomal miRNA in preterm birth compared with normal term birth, revealing that miRNA targeted pathways are involved in p53, TGF-b, and glucocorticoid receptor signaling.[60]

Interestingly, recent research has demonstrated that infections, such as malaria or HIV, can alter trophoblast EV composition.[61] Trophoblastic exosomes exhibit a strong

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Table 3. Exosomal RNA Markers in Maternal Diseases

Complications	Exosomal biomarker	Analysis/validation method	Reference
PE	12 miRNAs, notably miR-486-1-5p↑ and miR-486-2-5p↑ (other: miR-423-5p↑, miR-451a↑, miR-107↑, miR-15a-5p↑, miR-335-5p↑, miR-92a-2-3p↑, miR-103a-1-3p↑, miR-103a-2-3p↑, miR-92a-1-3p↑, miR-126-3p↓)	NGS	64
PE	miR-210↑	qPCR	79
PE	miR-15a-5p↑	qPCR	80
PE	miR-155↑	qPCR	81
PE	120 mRNAs: CGA↑ and KISS1↑ are validated 248 lncRNAs: NR_120647↑, lnc-WDR60-5:1↑, lnc-SOAT1-3:2↓, and NR_110816↓ are validated	Microarray, qPCR	82
PE	Early onset: miR-3605-3p↓ and miR-122-5p↑ Late onset: miR-3605-3p↑ and miR-122-5p↓	Microarray	83
PE	miR-134↑, miR-196b↑, miR-302c↓, miR-346↓, miR-376c↑, miR-486-3p↑, miR-590-5p↑, and miR-618↓	Microarray	84
PE	miR-153-3p↓, miR-222-3p↑, miR-224-5p↑, miR-325↓, miR-342-3p↓, miR-532-5p↑, miR-653-5p↓	qPCR	85
Severe PE	29 miRNAs, notably miR-125b-5p↑, miR-199a-5p↑, miR-200c-3p↑, miR-203a-3p↑, miR-215-5p↑, miR-340-5p↑, miR-483-5p↓, miR-335-5p↓, miR-543↓, and miR-654-5p↓	NGS	86
Gestational hypertension and PE	miR-517-5p↓, miR-520a-5p↓, and miR-525-5p↓	qPCR	87
GDM	miR-516-5p↓, miR-517-3p↓, miR-518-5p↓, miR-222-3p↓, and miR-16-5p↓	qPCR	88
GDM	9 up-regulated miRNAs (miR-125a-3p, miR-224-5p, miR-584-5p, miR-186-5p, miR-22-3p, miR-99b-5p, miR-433-3p, miR-197-3p, and miR423-3p) and 14 down-regulated miRNAs (miR-208a-3p, miR-335-5p, miR-451a, miR-145-3p, miR-369-3p, miR-483-3p, miR-203a-3b, miR-574-3p, miR-144-3p, miR-6795-5p, miR-550a-3-3p, miR-411-5p, miR-550a-3-3p, and miR-140-3p)	NGS, qPCR	41
GDM	10 up-regulated miRNAs (miR-122-5p, miR-132-3p, miR-1323, miR-136-5p, miR-182-3p, miR-210-3p, miR-29a-3p, miR-29b-3p, miR-342-3p, and miR-520h)	qPCR	89
GDM	miR-518a-5p↑, miR-518b↑, miR-518c↑, miR-518e↑, miR-520c-3p↑, and miR-525-5p↑	qPCR	90
GDM	84 mRNAs: TIMM8B↑, SIRT1↑, MIEF2↑, SIRPB1↑, CRYBG3↓, GRIN2D↓, CLDN1↓, ARID1B↓, and ABCD1↓ are validated 256 lncRNAs: NONHSAT034253.2↑, lnc-CCNH-5:2↑, ENST00000509911.2↑, lnc-RXYLT1-3:2↑, lnc-TFDP2-7:2↑, lnc-COX17-2:3↑, lnc-ZBTB46-3:6↑, lnc-NTN4-3:1↓, lnc-NDUFAF4-3:1↓, lnc-TBC1D30-4:1↓, ENST00000596839.1↓, lnc-ZW10-2:1↓, lnc-ZNF800-1:1↓, lnc-EIF4ENIF1-1:1↓, and lnc-ATP8B3-3:1↓ are validated	Microarray, qPCR	91
GDM and macrosomia	98 mRNAs, 372 lncRNAs, and 452 circRNAs: GDF3↑, PROM1↑, AC006064.4↑, lnc-HPS6-1:1↓, circ_0014635↑, and lnc-ZFHX3-7:1↑ are validated	Microarray, qPCR	92
Preterm	173 miRNAs	NGS	60
Preterm	miR-100-5p↓, miR-141-3p↓, miR-194-5p↑, miR-377-3p↓, miR-515-5p↓, miR-517a-3p↓, miR518e-5p↓, and miR-525-5p↓	NGS, qPCR	93
Infection	chromosome 19 miRNA clusters	qPCR	61

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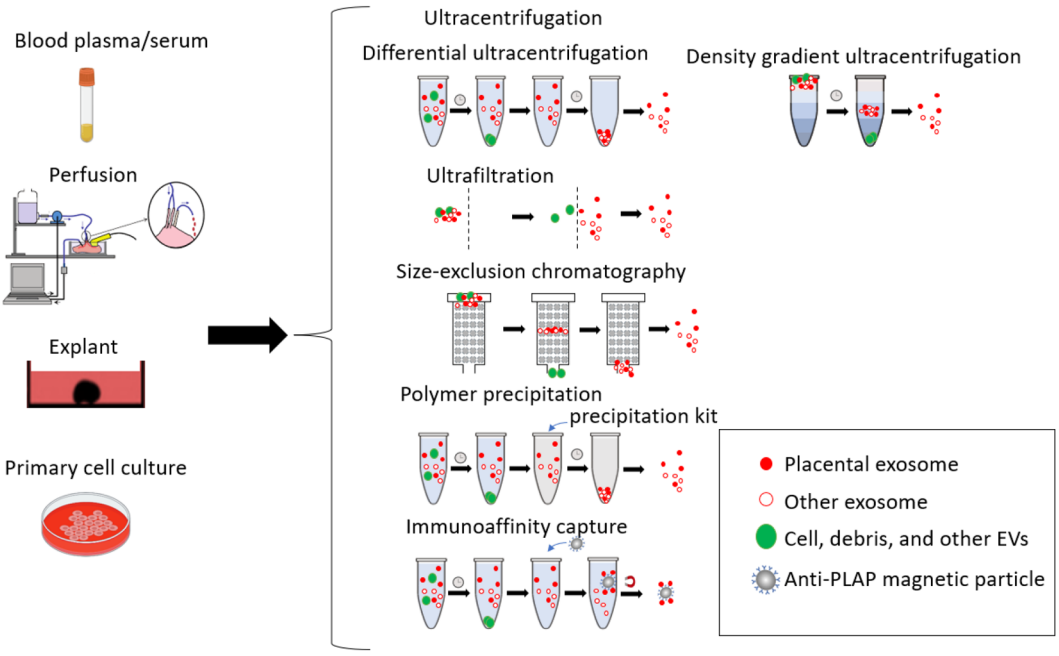

Figure 4. Enrichment of placental exosomes in maternal health research.

Table 4. Commercial Kits for Exosome Isolation

Method	Supplier	Products	Time	Cost	Scalability	Recovery	Specificity
Size-exclusion chromatograhy	iZON	qEV columns according to sample loading volume and optimal recovery range of particle size	+	+	+	+	+++
	Norgen Biotek	Separate reagents for plasma/serum, urine, saliva, cell culture media	+	+	+	+	+++
	Creative Bioarray	ExoQuali Exosome Chromatography Columns	+	+	+	+	+++
Polymer precipitation	Invitrogen	Total exosome isolation kits for cell culture media, serum, plasma, and urine	+++	++++	++++	++++	+
	Cell guidance systems	ExoSpin Exosome Purification Kit for cell culture media/urine/saliva and other low-protein biological fluids	+++	++++	++++	++++	+
	Qiagen	miRCURY Exosome Serum/Plasma Kit and miRCURY Exosome Cell/Urine/CSF Kit	+++	++++	++++	++++	+
	System Biosciences	ExoQuick and ExoQuick-TC for biofluids and tissue culture media	+++	++++	++++	++++	+
	Macherey-Nagel	Exosome precipitation solutions for serum/plasma and urine	+++	++++	++++	++++	+
	Creative Bioarray	ExoQuali exosome isolation kits for cell culture media, stem cell media, urine, plasma, serum, and other body fluids (all in one)	+++	++++	++++	++++	+
Immuno affinity capture	Invitrogen	Dynabeads Exosome-Human CD63 isolation, human CD9 isolation, and human CD81 isolation	++++	+++	+++++	+++	++++
	Miltenyi Biotec	Exosome isolation kit CD9, CD81, CD63, pan	++++	+++	+++++	+++	++++
	Inoviq	Exo-Net pan-exosome	++++	+++	+++++	+++	++++
	Creative Bioarray	ExoQuali Overall Exosome Isolation Immunobeads from cell media, biofluids	++++	+++	+++++	+++	++++

antiviral property, containing miRNAs from the chromosome 19 miRNA cluster.[56,100]

Kumpel et al. studied mRNA expression in STB microparticles isolated from human placenta,[28] demonstrating that these microvesicles contain transcripts of trophoblast-specific proteins (HPL, HCG, and GPIIIa). However, changes in placental exosomal mRNA expression are still under exploration. In an effort to analyze exosomal transcriptomics under pathological conditions, Hu et al. identified 120 differentially expressed mRNAs in exosomes isolated from cord blood of PE patients,[82] while Cao et al. identified 84 changes in mRNA expression and 256 changes in lncRNA expressions for GDM patients.[91] Yuan et al. found a total of 98 mRNAs, 372 lncRNAs, and 452 circRNAs that are differentially expressed in cord blood exosomes of pregnancies with GDM and macrosomia; then, they constructed a good prediction model using exosomal expression of GDF3 and AC006064.4, along with maternal age, fasting plasma glucose, and 2-h plasma glucose.[92]

EXOSOME ENRICHMENT AND PURIFICATION METHODS USED IN STUDYING MATERNAL HEALTH DISEASES

There are various sources of exosomes such as trophoblast cell culture (primary cells and established cell lines), maternal blood, placental perfusate, and placental explant culture; see Figure 4. Several methods for the isolation of placental exosomes have been introduced with certain differences in

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subpopulation of isolated EVs, including ultracentrifugation (differential ultracentrifugation and density-gradient centrifugation), ultrafiltration, size-exclusion chromatography, polymer precipitation, and immunoaffinity capture. Table 4 is a summary of some commercially available products for exosome isolation. Comparative analysis can be compromised, and data interpretation may be confounded when using nonstandardized and/or poorly characterized vesicle preparations. Therefore, standardization is needed to facilitate the discussion of the results.

Ultracentrifugation. Ultracentrifugation is the most commonly used method for exosome isolation, and it is sometimes considered the gold standard for this purpose. Two types of ultracentrifugation are available: differential (sequential) centrifugation and density gradient ultracentrifugation.

The principle of differential centrifugation is based on different sedimentation rates of particles with different sizes, shapes, and densities under centrifugal force. This type of ultracentrifugation usually requires multiple steps to sequentially remove cell and debris, apoptotic bodies, large vesicles, and microvesicles and finally precipitate exosomes with high-speed centrifugation (≥100,000*g*). This method can isolate a large amount of starting material at a relatively low cost (except for the initial cost of expensive ultracentrifugation equipment). A detailed protocol can be adopted in ref 101. In particular, differential centrifugation (involving sequential centrifugation of supernatant at 300, 2000, 12,000, and 100,000*g*) has been employed to isolate a mixed population of both microparticles and nanoparticles from biological fluids or conditioned media. These procedures are often called STB microparticle preparations, although the isolated EVs are often a result of co-sedimentation of exosomes with other vesicles. For instance, when isolating exosomes from placental explant culture supernatant, the sample is subjected to centrifugation at 4 °C and 800*g* for 10 min to remove cell and debris, at 10,000*g* for 5 min to remove apoptotic bodies and ectosomes, and finally at 100,000*g* for 60 min to receive exosome pellets.[28,102] Similarly, Baig et al. sequentially centrifuged culture supernatant at 4 °C at 1000*g* for 10 min, 10,000*g* for 10 min, and 100,000*g* for 60 min for the isolation of exosomes.[78]

Sometimes, an additional filtration step is added to the protocol to increase the exosome purity. For example, after sequential centrifugation at 4 °C and 300*g* for 10 min, 2000*g* for 10 min, and 10,000*g* for 30 min to eliminate cells, dead cells, and cell debris, supernatant was filtered through a 0.22 μm filter; then, exosomes were pelleted by ultracentrifugation at 100,000*g* for 70 min at 4 °C.[103] Diluted plasma sample was subjected to a similar procedure, with the last ultracentrifugation at 120,000*g* for 75 min instead.[86] Similarly, blood samples were sequentially centrifuged at 4 °C and 3000*g* for 15 min and 12,000*g* for 30 min, filtered, and then ultracentrifuged at 100,000*g* for 180 min.[72,82,91,92] For maternal perfusate, samples were centrifuged twice at 1500*g* for 10 min and 10,000*g* for 30 min, filtered, and ultracentrifuged at 150,000*g* to achieve enriched exosomes.[104]

Several studies have documented the achievement of a purer population of exosomes by incorporating density gradient ultracentrifugation after the final ultracentrifugation step. This technique involves the separation of vesicles based on their variation in flotation densities (buoyant density), enabling them to travel in an overlaid sucrose or iodixanol (such as OptiPrep) gradient centrifugation. For instance, after differential centrifugation (300*g*, 1200*g*, 10,000*g*, filter, 100,000*g*), the exosome pellet is subsequently ultracentrifuged on top of a 30% sucrose/D_2O density cushion at 100,000*g*.[56,66] The exosome-containing phase was collected and resuspended in phosphate-buffered saline (PBS) and then ultracentrifuged again at 100,000*g* for 1 h. In a similar manner, after centrifugation at 500*g* and 2500*g*, the supernatant was filtered through a 0.22 μM filter and centrifuged at 100,000*g* overnight; then, the pellets were prepared in OptiPrep gradient and ultracentrifuged at 100,000*g* for 22 h.[85,100]

Another research group slightly modified the sequences of the centrifugation protocol. After centrifugation at 300*g* for 15 min, 2000*g* for 30 min, and 12,000*g* for 45 min, the resulting supernatant was filtered through a 0.22 μm filter sterilizer Steritop (Millipore) and subsequently subjected to further centrifugation at 120,000*g* for 70 min. The resulting pellet was resuspended in PBS, washed, and subjected to another round of centrifugation at 120,000*g* for 75 min. The pellet was again resuspended in PBS, layered on a cushion of 30% (w/v) sucrose, and centrifuged at 110,000*g* for 75 min. The fraction containing exosomes was recovered and then ultracentrifuged at 110,000*g* for 70 min.[48] Minor changes to protocol have been adopted by the same research group,[31,49] such that the sample was diluted with PBS and exosomes were isolated using a combination of differential centrifugation, microfiltration, and buoyant density ultracentrifugation. Initially, centrifugation was carried out at 2000*g* and 4 °C for 30 min, followed by 12,000*g* at 4 °C for 45 min to sediment cell nuclei, mitochondria, and debris. The supernatant was ultracentrifuged at 100,000*g* at 4 °C for 2 h. The pellet was suspended in PBS and filtered through a 0.22 μm filter. The filtrate was centrifuged at 200,000*g* at 4 °C for 70 min, and the pellet was resuspended in 2.5 M sucrose. A continuous sucrose gradient (0.25−2.5 M) was made and centrifuged at 110,000*g* for 20 h. Fractions were automatically collected using a peristaltic pump and then centrifuged at 200,000*g* for 70 min. Later on, this group applied an OptiPrep gradient to their ultracentrifugation procedure for the enrichment of exosomes,[41,52,64,67,105] probably because of the outperformance of this method in terms of purity,[106] which is a prerequisite for reliable omics data. Alternatively, after differential centrifugation (2000*g* for 30 min, 12,000*g* for 45 min, and 100,000*g* for 2 h), the exosomes could be loaded in gradient iodine yellow solution (from 40% w/v to 5% w/v) and then ultracentrifuged at 100,000*g* for 20 h.[80]

Despite the popularity, such preparations have major drawbacks of sample loss and fragmentation of exosomes due to filtration and centrifugation force. Time-consuming, tedious labor work and the requirement for large sample volumes should also be considered as disadvantages of these protocols.

Size-Based Isolations. Size-based isolation methods, such as ultrafiltration and size-exclusion chromatography (SEC), have also been used to isolate exosomes with less contamination from other EV subtypes.

Although filtration was introduced as a standalone method, it is often employed as a supplement to ultracentrifugation (see the section above). This technique utilizes porous membranes to trap larger particles, while allowing only smaller particles to pass through. Compared with other methods, ultrafiltration requires significantly less time, but there is a risk of filter clogging (especially for highly viscous samples) that reduces the lifetime of the membranes. Large-scale isolation of exosomes with high purity can be achieved with cross-flow/tangential-flow filtration.[107] In this approach, pressure is not

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applied orthogonally to the membrane but instead passes sample tangentially to the membrane.

SEC is another size-based isolation technique. Several groups used iZON's qEV column[71,74,93] or Norgenbiotek's exosome isolation column[88] to isolate exosomes based on size exclusion chromatography without ultracentrifugation. These commercial prepacked columns are convenient and reproducible and can be applied to many sample types without the need of a chromatography system as they are compatible with a standalone pump. The columns used in SEC are filled with a porous matrix (stationary phase) that allows tiny particles to pass through. Particles that are larger than the cutoff size of the porous matrix are eluted faster than smaller particles. The cutoff size is dependent on the choice of material for matrix; for example, Sepharose-2B (which has a pore size of ~60 nm) was used by Salomon's group. Exosomes purified by this method maintain their structure, integrity, and biological function as shear force is avoided during the process, which is favorable for later applications such as SWATH-MS proteomic analysis and in vivo experiments.[42,59]

Polymer Precipitation. Exosome isolation can be performed with low-speed centrifugation using polymer-based precipitation. The most common polymer for this purpose is polyethylene glycol (PEG) with MW from 6000 to 20,000 Da. The highly hydrophilic polymers can induce the formation of a hydrophobic microenvironment by interacting with water molecules surrounding exosomes, leading to exosome precipitation out of the aqueous phase. Commercial kits such as Invitrogen total exosome isolation kit, ExoSpin Exosome Purification Kit (Cell Guidance Systems), miRCURY exosome isolation kit (Qiagen),[83,87] ExoQuick (System Biosciences),[73,84] and Macherey-Nagel's exosome precipitation solution[79] have been used for exosome isolation in maternal health research.

These procedures isolate EVs from less sample volume in a rapid, simple, and low-cost manner. However, the finalized preparation of EVs often faces challenges such as co-isolation of other EV subtypes and protein complexes, as well as reduction in stability.[108] Hence, this method is ineffective in clinical settings but is useful in other situations. In an effort to obtain purified exosomes, Shen et al. implemented a protocol with a series of centrifugation steps combined with PEG precipitation.[81] Briefly, serum was initially centrifuged at 3000*g* for 20 min at 4 °C, after which the resulting supernatant was filtered through a 0.22 μm filter followed by an overnight incubation with 8% PEG 6000. The mixture of 8% PeG 6000-serum was subjected to further centrifugation at 10,000*g* for 1 h at 4 °C, and the mixture precipitate was resuspended in 0.25 m sucrose. Subsequently, the suspension was layered onto a linear sucrose gradient, ultracentrifuged at 100,000*g* for 5 h at 4 °C, and divided into fractions with density ranging from 1.03 to 1.18 g/mL. These fractions were incubated with 8% PEG 6000 overnight again and were then centrifuged at 10,000*g* for 1 h at 4 °C. Finally, the collected exosomes were suspended in PBS.

Immune Affinity Capture. This method is based on the specific binding between proteins (antigens) and their corresponding antibodies. The antibody-based isolation employs exosome surface markers, especially transmembrane proteins such as CD9 and CD63, to isolate exosomes from samples. Placental-specific markers, such as PLAP, are also used for the isolation of placental exosomes. Immunological-based capture provides specificity to the isolation process, but it is not suitable for large-scale analysis of exosomes due to the cost of required antibodies, which is dependent on the sample volume. Therefore, this approach is often used after exosome isolation by using other methods. For instance, this sample procedure has also been utilized by Sabapatha et al.[25] After plasma samples were separated by size exclusion chromatography followed by ultracentrifugation, the pellet was resuspended and incubated with anti-PLAP antibody agarose microbeads. The exosome/microbead complex was subsequently separated through centrifugation, allowing bound exosomes to be isolated. After elution to release PLAP-positive exosomes from microbeads, microbeads were removed by centrifugation. Isolated placental exosomes were then concentrated by another ultracentrifugation for further analysis.

Luo et al. isolated exosomes from BeWo cell culture media by immune capture with Dynabeads.[109] After removal of cell and debris, concentrated culture media was subjected to incubation with Dynabeads M280 that were prebound with an anti-mouse IgG/anti-CD63 mouse monoclonal antibody at 4 °C overnight. After several washings, the captured exosomes could be used for RNA extraction and analysis.

Our group recently developed a simple and rapid method to directly capture exosomes from cell culture media using superparamagnetic gold-loaded ferric oxide nanocubes (Au-NPFe_2O_3NCs).[110] Au-NPFe_2O_3NCs were prepared with anti-CD63 antibody and then incubated with conditioned media for 1 h at room temperature. After magnetic washing, the nanocube-captured exosomes were ready for further analysis. This is a promising material to avoid preisolation steps for placental exosome preparation.

PLACENTAL EXOSOME CHARACTERIZATION METHODS

Placental exosomes can be characterized physically (i.e., shape and size distribution) using electron microscopy, nanoparticle tracking analysis, and dynamic light scattering. Biological characterization of exosomes involves identifying the molecular components of exosomes, which can confirm the placental origin.

Physical Characterization. Electron microscopy (EM) is a widely used technique for imaging exosomes and is regarded as the gold standard for this purpose.[16] It provides direct evidence of the presence of exosomes and their size and morphology. The resolution for EM imaging is typically around 1−5 nm, and it can be used to characterize the morphology of exosomes, although the cup-shaped morphology often observed in EM imaging is an artifact caused by the collapse of the sample during the drying process.[111] EM can also be used for biological characterization of exosomes using the "immuno-gold-labeling" technique, though this has not yet been used in placental exosome research.

Nanoparticle tracking analysis (NTA) is an optical particle tracking method for obtaining the concentration and size distribution of exosomes. NanoSight is the commercial instrument name of NTA. In light scatter mode, nanoparticles in the sample scatter a laser beam, and the average velocity of each particle is determined by using the Stokes−Einstein equation. This equation utilizes the recorded Brownian motion of the particle captured by a CCD camera to calculate the particle's hydrodynamic diameter. Analysis of fluorescent-labeled exosomes (for example, by using nanocrystal Qdot605-conjugated antibody[59,67,104]) is also possible with fluorescent

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mode, allowing analysis of biochemical composition and origin of exosomes in addition to physical characterization.

Dynamic light scattering (DLS) is a technique used to determine the average hydrodynamic diameter of isolated particles that exhibit a relatively uniform size distribution. This technique utilizes the analysis of light scattering patterns to calculate the average size of particles in a sample. In a study about the effect of exosomal miR-155 to endothelial cells, Shen et al. used this technique to characterize the size distribution of placental exosomes.[81] However, the presence of particles with various sizes and characteristics can affect the accuracy and reliability of measurements obtained through DLS; therefore, this technique is less suitable for the analysis of heterogeneous EV populations.[112]

Biological Characterization. *Western Blot (WB).* WB is a widely used laboratory technique for detecting specific proteins in a mixture of proteins extracted from biological samples. The principle of WB involves the separation of the proteins based on their molecular weight using electrophoresis, followed by transfer onto a membrane. The membrane is then probed with a specific primary antibody that recognizes and binds to the target protein of interest. Subsequently, the bound antibody is visualized by various detection methods, such as enzyme-linked or fluorescent-labeled secondary antibodies. Conventional Western Blot has been used to demonstrate the presence of a specific protein in exosome lysates, such as exosome markers including tetraspanins (CD9, CD63, CD81), TSG101, Alix, syntenin, and placental marker (PLAP).

ExoELISA. ExoELISA (System Biosciences) enables the sensitive detection and quantification of specific exosomal subpopulations. The exosome particles and their associated proteins are directly immobilized onto the wells of the microtiter plate. After that, a detection antibody specific to a target antigen (e.g., CD63) is added to the wells. For quantification, an extrasensitive tetramethylbenzidine substrate is used. ExoELISA kits provide exosome standards for the creation of calibration curves. The ExoELISA-ULTRA kit offers increased sensitivity, detecting down to 1 μg of protein equivalent, and reduces the total assay time to only 4 h.

DETECTION METHODS

Specific Marker Detection with Conventional ELISA and Western Blot. The most common methods to show the presence of a specific protein in exosomes are ELISA and Western blot, which were discussed above. Western blotting is a semiquantitative technique that is powerful for detecting and characterizing specific proteins in a sample. However, it requires more time and skill than ELISA. On the other hand, ELISA is a quantitative technique that provides an accurate measure of the amount of protein present in a sample and has better sensitivity (typically down to 10 pg).

Interestingly, Göhner et al. developed an enzyme-linked sorbent assay (ELSA) assay to quantify STB-EVs in biological fluids down to 93.75 particles/mL.[30] In brief, STB-EVs are captured through the binding of phosphatidylserine to immobilized annexin V. Then PLAP enzymatic activity is measured by a colorimetric reaction involving NADPH and the formation of detectable formazan. The described assay is similar to a conventional ELISA but does not rely on an immune-active component. However, it is possible that the assay is interfered with by phosphatase activity from particles other than PLAP-positive placental EVs.

Fluorometric Detection. While fluorescent NTA has been used for placental exosome detection,[59,67,104] it has a limitation on the number of fluorescent channels. Conventional flow cytometry is often configured with a higher number of fluorescent channels, but its usage is limited due to the small size and low refractive index of exosomes. To address this problem, latex beads have been used to overcome the challenges in the usage of flow cytometry in placental exosome study.[73] These methods based on fluorescently labeled antibodies detect specific markers on the surface of intact exosomes, giving information that conventional ELISA and Western blot cannot provide.

Mass Spectrometry. This high-throughput analytical technique provides in-depth information about the protein composition of exosomes. This information can help researchers understand the functions and biogenesis pathways of exosomes and identify potential biomarkers. Mass spectrometry has several advantages over antibody-based methods such as ELISA and Western blotting, including better specificity, linearity, reproducibility, and typical limits of quantification down to the low fmol range while avoiding the issue of antibody specificity as peptides unique to each protein are measured.[113] Despite its limitations such as being costly and requiring pure samples and a skilled technician, mass spectrometry is a valuable tool in the analysis of placental exosomes, offering insights into the complex molecular mechanisms involved in maternal health diseases. To date, 9769 proteins found in exosomes have been reported in ExoCarta,[114] but only a few hundred proteins are often found in exosome studies in maternal diseases.[42,59,71]

Electrochemical Sensing. In general, electrochemical sensing has several advantages over ELISA, including ultrasensitivity, wider dynamic range, better resolution, low sample volume, and potential for portable instrumentation. Advances in electrochemical sensing have been used as methods for the ultrasensitive detection of placental exosomes. For example, electrochemical biosensors have been developed using antibodies directed against CD63 (exosomal marker) and PLAP (placental marker) for direct exosome isolation and detection of placental exosomes with chronoamperometry.[110] Interestingly, the nanocube used in this study for capture of exosomes has HRP enzyme mimicking activity, which can be applied for colorimetric detection in a manner similar to ELISA and Western blot.[115] Further exploration about applications of this nanostructure material needs to be carried out in the study of maternal diseases.

RNA Detection. Analysis of exosomal RNA can be performed using several different techniques, including microarray (for example, NanoString), quantitative PCR (qPCR), and RNA-seq (which used next-generation sequencing for RNA detection and quantification).

Microarray technology enables the simultaneous assessment of the expression levels for a vast number of RNAs. It is a robust and affordable solution for whole transcriptome differential gene expression analysis when a good reference sequence for the organism of interest is available. However, microarrays can detect sequences that are homologous to what is on the array.

qPCR is a highly sensitive and quantitative method for measuring nucleic acid levels. It is best suited for analyzing the expression of a small number of genes with known sequences up to about 30 targets. Compared with microarrays or RNA-seq, qPCR offers a broader dynamic range, lower limit of

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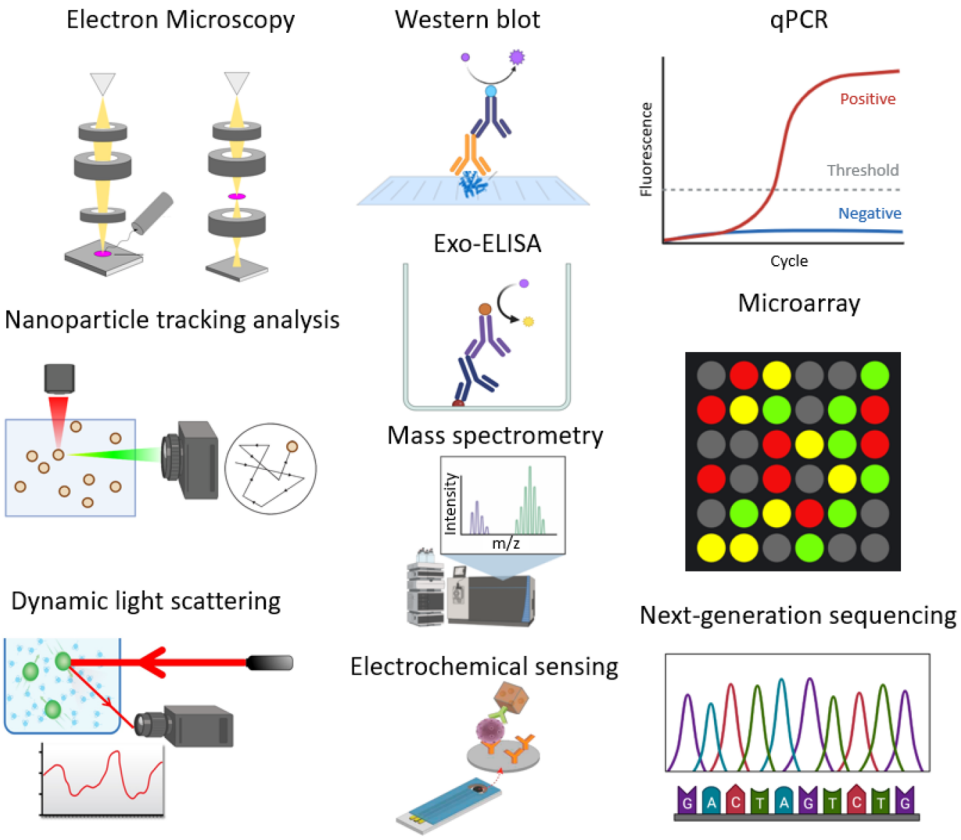

Figure 5. Placental exosome characterization and detection methods. The representative figure for electrochemical sensing was adapted with permission from ref 110. Copyright 2019 American Chemical Society. Other figures were created with Biorender.com.

detection, and more unbiased results. Moreover, it requires a very small amount of starting material and is relatively inexpensive for analysis of a small number of targets.

RNA-seq (or next-generation sequencing) is a hypothesis-free approach that does not rely on prior sequence information. It offers the capability to detect novel genes with good sensitivity to quantify rare variants and transcripts.[116] RNA-seq is particularly advantageous for studies involving numerous targets or samples, as it can identify variants with single-base resolution in a single experiment.[116]

It should be noted that qPCR and microarray data can have good correlation for only RNA exhibiting a fold-change greater than 2 or less than 0.5.[117] Discrepancies may occur between results from these methods due to a low concentration of miRNA or high sequence similarity with other family members.[93] Other notes on exosomal RNA analysis technology can be found in the International Society for Extracellular Vesicles position paper.[118]

Figure 5 provides typical characterization and detection methods used in placental exosome research.

CHALLENGES IN PLACENTAL EXOSOME ISOLATION, CHARACTERIZATION, AND DETECTION METHODS

Although several isolation, characterization, and detection methods have been established for different biomarkers associated with maternal diseases, there remain biological and technical challenges that need to be addressed.

Biological Challenges. *Sample Heterogeneity.* Placental exosomes exhibit heterogeneity in size, density, and molecular composition, influenced by factors such as cellular origin, pregnancy stage, and external factors. The detection of exosome biomarkers can be influenced by various genetic, physiological, demographical, and environmental factors, making it challenging to select an appropriate control for a diverse sample cohort. To address this challenge, it is necessary to conduct regular experiments to assess the impact of sample heterogeneity on the biomarker quantity, development, and function. Additionally, a control cohort encompassing a broad range of variants could help to overcome challenges related to sample heterogeneity.

Clinical Sample. It is widely accepted that pre-eclampsia develops in two stages.[119] During the first stage, there is abnormal placentation and insufficient trophoblast invasion, which results in oxidative stress and excessive inflammation at the maternal−fetal interface. In the second stage, the injured placenta releases imbalanced angiogenic factors that alter the levels of inflammatory cytokines and lead to changes in immune cell recruitment, eventually leading to the development of maternal syndromes and multiorgan failures. Thus, effective recognition of early stages will timely guide the intervention to restrict the development of pre-eclampsia. However, it should be noted that a majority of clinical samples used in biomarker discovery studies are not collected during the early gestation period. This is because most of the time maternal health diseases, such as PE or GDM, are not diagnosed or established until later stages of pregnancy, typically after 20 weeks of gestation. Therefore, further studies about placental exosomes need to be carried out at early stages

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of pregnancy to ensure its utility as a diagnostic or prognostic tool.

Technical Challenges. *Presence of Contaminating Molecules.* Most exosome preparations are contaminated with other EVs and protein aggregates due to the overlapped size and density. Evaluating the purity of isolated exosomes can be challenging as there is no universally accepted method for that. As mentioned in ref 71, the purity of isolated exosomes in a sample can be measured using the ratio of particle number to protein amount (i.e., sEV number/μg of protein), where ratios greater than 3×10^{10} are considered high purity. The purity can also be assessed using tandem mass spectrometry to identify exosomal protein markers from a reference set of 34 exosomal markers.[23] A sample is considered acceptable for analysis if it contains at least 9 exosomal marker proteins (25% of the reference list), and this quality criterion should align with the sEV number/μg of protein ratio.

Limited Comparability of Isolation Methods. The process of isolating placental exosomes is not consistent among different research groups. This makes it difficult to validate the promising findings from these studies. Factors such as the type of placental tissue used, timing of sample collection, storage, and anticoagulation reagents can all impact the purity and composition of isolated exosomes. To ensure accurate results, researchers should follow the established guidelines in ref 20 for exosome isolation before conducting experiments.

Sample Source and Preparation. There are also several challenges in the detection and quantification of specific RNA in placental exosome cargo. Low concentrations of RNA and high similarity of miRNAs within the family present inconsistent data. Additionally, there is a lack of publication using normalization to reference RNA in exosomal RNA, opening the chance for false biomarker discovery.

Preanalytical factors, such as sample collection, processing, the duration between sample collection and centrifugation, storage conditions, centrifugation force, and cryopreservation conditions, can cause changes in biomarker levels. As a result, it is necessary to consider these factors when conducting biomarker analyses. Factors such as the type of placental tissue used, timing of sample collection, storage, and anticoagulation reagents can all impact the purity and composition of isolated exosomes. To date, ultracentrifugation is the most frequently used method for exosome isolation from body fluids. However, this method can be time-consuming, costly, and cumbersome. Furthermore, the high-pressure environment created during ultracentrifugation can expose exosomes to damage, leading to less specificity during precipitation. It can also be challenging to achieve a consistent and reproducible isolation across different settings and locations. To ensure accurate results, researchers should follow the established guidelines in ref 20 for exosome isolation before conducting experiments.

MICROFLUIDIC DEVICES FOR EXOSOME RESEARCH

Recent advancements in microfabrication technology have introduced cutting-edge lab-on-a-chip microfluidic devices that can address the challenges in exosome studies. These devices are capable of integrating all of the steps of exosome isolation, characterization, and detection into a single, streamlined process. They offer several advantages, including their ability to handle small sample volume, cost-effectiveness, precise manipulation of biological particles, high sensitivity and specificity, reliable reproducibility, and stability.[120]

Currently, two main approaches have been explored for microfluidic-based exosome isolation techniques. The first approach uses physical properties, of exosomes, such as size, density, and electric properties, to achieve label-free isolation. The second approach uses affinity-based isolation, where specific probes are employed to capture EVs. A comprehensive overview of both approaches has been provided in ref 121. Table 5 provides a summary of EV isolation techniques with remarkable microfluidic devices.

Several microfluidic platforms with the integration of characterization and detection of exosomes have been developed. These platforms employed various signal amplification and detection strategies such as thermophoretic fluorescence sensing, surface plasmon resonance, surface-enhanced Raman spectroscopy, and magnetic detection.[120] One notable microfluidic device is the ExoChip, developed by Kanwar et al.[149−151] The ExoChip is a low-cost and simple microfluidic platform that can capture and simultaneously quantify exosomes in a single device. The authors demonstrated that ExoChip can recover exosomes with intact RNA, allowing for the profiling of exosomal-microRNAs through OpenArray analysis. This has the potential for biomarker discovery applications. Another interesting platform is the ExoPCD-chip, developed by Xu et al.[152] This two-stage microfluidic platform combined on-chip isolation of exosomes from serum and in situ electrochemical analysis. It can detect $CD63^+$-exosomes at concentrations as low as 4.39×10^3 particles/mL with a linear range spanning 5 orders of magnitude, surpassing existing methods. Recently, Zhou et al. demonstrated a plasma separation and EV detection (PS-ED) chip, which is an integrated microfluidic device that can separate plasma and quantify and perform high-throughput protein analysis of EVs directly from whole blood.[153] This device has two modules: the PS module for plasma separation and the ED module for EV detection. The PS module uses a six-loop microchannel to rapidly separate plasma from whole blood under an inertial force, preserving the quantity of EVs without causing damage to blood cells or interference from cellular debris. The ED module enables protein analysis (CD81, CD24, and EpCAM) and quantification of EVs, with a dynamic range from 2.5×10^2 to 2.5×10^8 particles/μL and a lower limit of detection of 95 particles/μL. Moreover, clinical analysis shows that this PS-ED chip can be utilized for precise diagnosis and monitoring of ovarian cancer.

Despite not yet being adopted as a method for placental exosome research, this technology holds great promise for future applications, particularly in combination with automation to improve the reproducibility of data. Figure 6 provides a visual representation of this exciting technology and the possibilities it offers.

CONCLUSION AND FUTURE DIRECTION

In this review article, we provided a comprehensive overview of placental exosomes and their potential use as biomarkers for maternal health diseases such as PE and GDM. We discussed the importance of clarifying the nomenclature used for placental exosomes, as the use of consistent terminology is crucial for the advancement of the field. Next, we provide a general overview of the role of placental exosomes in maternal health diseases. The placenta, as the main organ connecting the mother and fetus, plays a vital role in maintaining a healthy pregnancy. It has been suggested that placental exosomes could act as biomarkers for maternal health diseases. Then we

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Table 5. Summary of Microfluidic Devices Used for Exosome Isolation

Techniques and working principle	Advantages and disadvantages[121]	EV samples	References
Filtration: Filter acts as a barrier that allows small particles to pass through while retaining larger particles.	• High yield, field-free, easy handling	Cell culture, urine	Exodisc[122]
		Whole blood, plasma	Exodisc-B and Exodisc-P[123]
	• Problem with membrane clogging and EV aggregation	Cell culture, plasma, urine, and lavage	ExoTIC[124]
		Cell culture, plasma	125
Inertial isolation: Inertial lift forces occur when particles flow through microchannels at high velocities. These forces cause larger particles to experience higher inertial migration speed and focus on equilibrium positions farther away from the channel walls.	• Simple device, high throughput, field-free	Cell culture, whole blood	HiDFF[126]
	• Low size resolution	Cell culture, serum	127
Viscoelastic isolation: Viscoelastic lift forces occur when particles flow through microchannels in viscoelastic fluids, causing smaller particles to migrate toward the channel walls.	• Low size resolution, simple device, flexible flow rate, high size resolution, field-free	Whole blood	128
	• Use polymer additive	Serum	129
Deterministic lateral displacement: As particles flow through the channel, smaller particles are guided to follow the fluid streamlines and pass through the gaps between the obstacles, while larger particles are displaced laterally and deviate from the main flow, resulting in their separation from the smaller particles.	• High size resolution, minimal damage to EVs, field-free	Urine, serum	NanoDLD array[130]
	• Low throughput, risk of device clogging, requires precise device fabrication		
Pinched flow fractionation: As particles flow through a constricted channel, larger particles experience increased resistance and are pushed toward the channel walls, while smaller particles are less affected and remain closer to the centerline of the flow.	• Simple device, field-free	Cell culture	131
	• Requires large sheath-to-sample flow rate ratio		
Asymmetric flow field-flow fractionation: Sample is injected into a thin and elongated channel where a crossflow is applied perpendicular to the main flow direction. This crossflow causes the particles to undergo lateral migration, with larger and more elongated particles migrating closer to the channel walls.	• High size resolution, minimal damage to EVs, field-free	Urine	132
	• Requires precise device fabrication and relatively purified input	Cell culture	133
Dielectrophoresis: Uses nonuniform electric fields to manipulate and separate particles based on their dielectric properties.	• Controllable capture and release of EVs, integration of in situ detection	Plasma, whole blood	ACE microarray[134,135]
	• Electric/thermal damage to EVs, requires complex device fabrication	Cell culture	136
Acoustic isolation: The acoustic forces generated by the waves can exert pressure gradients, acoustic radiation forces, and acoustic streaming, enabling the isolation of EVs based on their size, density, and acoustic properties.	• Biocompatible, contact-free, isolation via varied physical properties	Whole blood, plasma	137
	• Complex device fabrication and external control		
Magnetic field: Diamagnetic bioparticles experience a magnetophoretic force opposite to the magnetic field gradient (negative magnetophoresis) due to their lower polarizability compared to the ferrofluid. This force, proportional to the particle volume, enables the active separation and migration of particles in the ferrofluid system.	• High throughput, simple device	Cell culture, serum	FerroChip[138]
	• Low size resolution	Cell culture	139
Immunoaffinity isolation: Particles are captured based on the specific binding between antibody/aptamer and antigen.	• High purity	Cell culture	140
		Cell culture, plasma	141
		Serum, plasma	ExoTENPO[142]
	• Rely on specific antibody/aptamer; requires harsh conditions to release EVs	Cell culture, plasma	nano-HB[143]
		Cell culture	144

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Table 5. continued

Techniques and working principle	Advantages and disadvantages[121]	EV samples	References
Lipid-based isolation: Particles are captured based on affinity between lipophilic compound and the enriched lipid composition on the outer surface of EVs.	• Gentle EV retrieval, high purity	Cell culture	145
		Cell culture, plasma	^{new}ExoChip[146]
	• Lower specificity than immunoaffinity isolation, lack of surface antigen information	Cell culture, plasma	^{HB}EV-Chip[147]
		Cell culture, plasma	148

describe the main methods for exosome enrichment, characterization, and detection, along with the challenges faced in these processes. Finally, we emphasized the need for further validation of placental exosomes as biomarkers in maternal health diseases, specifically at early stages of pregnancy, where the disease has not yet been established. The use of a consistent and uniform methodology for exosome isolation, as well as the use of appropriate validation techniques such as tandem mass spectrometry and quantitative PCR, will be crucial in advancing the field and unlocking the full potential of placental exosomes as biomarkers. Lastly, we strongly advocated for the application of state-of-the-art microfluidic chips for exosome study in maternal health.

In recent years, extensive research on placental exosomes has shed light on their potential as valuable biomarkers for maternal health and their involvement in various processes during pregnancy. These small extracellular vesicles have gained attention for their ability to carry a diverse cargo of biological molecules, reflecting the physiological and pathological status of the placenta and its interaction with maternal tissues. They hold exciting prospects and clinical implications, particularly in the areas of modulating immune responses, influencing placental development and function, and early detection and prediction of maternal diseases. Furthermore, exosomes can be loaded with therapeutic molecules, such as small interfering RNAs or growth factors, and used for targeted delivery to specific tissues or cell types.[154] By reduction of off-target effects and increase of the concentration of the therapeutic substance at the intended site of action, the efficacy and safety of drug delivery are enhanced. This strategy opens up possibilities for modulating immune responses, promoting tissue regeneration, and improving pregnancy outcomes.

Future research on exosomes and EVs in maternal health should aim to deepen our understanding of their role in pregnancy-related disorders and their potential as diagnostic markers. Integration of advanced technologies such as machine learning and high-throughput omics approaches can provide a more comprehensive analysis of exosomal content and enhance our knowledge of their functions. High-throughput omics techniques enable large-scale profiling of exosomal cargo including gene expression, protein abundance, and metabolite profiles. By characterizing the molecular landscape of exosomes, we can identify specific molecules, pathways, and networks associated with maternal health conditions, leading to new targets for diagnosis, treatment, and monitoring. Machine learning algorithms can analyze extensive data sets, revealing hidden patterns, correlations, and predictive models that human researchers may overlook. An example of combining machine learning with an exosomal mRNA data set for diagnostic purposes can be found in ref 142. Integrating proteomics, genomics, and metabolomics data with machine learning algorithms can uncover complex relationships and develop predictive models for maternal diseases. This integrated approach facilitates the discovery of new biomarkers and provides valuable insights into the underlying mechanisms of placental exosomes in maternal health.

Studies could also aim to identify specific EV subpopulations that are associated with specific pregnancy-related disorders as well as to develop more sensitive and specific detection methods. Additionally, research could explore the potential for using exosomes as a delivery system for therapeutic agents, such as small interfering RNAs, to treat pregnancy-related

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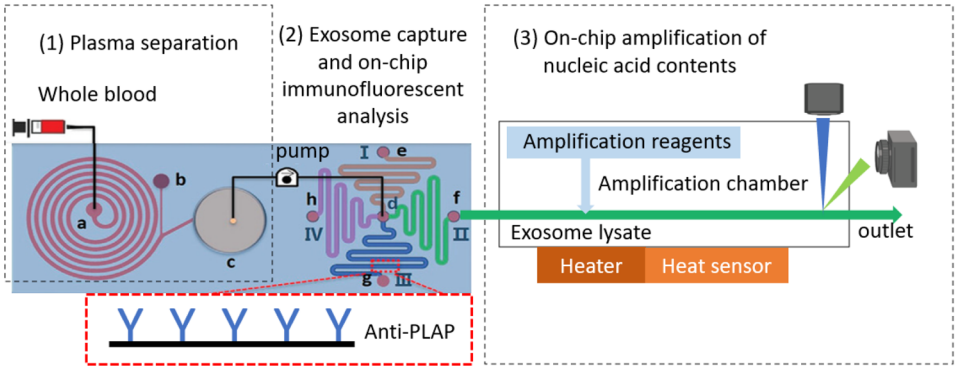

Figure 6. A proposed model of fully integrated microfluidic devices for placental exosome isolation, on-chip immunofluorescent analysis, and nucleic acid biomarker detection from whole blood. (1) Plasma separation module, (2) exosome detection module using a specific marker for placental exosome and immunofluorescent analysis, (3) nucleic acid amplification module. Adapted with permission from ref 153. Copyright 2020 American Chemical Society.

complications. This could involve manipulating the content or targeting the delivery of exosomes or EVs to specific cells to impact their behavior and functions. Overall, a continued exploration of the biology and function of exosomes in maternal health has the potential to advance our understanding of these complex diseases and lead to new diagnostic and therapeutic approaches.

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Notes

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