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**Title:**

Extracellular vesicles as markers and mediators of pregnancy complications: gestational diabetes, pre-eclampsia, preterm birth and fetal growth restriction.

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**Abstract:**

In high income countries, approximately 10% of pregnancies are complicated by pre-eclampsia (PE), preterm birth (PTB), fetal growth restriction (FGR) and/or macrosomia resulting from gestational diabetes (GDM). Despite the burden of disease this places on pregnant people and their newborns, there are still few, if any, effective ways of preventing or treating these conditions. There are also gaps in our understanding of the underlying pathophysiologies and our ability to predict which mothers will be affected. The placenta plays a crucial role in pregnancy and alterations in placental structure and function have been implicated in all of these conditions. As extracellular vesicles (EVs) have emerged as important molecules in cell-to-cell communication in health and disease, recent research involving maternal- and placental-derived EVs (pEVs) has demonstrated their potential as predictive and diagnostic biomarkers of obstetric disorders. This review will consider how placental and maternal EVs have been investigated in pregnancies complicated by PE, PTB, FGR and GDM and aims to highlight areas where further research is required to enhance the management and eventual treatment of these pathologies.

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**Abstract figure legend** Graphical abstract summarising the salient points covered within the topical literature review.**Extracellular vesicles as markers and mediators of pregnancy complications: gestational diabetes, pre-eclampsia, preterm birth and fetal growth restriction.**

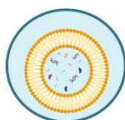
Rachel Farrelly, Margeurite Gina Kennedy, Rebecca Spencer, Karen Forbes



In high income countries, approximately 10% of pregnancies are complicated by pre-eclampsia (PE), preterm birth (PTB), fetal growth restriction (FGR) and/or macrosomia resulting from gestational diabetes (GDM).



Yet, there are still few, if any, effective ways of preventing or treating these conditions.



Recent research involving maternal- and placental-derived EVs (pEVs) has demonstrated their potential as predictive and diagnostic biomarkers of obstetric disorders.



This review considers how EVs have been investigated in pregnancy and to highlights areas where further research is required to enhance the management and eventual treatment of these pathologies.



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## Introduction

In high income countries, approximately 10% of pregnancies are complicated by pre-eclampsia (PE), preterm birth (PTB), fetal growth restriction (FGR) and/or large-for-gestational age (LGA) infants resulting from gestational diabetes (GDM) (Table 1). Despite the burden of disease they place on pregnant people and their newborns, there are still few, if any, effective ways of preventing or treating these conditions. Current management relies on clinical surveillance and optimising the time, place and route of delivery (Alberry & Soothill, 2007; Goldenberg *et al.*, 2008; Burton *et al.*, 2019; Quintanilla Rodriguez & Mahdy, 2022). These obstetric conditions are not only responsible for maternal, fetal and neonatal morbidity and mortality; they are also associated with long-term increased risks of cardiometabolic disease in the mothers and children (Lees *et al.*, 2013; Colella *et al.*, 2018; Graves *et al.*, 2019; Bendix *et al.*, 2020).

There is a growing body of evidence suggesting extracellular vesicles (EVs) play an important role in communication between the mother, placenta and fetus (Tong & Chamley, 2015; Chiarello *et al.*, 2018). This makes them a promising means of investigating the mechanisms underlying obstetric diseases, as well as a potential source of predictive, diagnostic and prognostic biomarkers (Familar *et al.*, 2017; Miranda *et al.*, 2018a). It may also be possible to harness their capacity for inter-organ communication for use in future therapeutics (Merino-Gonzalez *et al.*, 2016; Keshtkar *et al.*, 2018). This review aims to highlight the recent advances in the study of extracellular vesicles in pregnancy with a particular focus on gestational diabetes, pre-eclampsia, preterm birth and fetal growth restriction. It also aims to expose gaps in our current understanding and potential areas for future study in the pursuit of treatment for these pathologies.

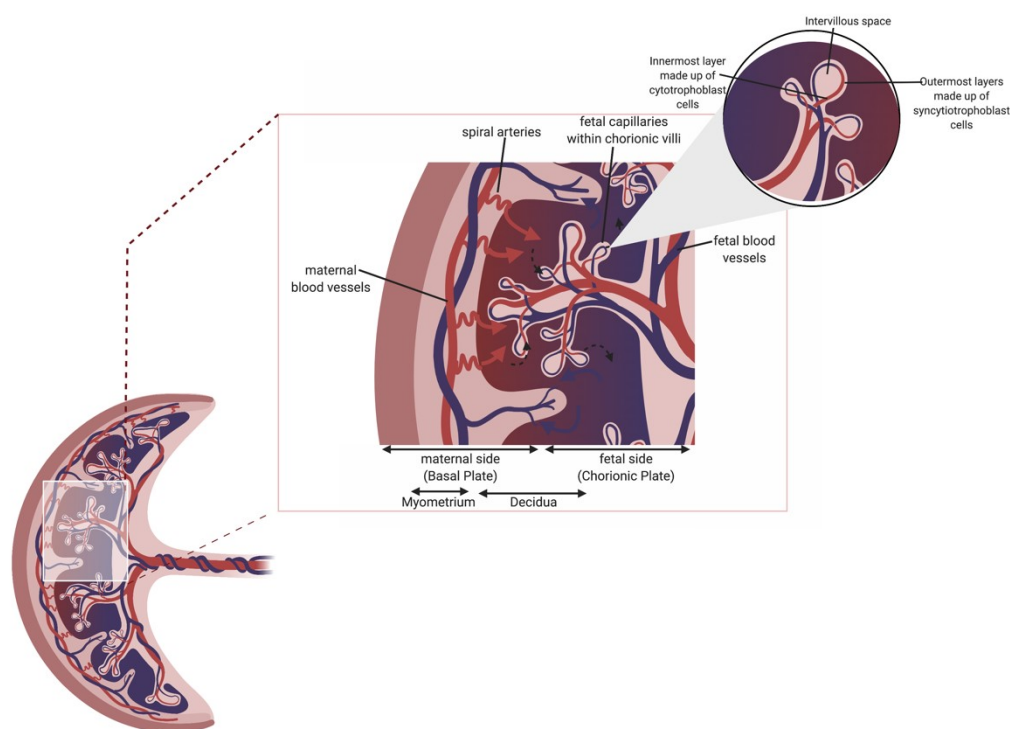
**Table 1.** Brief overview of the obstetric conditions discussed in this article. The definitions and diagnostic criteria provided are widely used but not universally agreed.

Obstetric Condition	Definition	Diagnosis
Gestational diabetes	Glucose intolerance above an agreed threshold that develops during pregnancy and usually resolves after delivery	Elevated fasting plasma glucose and/or elevated one or two hour plasma glucose following a glucose tolerance test (thresholds vary) (National Institute for Health and Care Excellence, 2015 (updated 2020); Kapur <i>et al.</i> , 2020)
Pre-eclampsia	A multisystem disorder characterised by new-onset hypertension at 20+0 weeks of pregnancy or later with one or more additional features	Blood pressure $\geq 140/\geq 90$ mmHg with proteinuria and/or evidence of maternal acute kidney injury, liver dysfunction, neurological features, haemolysis or thrombocytopenia, and/or fetal growth restriction (Brown <i>et al.</i> , 2018; American College of Obstetricians and Gynecologists, 2020)
Preterm birth	Birth before 37 completed weeks of gestation	Birth before 37 completed weeks of gestation
Fetal growth restriction	A failure of the fetus to reach its growth potential. (Malhotra <i>et al.</i> , 2019)	Estimated fetal weight (EFW) or abdominal circumference (AC) $< 3^{\text{rd}}$ centile or EFW or AC $< 10^{\text{th}}$ centile with abnormal Doppler velocimetry and/or slowing of fetal growth (Gordijn <i>et al.</i> , 2018)

## The Placenta

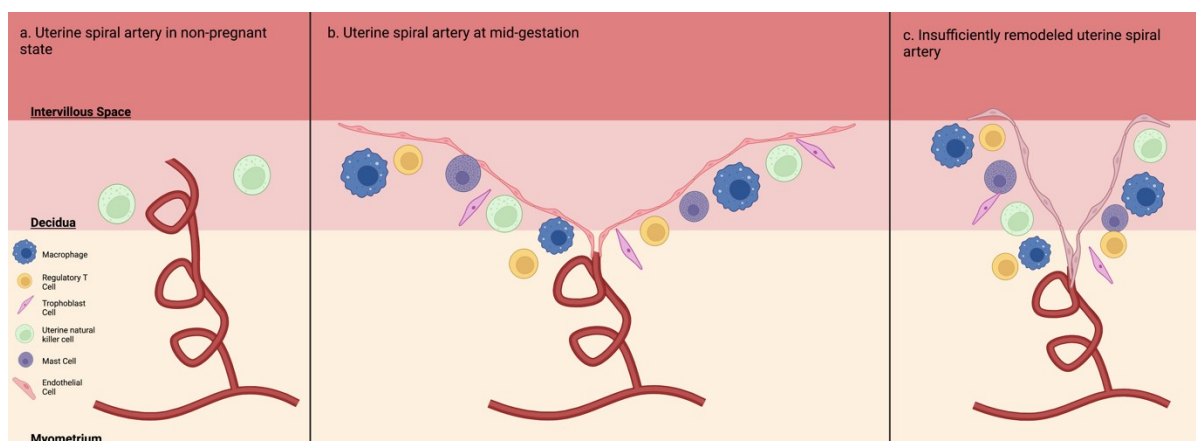
The placenta plays a central role in healthy pregnancy and is both a source and recipient of EVs. From 16 weeks of gestation the placenta comprises a supporting mesodermal core, containing an

extensive network of fetal blood vessels (Figure 1) (Kingdom *et al.*, 2000). These form a branching, villous structure overlaid by the syncytiotrophoblast, a continuously replenished syncytium that sheds fragments into the maternal circulation. Placental angiogenesis and vasculogenesis is heavily influenced by members of the vascular endothelial growth factor (VEGF) family, including placental growth factor (PlGF) and VEGF-A, and their receptors (Umapathy *et al.*, 2019).



**Figure 1. Anatomical cross-section of the human placenta adapted from (Jansen *et al.*, 2020). Created in Biorender.com**

Maternal blood enters the intervillous space via the spiral arteries to supply the fetus and placenta with oxygen and nutrients. During pregnancy the spiral arteries are remodelled following extravillous trophoblast invasion, under the influence of decidual immune cells, to reduce vascular resistance and increase flow velocity (Cartwright *et al.*, 2010b; Williams *et al.*, 2009). Incomplete spiral artery remodelling has been associated with early-onset fetal growth restriction and pre-eclampsia (Figure 2) (Cartwright *et al.*, 2010a) due to impaired placental function, however and the precise mechanisms responsible for this remain elusive.



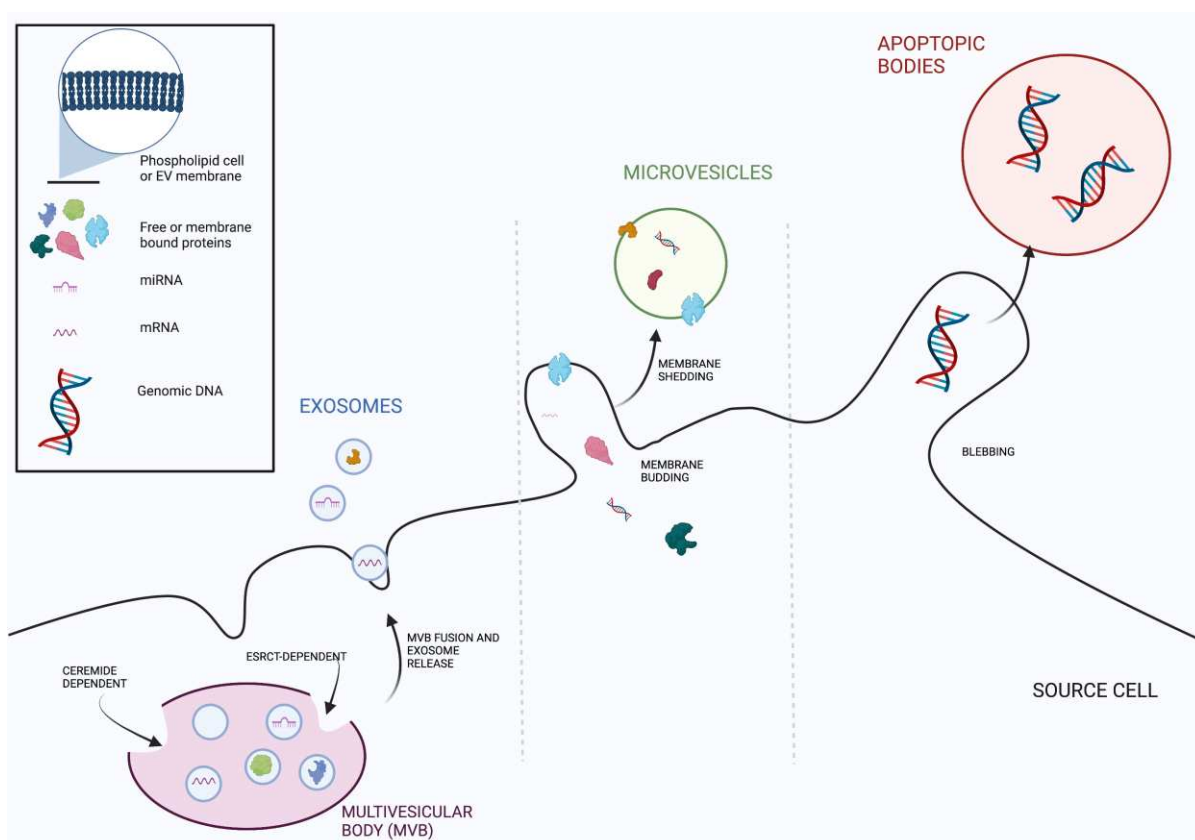
**Figure 2. Schematic diagram of the spiral artery in non-pregnant state, healthy pregnancy, and pregnancies with insufficiently remodelled spiral arteries. A number of other cells in addition to trophoblast cells are thought to be involved in the vascular remodelling of the spiral artery. These include macrophages, uterine natural killer cells (uNK) and uterine mast cells. Adapted from (Schumacher et al., 2018). Created in Biorender.com**

One of the major challenges in studying normal and abnormal placental function is the inaccessibility of the placenta during pregnancy. Placental sampling during pregnancy carries a risk of miscarriage or preterm birth, so placentas from uncomplicated pregnancies can only be analysed following term deliveries. Therefore, placentas from spontaneous or iatrogenic preterm deliveries cannot be compared to 'normal' placentas of the same gestation. Analysis of placental EVs offers a possible solution to this challenge.

## Extracellular Vesicles

Extracellular vesicles are a non-replicating, lipid bilayer delimited particles produced by all cells for cell-to-cell communication (Yáñez-Mó *et al.*, 2015). They are transported within extracellular spaces, in biofluids such as amniotic fluid (Balbi *et al.*, 2017; Hell *et al.*, 2017) and in plasma (Arraud *et al.*, 2014). EVs are characterised by their pathway of release from cells into three subtypes of overlapping sizes: exosomes (~40-150nm), microvesicles (~100-1000nm) and apoptotic bodies (~500-2000nm; Figure. 3 (Skotland *et al.*, 2017). Each of these subtypes is distinguished by its mechanism of biogenesis, exosomes, for example, are generated from the inward budding of the endosomal membrane to form a multivesicular body (MVB) (Skotland *et al.*, 2017; McVey & Kuebler, 2018). The MVB then releases these intraluminal vesicles as exosomes upon fusion with the plasma membrane. Microvesicles, on the other hand, are derived from the outward budding of the plasma membrane and are typically released under conditions of cellular stress and activation (Skotland *et al.*, 2017; McVey & Kuebler, 2018). Lastly, apoptotic bodies are formed during apoptosis, where cellular components are fragmented and packaged into EVs (Kalra *et al.*, 2016)

Due to the overlapping size distributions of the subtypes, in combination with the lack of a definitive specific marker for each subtype, the field is now moving away from using these terms to describe EVs in publications (Lancaster & Febbraio, 2005; Théry *et al.*, 2018). The ‘Minimal Information for Studies of Extracellular Vesicles’ set by the International Society for Extracellular Vesicles (ISEV) instead recommends that EVs are described by their physical characteristics, ergo, their size with defined ranges that do not overlap (Théry *et al.*, 2018). For example, EVs <200nm in diameter are now referred to as small EVs (sEV) and EVs >200nm are large EVs (LEVs).



**Figure 3. Biogenesis of Exosomes, Microvesicles and Apoptotic Bodies (small, medium and large EVs (El Andaloussi *et al.*, 2013)).** Exosomes range from 30-150nm in diameter and are released from the multivesicular body through the fusion with the cell membrane, they show the same membrane orientation as the source cells. Whilst this is also the case with microvesicles (100nm-1µm in diameter), they are formed from a heterogenous process involving the budding of a cell membrane around the intended contents and the shedding of this membrane. Apoptotic bodies range from 50nm-5000nm in diameter, (Doyle & Wang, 2019) They are formed by the separation of source cell plasma from the cytoskeleton as a reaction to increased hydrostatic pressure after cell contraction (Wickman *et al.*, 2012). Created with Biorender.com



## Extracellular Vesicle Cargo

All EVs transport a range of cellular cargo, including phospholipids, miRNA, mRNA, DNA, and transmembrane as well as cytosolic proteins (Kalra *et al.*, 2016; Sáez *et al.*, 2018). In 2007, a role for EVs as mediators of cell-cell communication was first described when Valadi *et al.* demonstrated that EVs can transfer mRNA from one cell to another, leading to protein transcription and hence a potentially functional effect (Lotvall & Valadi, 2007; Valadi *et al.*, 2007). It is now well established that in addition to mRNA, EVs transport their other cargo, including protein, lipid and non-coding RNAs between cells from different areas of the body and as such, they are key regulators of cellular communication (Boon & Vickers, 2013; McVey & Kuebler, 2018).

In addition to their key roles in cell-cell communication, the stability of EVs and their ability to protect their cargo, specifically miRNAs, from degradation (Ge *et al.*, 2014) has sparked research interest into the use of EVs as diagnostic biomarkers (Simeone *et al.*, 2020; Zhao & Yang, 2021), as indicators of disease progression (Lee *et al.*, 2021; Simionescu *et al.*, 2021) and the preliminary exploration at targeted therapies (D'Arrigo *et al.*, 2019; Pezzana *et al.*, 2021). The benefit of EVs over non-vesicular methods of transportation is that the phospholipid bilayer protects cargo and could allow for tissue-specific delivery (Hoshino *et al.*, 2015; Jiao *et al.*, 2017; Manier *et al.*, 2017).

Whilst the role of EVs as biomarkers and functional mediators of cell-cell communication in different physiological and pathological conditions, including in pregnancy, has only emerged in recent years, the production of EVs from the placenta has been long established. During normal pregnancy the syncytiotrophoblast releases EVs of different sizes, including macro-vesicles (such as syncytial nuclear aggregates (SNAs), micro-vesicles (MVs) and exosomes, into maternal circulation (Out *et al.*, 1991; Heazell *et al.*, 2007; Warrander *et al.*, 2012). Much of the original work that examined EVs in pregnancy primarily focussed on large EVs such as MVs and SNAs; establishing that levels increase across gestation and that they have key roles in the pathogenesis of pre-eclampsia by influencing the maternal immune response (Holder *et al.*, 2012; Holder *et al.*, 2016). In more recent years, the focus has shifted to study small EVs in obstetric complications

The role of these large EVs in pregnancy is well reviewed (Redman & Sargent, 2008; Redman *et al.*, 2012) and the study of small EVs continues to be the most commonly studied EV subtype across multiple conditions including cancer (Azmi *et al.*, 2013; Xu *et al.*, 2018 ; Lee *et al.*, 2021 ; Hoshino *et al.*, 2015), heart disease (Bei *et al.*, 2017; Saheera *et al.*, 2021; Akhmerov *et al.*, 2022) and obstetric complications. Therefore, whilst some of the discussed work may include large EVs, due overlap in size categorisation between small EVs and MVs, the main focus will be on sEVs.

## Extracellular vesicles in healthy pregnancy

Endometrial, embryonic and placental EVs all contribute to early pregnancy development, influencing implantation, immunomodulation and spiral artery remodelling (Andronico *et al.*, 2019; Zhang *et al.*, 2020; Chen *et al.*, 2022; Morelli & Sadovsky, 2022). Endometrial epithelial EVs are present in the uterine fluid of humans and other animals (Li *et al.*, 2021; Mishra *et al.*, 2021). EVs from cultured endometrial epithelium are able to enter trophoblast cells (Evans *et al.*, 2019) and have been shown to increase blastocyst adhesion and invasion and promote embryo development (Mishra *et al.*, 2021). Conversely, EVs from cultured embryos can enter endometrial epithelial cells (Giacomini *et al.*, 2017) and trophoblast EVs are readily taken up by monocytes, increasing migration and altering cytokine production (Atay *et al.*, 2011). Placental EVs may also be responsible for presenting paternal minor histocompatibility antigens to maternal T and B cells in such a way that they produce immunotolerance (Morelli & Sadovsky, 2022).

As discussed, spiral artery remodelling is important for meeting the metabolic demands of the fetus and placenta. Salomon *et al.* have demonstrated the capacity for extravillous trophoblast-derived EVs to increase vascular smooth muscle cell migration (Salomon *et al.*, 2014b), while Jia and colleagues found that both maternal and umbilical cord derived porcine EVs could significantly enhance the migration and proliferation of human umbilical vein endothelial cells (Jia *et al.*, 2018). Placental EVs have also been found to contain and release vascular endothelial growth factor A (VEGF-A), a member of the VEGF family and one of the major contributors to angiogenesis and vasculogenesis (Figure 2) (Patton *et al.*, 2015; Condrat *et al.*, 2021).

## Bidirectional Extracellular Vesicle Trafficking via the Placenta

The placenta releases EVs into the maternal and fetal circulations, takes up maternal EVs and allows transit of maternal and exogenous EVs to the fetus (Chiarello *et al.*, 2018; Buca *et al.*, 2020). The capacity for pEVs to enter maternal cells has been demonstrated by Tong *et al.* who found uptake of fluorescently-labelled EVs from cultured placenta localised to maternal lungs, liver and kidneys after venous administration in pregnant mice (Tong *et al.*, 2017). The concentration of placental EVs (pEVs) in maternal plasma increases over the course of pregnancy, as indicated by exosomal placental alkaline phosphatase (PLAP) concentration (Salomon *et al.*, 2014a), with 12-25% of all maternal circulating EVs being of placental origin (Elfeky *et al.*, 2017). Bidirectional trafficking of placental specific chromosome 14 and chromosome 19 cluster miRNAs into maternal and fetal compartments has been demonstrated both *in vivo* in mice, as well as in matched patient placental biopsies, maternal and fetal plasma (Chang *et al.*, 2017 2018; Paquette *et al.*, 2018).

Holder et al.'s visualisation of the internalisation of fluorescent-labelled maternal macrophage-derived EVs by the placenta shows that this EV-mediated communication between maternal tissues and the placenta is bidirectional. They also found maternal macrophage EVs that modulate placental cytokine production were actively transported into the placenta by clathrin-mediated endocytosis (Holder, 2016). This bidirectional communication is supported by the ability of maternal adipose tissue EVs to influence glucose metabolism in the placenta, by upregulating genes involved in glycolysis and gluconeogenesis (Jayabalan, 2019). EVs cargo from dietary origin have also been shown to enter into and influence events in the placenta, highlighting the potential for dietary-derived EVs/cargo to reach even distal organs (Timms *et al.*, 2022).

## Extracellular vesicles in obstetric diseases

Given the increasing evidence for EV function in healthy pregnancy, it is unsurprising that interest in the roles of EVs in obstetric diseases has also grown. This includes both their possible actions as mediators of pregnancy complications and their potential as a source of protein and RNA biomarkers to predict and diagnose disease.

### Extracellular vesicles in Gestational Diabetes

Gestational diabetes mellitus (GDM) affects 6% of pregnancies worldwide and is defined as a glucose intolerance that develops during pregnancy and resolves post-partum (Coustan, 2013; Mack & Tomich, 2017). Pregnancies complicated by GDM have been shown to have higher concentrations of circulating EVs than uncomplicated pregnancies, and that a large percentage of these are PLAP-positive, suggesting that there may be increased pEV biogenesis and release in GDM pregnancies (Salomon, 2016). pEVs have been shown to interact with maternal organs, influencing skeletal muscle biology (Kupper & Huppertz, 2022) and contributing to insulin resistance in the mother (Kandzija *et al.*, 2019; Palma *et al.*, 2022). Whilst the importance of pEVs in the pathogenesis of GDM is of obvious importance, there is also evidence that EVs in maternal circulation, and their miRNA cargo, could potentially influence placental development and dysfunction in GDM (Kennedy *et al.*, 2019; Quilang *et al.*, 2022). Gillet *et al.* (2019), detected 10 maternal serum EV miRNAs that were upregulated in GDM at early gestation (6-15 weeks) (Gillet *et al.*, 2019). Whilst the functional role of these was not reported, eight of the altered miRNAs have predicted functions related to vascular development. Given that placental vascular dysfunction is a feature of GDM, it is possible that these circulating EV-enclosed miRNAs may contribute to placental dysfunction in GDM. Indeed our recent observation that vascular regulatory miRNAs are also altered in both EVs in maternal circulation, and in placenta in GDM pregnancies that go on to deliver LGA babies (Kennedy *et al.*, 2019), supports this hypothesis. Although further work is required to confirm this, a potential role

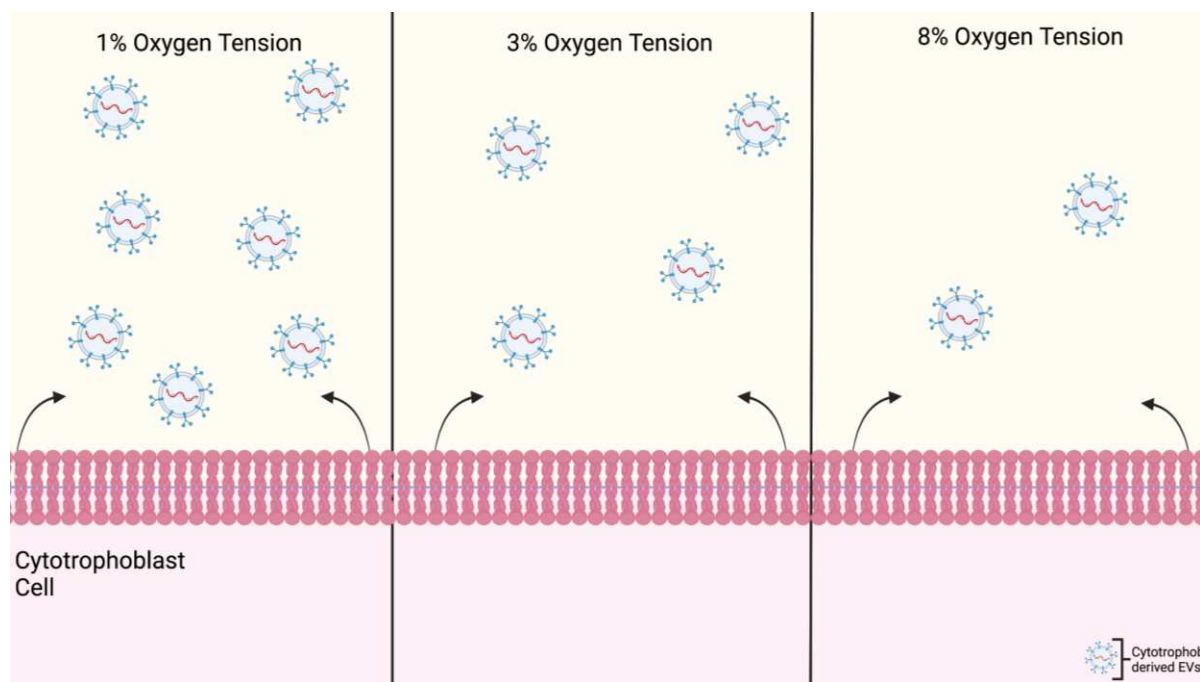
for EV miRNAs in pathogenesis of GDM and associated fetal growth, could provide both novel diagnostic and therapeutic opportunities to reduce clinical burden associated with GDM.

### Extracellular vesicles in Pre-eclampsia

Pre-eclampsia (PE) is a multisystem disease characterised by new-onset hypertension after 20 weeks of gestation and one or more additional indicator of organ dysfunction, such as proteinuria (Brown *et al.*, 2018; American College of Obstetricians and Gynecologists, 2020). It's incidence ranges from two to five percent and it can result in both fetal and maternal morbidity and mortality (Huppertz, 2008; Lisonkova & Joseph, 2013; Jin & Menon, 2018). To date, pre-eclampsia is the obstetric complication in which EVs have been most extensively studied.

Several studies have suggested pEVs provide a link between placental damage and the maternal phenotype in PE. Dutta *et al.* demonstrated that the injection of EVs from trophoblast cultured in hypoxic (1% oxygen) conditions into the tail veins of pregnant rats increased the mean systolic blood pressure more than the administration of trophoblast derived EVs produced in normoxic (8% oxygen) conditions (Dutta *et al.*, 2020). Similarly, Han *et al.* found EVs from freeze-thaw injured placenta induced hypertension and proteinuria when administered to non-pregnant mice, disrupted endothelial integrity and induced vasoconstriction (Han *et al.*, 2020). Powell *et al.* demonstrated the uptake of sEVs from human maternal plasma into the vessel wall of mouse mesenteric arteries (Powell *et al.*, 2022). Exposure to sEVs isolated from the plasma of individuals with PE produced significantly more vasoconstriction and less endothelium-induced relaxation in these vessels than sEVs from plasma of individuals without PE.

In addition to peripheral vascular changes, pre-eclampsia is also characterised by neurological changes, manifesting as hyperreflexia and potentially seizures (eclampsia). Leon *et al.* found that human brain endothelial cells, used as an *in vitro* model of the blood-brain barrier, showed increased permeability and reduced transendothelial electrical resistance when exposed to plasma EVs from mothers with PE compared to plasma EVs from mothers without PE (Leon *et al.*, 2021). A similar response was seen to EVs from placenta cultured in hypoxic conditions (1% oxygen) compared with EVs from placenta cultured in normoxic conditions (8% oxygen) (Figure 4). These changes were mitigated by co-administration of magnesium sulphate, a drug that is used clinically to prevent seizures in pre-eclampsia.



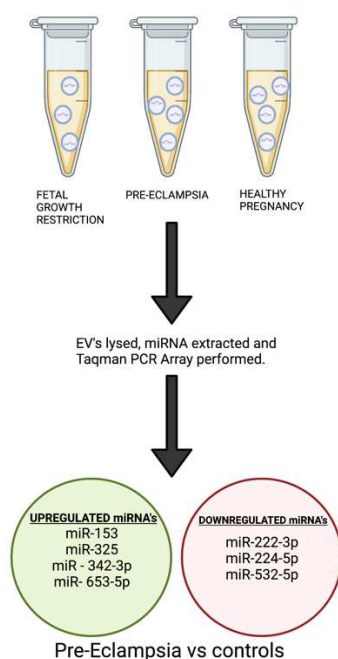
**Figure 4. Oxygen tension in pEV biogenesis and bioactivity.** Saloman et al. carried out a study by which they treated cytotrophoblast (CT) cell cultures with different levels of oxygen tension. They found that in the higher oxygen tensions, fewer EVs were secreted by CT cells than in the lower oxygen tensions. They hypothesised that CT derived exosomes are formed under hypoxic conditions within early pregnancy in the placenta and that this may be an adaptive response to encourage trophoblast invasion (Salomon et al., 2015). This supports the further investigation into early pregnancy pEV analysis in their role in both healthy and abnormal pregnancies (Truong et al., 2017; Dutta et al., 2020). Created in Biorender.com

While placental EVs may negatively impact maternal health in the later stages of pre-eclampsia, there is also evidence to suggest maternal EVs may negatively impact the placenta earlier in pregnancy. Kohli et al. administered EVs from serum-starved cultured mouse endothelial cells to pregnant mice at days 10.5 and 11.5 of gestation (E10.5, E11.5), the time at which the mature mouse placenta is established (Kohli *et al.*, 2016). This resulted in significant increases in maternal blood pressure, proteinuria and soluble fms-like tyrosine kinase (sflt1) compared with a control media. Fetal and placental size were reduced, fetal survival was reduced and placental inflammasome activation was increased in pregnancies administered EVs versus control media. The placentas of EV-administered mice also showed structural changes consistent with malperfusion.

In 2020 Li et al. isolated EVs from maternal plasma to quantify their concentration and size, as well as determine whether components of their cargo were up or down regulated across 60 healthy, PE and FGR pregnancies. Their findings displayed a twofold upregulation of the miRNA cargos miR-153-3p and miR-325-3p in pre-eclamptic pregnancies compared with healthy pregnancies (Figure 5) (Li *et al.*, 2020). The overexpression of miR-153 in humans is hypothesised to inhibit cell proliferation and invasion and encourage apoptosis (Zeng *et al.*, 2017). Further to this, miR-153 has also been shown

to bind to the 3' untranslated region of Hypoxia Inducible Factor 1 (*HIF1*) and suppresses expression. This is associated with reduced tube formation in the endothelial cells of the primary human umbilical vein, as well as reduced VEGFA expression and thus inhibited angiogenesis.

One notable finding of Li's work was that the seven miRNAs that were differentially expressed in pEVs from healthy and PE pregnancies did not show differences in miRNA sequencing of the whole plasma free miRNA. This was further shown in a study by Hromadnikova *et al.*, in which the capacity of C19MC miRNAs to predict FGR was higher when expression was analysed from lysed circulating maternal exosomes rather than whole plasma miRNA (Hromadnikova *et al.*, 2019). Li *et al.* also demonstrated an association between pEV cargo and the placental transcriptome, thus providing evidence that pEVs isolated from maternal blood can act as a non-invasive placental biopsy (Tannetta *et al.*, 2017).



**Figure 5. Summary of method and findings of Li *et al.* 2020. Li *et al.* extracted maternal plasma from 60 mothers who had either a healthy pregnancy, or suffered from FGR and pre-eclampsia. They isolated EVs and lysed them to determine whether their miRNA cargo was altered between each control group and found a collection were up and downregulated in pre-eclampsia compared to the two control groups. Created in Biorender.com.**

As well as contributing to the development of pre-eclampsia, EVs may have potential as therapies. One example of this is decidual mesenchymal stem/stromal cell (DMSC) EVs. DMSCs have been shown to reduce endothelial cell dysfunction in response to oxidative and inflammatory damage when co-cultured with them (Alshabibi *et al.*, 2018). Zheng *et al.* hypothesised that the beneficial

effects of DMSCs may be mediated by EVs, which in turn could be used to mitigate the endothelial dysfunction seen in pre-eclampsia (Zheng *et al.*, 2020). This was supported by their finding of increased cell attachment and proliferation and decreased IL-6 expression and lipid peroxidation when human umbilical vein endothelial cells (HUVECs) were exposed to DMSC EVs in combination with serum from pre-eclamptic mothers compared with pre-eclamptic serum alone.

### Extracellular Vesicles in preterm birth

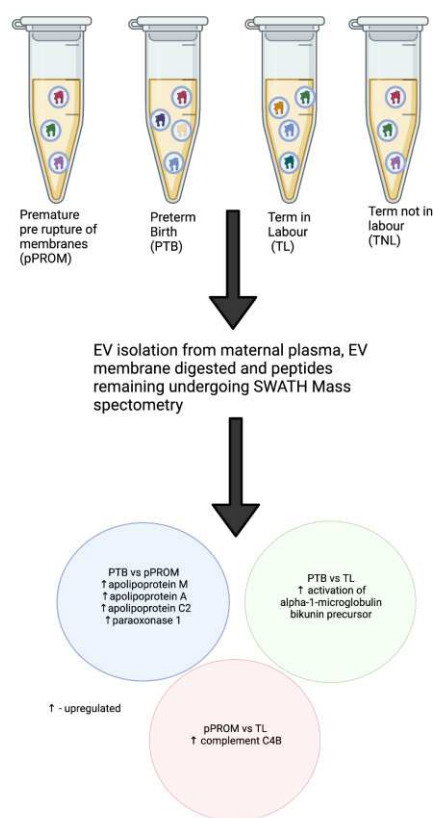
There are multiple mechanisms that lead to the same final common pathway of preterm birth, which in turn is the leading cause of perinatal mortality and morbidity in developed countries (Goldenberg *et al.*, 2008). EVs appear to contribute to the normal initiation of labour at term (Palomares *et al.*, 2021; Yadava *et al.*, 2021), making it plausible that they could form part of the mechanistic pathway in some cases of preterm birth.

In an *in vivo* mouse study (Sheller-Miller *et al.*, 2019), maternal plasma EVs at gestational day 18 (E18, late pregnancy) were found to contain proinflammatory cargo, thought to contribute to labour and delivery. Administration of E18 EVs to pregnant mice at E15 resulted in preterm birth in four out of five dams, while none of the dams administered E9 EVs delivered prematurely.

Intraperitoneally administered EVs were delivered to the uterine tissues regardless of gestational age, while E18 EVs were found to both prepare the uterus and cervix for parturition as well as promote prepartum proinflammation in fetal membranes. Whilst this is a murine model with a small sample of mice (n=15), the study highlights the importance of pEVs in paracrine signalling within pregnancies.

When studying human pregnancy, Menon *et al.* found fewer PLAP<sup>+ve</sup> (placental) EVs in maternal plasma from the first and second trimesters of pregnancies ending in preterm birth compared with pregnancies ending in term birth (Menon *et al.*, 2020). In contrast, the number and size distribution of PLAP<sup>-ve</sup> EVs was similar between the two groups. Using sequential windowed acquisition of all theoretical mass spectra (SWATH) mass spectrometry of pEV cargo, they identified 96 proteins which differed significantly across gestation between pregnancies ending in term and preterm birth. The highest scoring network for these proteins related to cell death and survival. Analysing total circulating EV miRNAs using next generation sequencing they also identified 173 miRNAs that differed significantly across gestation between pregnancies ending in term and preterm birth (Menon *et al.*, 2019). Signalling pathways targeted by these miRNAs included p53, fitting with the proteomic finding of differences relating to cell death and survival, TGF- $\beta$  signalling and glucocorticoid receptor signalling.

An additional cross-sectional study by Menon et al. compared the protein cargo of total circulating EVs in maternal plasma between pregnancies at term not in labour (TNIL), at term in labour (TL), with preterm premature rupture of membranes (pPROM) and ending in preterm birth (PTB). Canonical pathway analysis of the results of SWATH mass spectrometry showed similarities between TNIL and PTB EV cargo when compared with TL EVs. Differences were evident, however, between pPROM EV and PTB EV cargo, with increased concentrations of proteins related to inflammation, coagulation activation and response to oxidative stress in PTB EVs. Individual proteins related to cholesterol signalling, cell proliferation and classical immune activation showed significant differences between the four groups on pairwise comparison (Menon et al, 2019b) (Figure 6). These findings suggest further investigation of different preterm birth phenotypes may provide greater insights than studying preterm birth as a single entity. Menon's research team makes up the majority of the recent literature in this field, highlighting the novelty of this area and the resulting literature gaps. Further weight will be added to their findings if they can be replicated by other researchers.



**Figure 6. Summary of method and findings of Menon et al. 2019b. EVs isolated from maternal plasma were collected from 4 groups term not in labor (TNIL, n = 13); term in labor (TL, n = 11); preterm premature rupture of membranes (pPROM, n = 8); and group 4: preterm birth (PTB, n = 13). SWATH Mass spectrometry proteomics were performed displaying notable differences between the 4 groups. Created in Biorender.com**



McElrath et al. displayed the potential of using EVs isolated from maternal plasma in the first trimester of singleton pregnancies as a source of predictive biomarkers for PTB before 35 weeks of gestation (McElrath *et al.*, 2019). Using a 1:2 case-controlled study, a panel of five EV proteins was selected based on analysis of samples from a training set of 135 pregnancies. When tested on a further 126 pregnancies, their marker panel had an AUC of 0.74 (95%CI 0.63-0.81), with a positive likelihood ratio of 2.70 and a negative likelihood ratio of 0.27. Zhao et al. analysed EV lipids in second trimester maternal plasma and found higher levels of PS(34:0) in microvesicles from 27 pregnancies ending before 37 weeks of gestation compared with 66 full term pregnancies (Zhao *et al.*, 2020). When tested in a validation set of a further 83 pregnancies, microvesicle PS(34:0) had an AUC of 0.71 (95% CI 0.60-0.82) for predicting birth before 37 weeks. Although both of these findings have yet to be externally validated, they support the utility of EVs as a source of predictive markers in pregnancy.

### Extracellular Vesicles in FGR

There are fewer studies focussing specifically on FGR than the other obstetric conditions discussed in this review. However, there is considerable overlap, both in terms of pathophysiology and incidence, between early-onset FGR (<32 weeks of gestation) and early-onset pre-eclampsia (<34 weeks of gestation). This may mean some of the findings about EVs in pre-eclampsia may prove relevant to FGR in the future.

Miranda et al. demonstrated an association between the ratio of pEVs (CD63<sup>+ve</sup> and PLAP<sup>+ve</sup>) to total circulating EVs (CD63<sup>+ve</sup>) and fetal growth *in utero* (Miranda *et al.*, 2018b). They found the ratio of pEVs to total EVs was significantly reduced in small-for-gestational age (SGA) fetuses (EFW <10<sup>th</sup> centile) compared with appropriate-for-gestational-age (AGA) fetuses, with a further reduction in the ratio for fetuses with FGR (EFW<10<sup>th</sup> centile and abnormal Doppler studies or EFW <3<sup>rd</sup> centile). This raises the possibility of monitoring pEV quantity as a marker for placental insufficiency and thus fetal growth.

Ariyakumar et al. also found total EV concentration was significantly lower in maternal plasma from pregnancies with FGR (birth weight <10<sup>th</sup> with absent or reversed umbilical artery end-diastolic flow) than in normal pregnancies, with FGR maternal plasma EV concentrations similar to that of non-pregnant individuals (Ariyakumar *et al.*, 2021). The concentration of Fas ligand (FasL), which promotes immune tolerance through suppression of the NF- $\kappa$ B subunit p65, was lower also in EVs from FGR pregnancies than AGA pregnancies. This suggests a possible pathophysiological effect of EVs in FGR.

## Conclusion

Extracellular vesicles are a key mediator of cell-cell communication, carrying miRNA, proteins and surface antigens. It is perhaps unsurprising, therefore, that they appear to be involved in so many key areas of cross-talk between the mother, the placenta and the fetus. In healthy pregnancy this includes implantation, immunomodulation and the initiation of labour. In pregnancy complications it includes the production of maternal pre-eclampsia manifestations in response to EVs from hypoxic or otherwise damaged placenta.

Studying maternal, placental and fetal EVs in healthy and complicated pregnancies is providing novel insights into the causes and pathophysiology of obstetric diseases. It is also allowing researchers to identify novel biomarkers to predict which pregnancies are at higher risk of complications. In time we may be able to harness this knowledge to use EVs as a therapy, either by utilising naturally produced EVs that have beneficial effects, as in the case of DMSC EVs, or by using artificially created EVs as a drug delivery system. Given the lack of current effective treatments for many obstetric complications, this would be of great benefit to the health of pregnant people and their future children.

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