Extracellular vesicles and their potential role inducing changes in maternal insulin sensitivity during gestational diabetes mellitus

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Abstract

Gestational diabetes mellitus (GDM) is one of the most common endocrine disorders during gestation, and affects around 15% of all pregnancies worldwide, paralleling the global increase in obesity and type 2 diabetes. Normal pregnancies are critically dependent on the development of maternal insulin resistance balanced by an increased capacity to secrete insulin, which allows for the allocation of nutrients for adequate fetal growth and development. Several factors including placental hormones, inflammatory mediators, and nutrients have been proposed to alter insulin sensitivity and insulin response, and underpin the pathological outcomes of GDM. However, other factors may also be involved in the regulation of maternal metabolism and a complete understanding of GDM pathophysiology requires the identification of these factors, and the mechanisms associated with them. Recent studies highlight the potential utility of tissue-specific extracellular vesicles (EVs) in the diagnosis of disease onset and treatment monitoring for several pregnancy-related complications, including GDM. To date there is a paucity of data defining changes in the release, content, bioactivity and diagnostic utility of circulating EVs in pregnancies complicated by GDM. Placental EVs may engage in paracellular interactions including local cell-to-cell communication between the cell constituents.
of the placenta and contiguous maternal tissues, and/or distal interactions involving
the release of placental EVs into biological fluids and their transport to a remote site
of action. Hence, the aim of this review is to discuss the biogenesis, isolation
methods and role of EVs in the physiopathology of GDM, including changes in
maternal insulin sensitivity during pregnancy.

1. Introduction

Gestational diabetes mellitus (GDM) is glucose intolerance of lesser severity than
overt diabetes, which is first diagnosed during pregnancy [1], and affects around 6 –
15% of all pregnancies across World Health Organization (WHO) regions worldwide
[2]. GDM imposes both long and short-term impact on mothers and offspring. The
short-term consequences of GDM include increased risks of pregnancy
complications such as macrosomia or excess fetal adiposity despite normal
birthweight, shoulder dystocia, operative delivery, and neonatal hypoglycaemia,
whereas long term consequences include increased risk of developing type 2
diabetes, obesity and cardiovascular diseases in the future [3, 4]. Several risk factors
contribute to the development of GDM and the most important among them are
family history of type 2 diabetes or previous history of GDM, overweight or obesity,
advanced age, and ethnicity [5-7]. GDM is associated with changes in insulin
sensitivity and insulin response which includes defects in insulin signalling in major
glucose disposal organs such as skeletal muscle and adipose tissue, and inability of
pancreatic beta cells to compensate for the decrease in insulin sensitivity [8, 9]. Even
though attempts have been made to identify the factors (for example placental
hormones such as placental growth hormone and inflammatory mediators such as
TNF-α) that contribute to the reprogramming of maternal metabolism in pregnancy
and GDM [10, 11], the underlying mechanisms remain poorly understood.

Extracellular vesicles (EVs) have emerged as important mediators of intercellular
communication in health and disease (reviewed in [12]). Although studies have
shown an increasing involvement of EVs in the regulation of metabolism and
associated disorders (reviewed in [13]), whether they contribute to the maternal
physiological changes in pregnancy is unclear. Hence in this review, we will be
exploring evidence regarding EV biogenesis and signalling, and studies analysing
EVs in pregnancy, particularly in GDM. Also we will discuss the molecular
mechanism associated with metabolic reprogramming in pregnancy, and how EV profile and content changes in pregnancy influences GDM and regulates metabolic responses of target cells.

2. Extracellular vesicle: Biogenesis and Signalling

The term “Extracellular vesicle” or “EVs” in general refers to all the lipid-membrane-enclosed particles released from the cell and comprises diverse populations of vesicles released by different cellular mechanisms. One of the principal characteristics of EVs is their diversity and heterogeneity.

2.1 EVs heterogeneity and biogenesis

“Exosomes” are a type of EV that originate in the early endosomes, formed from the inward budding of the plasma membrane. The early endosomes mature to late endosomes and during that process, inward invagination of their membranes leads to the formation of intraluminal vesicles (ILVs), which have a size of 30 to 100nm, similar to the size of exosomes (reviewed in [14]). During the formation of ILVs, cellular cargo such as proteins and nucleic acids are packaged into them, and endosomes with ILVs are termed ‘multivesicular bodies’ (MVBs). Subsequently, fusion of MVBs with the cell membrane leads to the release of these ILVs into the extracellular space, where they are termed “exosomes” (reviewed in [15]). “Microvesicles or microparticles or ectosomes” are formed by the direct budding of the plasma membrane whereas “apoptotic bodies” are released as a result of cell apoptosis [14, 16, 17]. Even though different populations of EVs originate via different biogenesis pathways, they overlap to a great extent in terms of their physical and functional properties, and this makes isolation or separation of pure populations of EV subtypes difficult. Hence, the International Society of Extracellular Vesicles (ISEV) recommends using specific terms for EV subtypes that refers to their physical and biochemical characteristics. For example, based on size, they can be referred to as “small EVs” (sEVs) when less than 100 or 200nm in diameter or “large/medium EVs” (l/mEVs) when greater than 200nm, or they can be called “CD63+EVs”, “PLAP+ve EVs” etc. based on the specific marker(s) that they possess or as “oncosomes” “hypoxic EVs” etc. based on their cellular origin or specific conditions during which they originate [18].

2.2. EVs in intercellular communication

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EVs released into the extracellular space can mediate autocrine signalling by acting on their cell of origin [19], paracrine signalling by acting on a neighbouring recipient cell [20], and endocrine signalling by crossing the endothelial barrier and reaching various organs [20, 21]. The finding that EVs can transfer miRNAs and proteins to recipient cells triggered substantial interest in the field [22, 23] and several studies have identified EVs as powerful mediators of intercellular communication (reviewed in [24, 25]). EVs in general are a 'fingerprint' of their cells of origin, however, studies show specific enrichment of molecules in these vesicles compared to their parent cells [26-28]. One of the most critical questions in this regard is whether the cargo, such as proteins, miRNAs and lipids, are specifically sorted into these vesicles for packaging. RNA-binding proteins such as AGO2 (Argonaute2), hnRNPA2B (Heterogeneous Nuclear Ribonucleoprotein A2B) and Y-box protein 1 are suggested to be involved in sorting and loading of miRNAs into sEVs [27-29]. Likewise, post-translational modifications such as ubiquitination, sumoylation and addition of certain small polypeptide chains are reported in the selective sorting of proteins into the exosomes during biogenesis [30-32]. Hence, the presence of selective sorting mechanisms indicates that the content of EVs changes with the physiological state of the cell.

The mechanisms through which EVs are taken up by target cells, and elicit their biological response have been reported by several studies [33-37]. However, intravenously administered EVs in animal models show dynamic spatiotemporal localization from blood to various organs, predominantly spleen, followed by the liver [38]. EVs, particularly sEVs can bind to target cells via classical ligand-receptor interaction. The receptors and ligands are specific for the recipient cell type and cell of origin of EVs respectively. The cluster of differentiation (CD) proteins of the tetraspanin family, namely CD9, CD81 and CD82, ICAM-I (Intercellular Adhesion Molecule 1), LFA-I (Lymphocyte function-associated antigen 1), and integrins have been reported to be involved in the attachment of extracellular vesicles to the cell surface[39], reviewed in [40]. Further, the receptor-ligand interaction can lead to the following: (1) activation of downstream signalling (2) internalization of vesicles by clathrin or caveolin mediated phagocytosis or (3) direct fusion of the extracellular vesicle with the cell membrane and release of their contents within the cell (reviewed in [40]). Evidence shows that proteins delivered by sEVs can influence the signalling
pathways in the recipient cells and bring about changes in cellular response or behaviour [23]. Similarly, the miRNAs delivered by sEVs can suppress the mRNAs in target cells by binding to their 3’UTR region and alter gene expression [20, 41]. A summary of the biogenesis of EVs and their interaction with target cells is presented in Figure 1. The heterogeneity of EVs makes it difficult to standardize their enrichment and characterisation methods, and a variety of techniques and approaches have been used to determine the role of EVs during pregnancy. In the next section, we describe several studies using a variety of experimental approaches to study EVs during pregnancy. We suggest that the design and methods used to enrich EVs needs to be chosen after consideration of the hypothesis that is being tested. In the absence of a gold standard method to enrich EVs that allows for a fair comparison between studies, a full description of the methods is highly recommended.

3. Experimental approaches to study EVs in pregnancy and their impact on the field

Several experimental approaches have been used to understand the role of EVs in pregnancy, which include isolation of EVs from biological fluids such as maternal plasma/serum, placental cell culture (primary and cell lines), placental perfusion, and placental explants.

3.1 Analysis of placental EVs from biological fluids

Maternal plasma contains EVs that are derived from different cell types such as platelets, leucocytes, endothelial cells, and placental cells [42, 43]. Circulating EVs from different cellular origin present in maternal plasma can be studied by flow cytometry using antibodies against proteins specific for their cell of origin [42-45]. However, flow cytometry detects only a proportion of the EV population where as Nanoparticle Tracking Analysis (NTA), which can detect nanovesicles, is capable of enumerating total populations of EVs [46]. However, differences in the NTA devices, as well as instrument settings are reported to significantly affect the measurement quality and quantitation of particles, which affects the reproducibility [47-49]. Interestingly, nanoscale multiplex high-resolution flow cytometry is being developed as a method to accurately quantify cell and size specific EVs in different solutions (reviewed in [50]). Among EVs derived from different cellular origin, the most
commonly studied are the placenta-derived EVs, as they can provide important insights into placental physiology and help monitor placental health in normal pregnancy and pregnancy-related disorders. An earlier study used antibody ED822 against an unknown trophoblast antigen for the flow cytometric analysis of placental EVs in maternal plasma [51]. However, the most commonly used antibody is against the Placental Alkaline Phosphatase (PLAP) enzyme, which is a membrane protein, present mainly in the syncytiotrophoblast layer of the placenta [52]. Anti-PLAP antibody has been used to enumerate placental EVs using flow cytometric analysis [43, 51, 53] or fluorescent NTA [54-56]. Also, PLAP ELISA kits are available for the characterization of placental EVs [57-59]. An immunocapture technique for the enrichment of PLAP positive EVs from maternal circulation [60] and direct enrichment of PLAP+ EVs from blood, using gold loaded nanoporous nanocubes has been reported [61].

Apart from maternal plasma, other less widely used biological fluids to study EVs in pregnancy are maternal serum [62], urine [63], gingiculo-crevicular fluid [64], fetal amniotic fluid [65, 66] and fetal umbilical cord blood [67]. In contrast to maternal plasma, there are only a few studies on EVs in maternal serum. This is most likely due to serum containing abundant amounts of platelet-derived EVs compared to EVs from other sources. However, successful quantitation/enrichment of placental EVs from biological fluids depends on the surface expression/abundance of placental markers on EVs as well as the specificity of the antibody used. In this regard, experimental designs that use placental cell culture, placental perfusion and placental explants are advantageous as placental EVs can be directly enriched without the need for immunocapture. A summary of the studies that have analysed EVs of different cellular origin derived from different biological fluids is presented in Table 1.

### 3.2 Analysis of EVs from placental tissues, cells, explants and perfusion systems

Placental cell culture, placental explants and placental perfusion systems can be used as models to study the physiological state of the placenta and disorders of
placental origin. EVs obtained from these experimental models can give critical information on the mechanisms by which the placenta responds to extracellular cues and transfers biological signals to other cells or tissues. Specifically, these models have been used for understanding exosome biogenesis in pregnancy such as the factors or conditions regulating exosome release from the placenta, definition of contents of placental EVs (analysis of their protein, nucleic acid and lipid content), and their effects on target cell phenotype. For example, the role of trophoblast-derived sEVs in the process of placentation and response to placental hypoxia has been studied using sEVs enriched from primary cultures of cytotrophoblast cells grown under different oxygen concentrations (1%, 3% and 8%), and analysing their effect on extravillous trophoblast cells migration [68]. Similarly, sEVs derived from human first trimester extravillous trophoblast cell line HTR8/SVneo have been used for understanding the phenomenon of spiral artery remodelling during placentation [69]. Nevertheless, these in vitro systems can be used to mimic pathological pregnancies. EVs derived from placental explants treated with pre-eclamptic sera had an increased expression of DAMPs (Danger associated molecular patterns), which activated an inflammatory response in endothelial cells [70]. The effect of glucose and oxygen concentration on EV secretion was analysed by incubating primary trophoblast cells with 5 mM and 25 mM of D-glucose under 1%, 3%, and 8% oxygen for 48 hours. The trophoblast cells can respond to the changes in glucose and oxygen concentrations and released higher amounts of sEVs, which induced an increase in the secretion of cytokines from endothelial cells [71]. Even though these in vitro models are representatives of placental physiology, the efficacy of these models to accurately recapitulate the placental physiology/pathology should be critically evaluated.

### 3.3 Effect of placental EVs on biological systems

Placental EVs carry signals in the form of miRNAs [72-74], proteins [75-78], lipids [79], and DNA [80]. Further, placental EVs were analysed for their effect or bioactivity on target cells. Generally, in vitro functional analyses were performed by inoculating the placental EVs with target cells and identifying the changes in the physiological response of these cells. Studies have analysed the effect of specific miRNA [81-83] as well as specific proteins present in placental EVs in association with their impact on recipient cells [71, 77]. Substantial research has been conducted with these
experimental designs to analyse the immunomodulatory potential of placental EVs. Immunoregulatory molecules such as Fas ligand (Fas-L), TNF-related apoptosis-inducing ligand (TRAIL) and Natural Killer Group 2D (NKG2D) ligands were identified on the surface of sEVs derived from placental explant cultures and their interaction with immune cells studied [84, 85]. It has been reported that extravillous-derived sEVs secreted by the placenta are taken up by monocytes to increase their migratory capability, which was analysed by counting the number of monocytes migrating in response to different concentrations of sEVs [86]. Additionally, the trophoblast-derived sEVs increase the release of pro-inflammatory cytokines such as IL-6 and IL-1β from the monocytes [86, 87]. Furthermore, the role of these EVs in contributing to the inflammatory response, such as activation of monocytes and neutrophils in pregnancy disorders such as PE has been studied [88, 89]. Finally, another important downstream application of placental EVs is their functional effects in vivo [21, 90-92]. Rodents and non-rodents have been used for studying the physiology of human pregnancy and related disorders and the choice of the appropriate animal model is typically based on the research question. There are several advantages and limitations associated with each model, however, discussion of these is beyond the scope of this review. Nonetheless, murine models are commonly used for the study of EVs in pregnancy [21, 90-92]. For example, placental sEVs from human placental explant cultures were labelled with fluorescent dye and inoculated in pregnant mice, showing EV localization to lungs, liver and kidney. The fluorescence intensities were normalized to background florescence of organs from control mice, which were inoculated with EVs isolated from culture medium that had not been exposed to placental explants. These EVs were reported to interact with endothelial cells in vitro by different mechanisms, such as endocytosis, phagocytosis, and through cell surface receptors, and caused endothelium-dependent vasodilation of mesenteric arteries via the nitric oxide pathway [21].

An important question in regard is whether the cargo or contents of the EVs obtained from varying sources differ in their physical and chemical properties. A study performed by Tannetta et al., 2013 [93] analysed and compared the expression of two PE related antigens, namely Flt-1 and endoglin in sEVs enriched from two different preparations. They used ex vivo perfusion of placental cotyledons and
mechanical disruption of placental tissue, obtained from normal and pre-eclamptic patients. The mechanically derived syncytiotrophoblast-derived microvesicles (STBM) had decreased expression of placental marker PLAP and endoglin and placental perfusate STBMs had lower Flt-1, thereby indicating that the extent of damage caused by the method of preparation of EVs can alter their physical and chemical properties. A similar study by Gupta et al., 2005 [94] comparing three preparations of placental EVs, namely from mechanical dissection of placenta, placental explant and placental perfusion, identified differences in bioactivity in target cells. Mechanically derived EVs suppressed the proliferation of their target cells more and induced apoptosis in them. This study suggested that EVs derived from explant and perfusion may be formed from apoptosis of placental cells, where as mechanical disruption can cause damage to membrane integrity and lead to necrotic particle release. Other studies have reported this discrepancy in the functional activity of mechanically derived vesicles compared to EVs from placental explants and placental perfusion cultured under physiological conditions [88, 95]. To date, a comparison of the composition and functional activity between EVs derived from primary trophoblasts, trophoblast cell lines, explants and perfusion techniques has not been reported.

3.4 Method to enrich EVs and their limitations

Another hurdle in studying EVs in pregnancy or in general is the inability to isolate pure populations of EVs, owing to the technical limitations. The most common method for the enrichment of EVs is differential centrifugation followed by ultracentrifugation. An initial centrifugation at 2000-3000g removes cellular debris, followed by low-speed centrifugation of up to 20,000g yielding large EVs, including large microvesicles and apoptotic bodies [54, 75], whereas ultracentrifugation (100000-200000g) yields small EVs [21, 53, 96]. However, ultracentrifugation sediments EVs with other contaminants in the solution, such as protein complexes, and protein-miRNA complexes. Therefore, ultracentrifugation is generally combined with other purification methods such as density gradient centrifugation, size exclusion chromatography or immunoprecipitation. Density gradient centrifugation separates EVs from non-vesicular molecular aggregates and protein owing to their low buoyant density in a density gradient medium such as sucrose or iodixanol [59, 97]. Size exclusion chromatography separates EVs based on their size, giving
preparations of EVs with minimal contamination of proteins. However, contamination by particles of similar size such as lipoproteins cannot be overcome using this method [98]. Immunoaffinity capture of EVs using EV specific markers such as CD63 offers a promising way of isolating pure populations of EVs [28, 99]. This method can separate vesicles of different sub-types and specific cell origins. There is a lack of consensus regarding the actual cargo of exosomes due to limitations in the enrichment of pure populations of exosomes without contamination with other types of vesicles [18]. Enrichment of pure populations of vesicles is needed to identify whether the miRNAs and proteins are merely associated with the exosomes or are present within the vesicles, and to eliminate those associated with protein complexes that may co-precipitate with the exosome population. Generally the method chosen to isolate EVs or a specific type of EV such as sEVs is highly dependent on the research question and the aims of the experiments. Variability in the enrichment methods is a crucial problem in the field of EVs, and more data is required to understand whether the different methods to isolate EVs affect the reproducibility of the data, and perhaps their interpretation.

However, the overall progress in the field has been dampened by the complexities with the EV enrichment methods (such as ultracentrifugation, density gradient centrifugation, size exclusion chromatography etc.). The co-purification of other types of vesicles and non-vesicular extracellular matter (such as cellular debris, protein complexes and protein-miRNA complexes) with pure populations of EVs, has introduced variability with the EV composition and confounded data interpretation within the current body of literature. With regard to studies in pregnancy, different experimental designs used for EV enrichment (such as maternal plasma/serum, placental cell culture (primary and cell lines), placental perfusion, and placental explants) has also introduced variability in their composition and function. Therefore, proper and standardized methods should be developed to isolate pure populations of exosomes with minimal contamination, which can definitely characterise these vesicles within the heterogeneous extracellular population. This is important for the successful development of EVs as biomarker tools or therapeutic targets. In the context of pregnancy, development of novel biomarkers to predict pregnancy complications may replace complex and inconvenient diagnostic methods and ensure higher accessibility to health care systems in low income countries. Hence,
studies exploring the biomarker potential of EVs in predicting pregnancy complications, including GDM, is an interesting area of research. However, to understand the role of EVs in the regulation of maternal metabolism, one needs to know the changes in maternal glucose homeostasis occurring during pregnancy. Next, we will discuss the mechanisms associated with changes in insulin sensitivity during gestation, and the potential role of EVs in this phenomenon.

4. Glucose homeostasis during pregnancy and GDM – implications of placenta-derived molecules

Maternal metabolic adaptation is key to the proper growth and development of the fetus. The early stages of pregnancy are anabolic, characterized by accumulation of nutrient reserves in maternal tissues whereas late gestation is catabolic with increased mobilization of metabolites to the fetus. Pregnancy is associated with changes in glucose tolerance due to changes in glucose uptake by skeletal muscle and adipocytes, glucose production by liver and insulin secretion from pancreas [100].

4.1 Insulin sensitivity and insulin secretion in healthy pregnancy and GDM

A longitudinal analysis of insulin sensitivity across gestation shows that pregnancy in its latter stages, is associated with 50-60% reduction in insulin sensitivity, in healthy pregnant women compared to non-pregnant controls [101]. This leads to elevated levels of glucose and free fatty acids in the maternal circulation, promoting optimal fetal growth. In women with GDM, however, insulin sensitivity is further decreased in the third trimester of pregnancy when compared to normal healthy women [101]. The decline in insulin sensitivity in GDM women is present even before pregnancy but becomes more evident during late gestation[101]. However, insulin secretion increases across gestation both in healthy pregnant, and GDM women, with the second-phase insulin response significantly higher in the GDM group, indicating beta cell stress and risk of beta cell dysfunction [101]. Hence in GDM, the decrease in insulin sensitivity coupled with the inability of pancreatic beta cells to produce the additional secretory insulin to compensate this, is the key contributor to hyperglycaemia. The changes in insulin sensitivity in healthy pregnancy and GDM are associated with changes in insulin signalling in skeletal muscles and adipose tissue, which are the major sites of glucose uptake in the body.
Insulin-stimulated glucose uptake by skeletal muscles decreases by 32% in healthy pregnant women compared to non-pregnant women [9]. Moreover, in women with GDM, there is a further decrease to 54% compared to healthy pregnant women [9]. The defects or deregulations in the post-insulin receptor signalling in skeletal muscle and adipose tissue contribute to insulin resistance in healthy pregnancy and GDM [9, 102]. The first step in the insulin signalling pathway is the interaction of the insulin with the insulin receptor (IR) in the cell membrane, followed by phosphorylation of tyrosine residues in the IR and activation of insulin receptor substrate (IRS) protein (reviewed in [103]). In GDM, there is a decrease in the tyrosine phosphorylation of IR compared with NGT controls, whereas the abundance of IR is not affected by pregnancy or GDM [9, 104, 105]. Also, decreased tyrosine phosphorylation of IRS-1 and reduced abundance of this protein in skeletal muscle and sub-cutaneous adipose tissue is observed in pregnancy and this is further exacerbated in GDM [9, 8]. IRS-1 provides the docking site for phosphatidylinositol 3-kinase (PI3K) and activates the PI3K/AKT pathway for glucose uptake by mobilizing the glucose transporter, GLUT-4 to the cell membrane (reviewed in [103]).

In contrast to tyrosine phosphorylation, phosphorylation of the serine residues in IR and IRS-1 deactivates these proteins and downstream insulin signalling leading to decrease in insulin sensitivity (reviewed in [103]). In this regard, serine phosphorylation of IRS-1 mediated by mTOR/p70S6K1 pathway, which in turn is activated by excess nutrients has been identified to contribute to insulin resistance in skeletal muscles in GDM [106]. Another vital player in mediating insulin resistance is the PI3K molecule and its subunits. PI3K is a heterodimer with two subunits, a regulatory subunit p85 and catalytic subunit p110. The p85α subunit of PI3K is upregulated in skeletal muscle and adipocytes, in pregnancy, with a higher increase observed in GDM pregnancies compared to the NGT controls [10, 107]. The p85α subunit competitively inhibits the p85-p110 heterodimer of PI3K for the docking site of IRS-1 in a dominant negative manner and decreases the downstream signalling [108]. However, the expression of glucose transporter molecule, GLUT-4 is unaltered in skeletal muscle from GDM women compared to lean and obese normal healthy pregnant women [109]. Furthermore, the GLUT-4 expression in adipose tissue is reported to be lower in pregnant women compared to non-pregnant controls and further decreased in women with GDM [110]. In contrast, analysis of insulin
signalling pathways in skeletal muscle and sub-cutaneous adipose tissue collected
from healthy pregnant and GDM patients shows that GLUT-4 expression is
decreased in both skeletal muscle and adipose tissue in obese-GDM pregnancies
[111]. The same study reported PI3K-p85α to be upregulated in skeletal muscle and
adipose tissue in obese GDM pregnancies and that there is no significant difference
in their expression in skeletal muscle in non-obese GDM pregnancies [111]. Hence,
the expression of the insulin signalling molecules in healthy pregnancy and GDM
may vary between studies, depending on factors such as population demographics
and BMI of patients used in the study.

In a healthy pregnancy, the progressive insulin resistance can be compensated by
an increase in beta cell mass. The precise mechanism by which beta cells expand is
unclear, however, it involves beta cell proliferation, neogenesis, and increase in beta
cell size and a decrease in beta cell apoptosis (reviewed in [112]). Studies in rodents
have shown that beta cell mass returns to normal postpartum and beta cell
expansion is a reversible phenomenon occurring in pregnancy [113]. The beta cell
proliferation and function in pregnancy is affected by several factors. For example,
transcriptional factor Foxm1 which is anti-apoptotic to beta cells [114, 115] and
Cytokine-inducible SH2-containing protein (CISH) and suppressor of cytokine
signalling 2 (SOCS2) proteins which increases beta cell proliferation, are induced in
pregnancy in mice models [116]. In addition, growth factors such as nerve growth
factor and hepatocyte growth factor as well as serotonin, which increases beta cell
mass and insulin secretion, are increased in pregnancy [116-118]. In GDM
pregnancies insulin secretion increases in association with insulin resistance, but the
increase in insulin secretion relative to insulin sensitivity is less compared with
healthy pregnant women [101]. Also, this is not necessarily reversed post-partum in
GDM women, forming a potential link between GDM and future onset of type II
diabetes [119].

Altogether, the decrease in insulin sensitivity and beta cell defects leads to the
hyperglycaemic condition of GDM. Excess maternal glucose is transferred to the
fetus via the placenta, leading to fetal hyperinsulinaemia, which promotes glucose
uptake in fetal tissues. This results in macrosomia, increased fetal adiposity, and
neonatal hypoglycaemia, and programs the fetus for a future risk of developing type
2 diabetes and obesity [3, 120, 121]. However, restoration of maternal insulin sensitivity post-partum in healthy pregnancy as a result of enhanced skeletal muscle insulin signalling has been reported [122] and hence placenta-derived factors and molecules might play a significant role in regulating maternal glucose tolerance during pregnancy [123].

4.2 Placenta-derived factors in regulating maternal glucose tolerance

The placenta secretes various hormones including estrogen, progesterone, human placental lactogen (hPL), placental growth hormone (pGH), and human chorionic gonadotropin (hCG), which help in the maintenance of pregnancy [124]. The placental hormones can influence insulin sensitivity and insulin secretion in healthy pregnancy and GDM. For example, pGH is involved in the increased expression of p85α subunit of PI3K in skeletal muscles leading to insulin resistance [10, 107]. Lactogenic hormones like hPL and prolactin have a protective effect over beta cells by mediating anti-apoptotic effect and increasing beta cell proliferation and insulin secretion [125] [126-128]. Studies with human pancreatic islets and insulinoma cell lines of murine origin reveal that hPL has a protective role over the pancreatic beta cell by activating the PI3K/AKT pathway and counteracting apoptosis. This increases beta cell activity by upregulation of the transcription factor PDX-1, which binds to the enhancer region of the insulin gene and promotes insulin secretion [129]. Also, the JAK/STAT pathway has been identified to be involved in mediating the pro-survival effect of placental lactogens [130, 131]. Interestingly, GDM is associated with increased circulating progesterone levels and progesterone receptor knock out mice show increased beta cell mass and improved glucose tolerance [132]. However, more studies are needed to understand the mechanism by which placental hormones can influence maternal insulin sensitivity.

Placenta is a source of adipokines such as adiponectin and leptin, and cytokines such as TNF-α and IL-6 [133-135]. Elevated circulating adiponectin has been associated with improved insulin sensitivity [136, 137] whereas elevated leptin leads to insulin resistance in GDM [138, 139]. Most importantly, circulating levels of TNF-α significantly correlate with changes in maternal insulin sensitivity during late gestation [11]. TNF-α can impair insulin signalling by increasing the serine phosphorylation of IR and IRS-1 and lead to insulin resistance in skeletal muscle and
adipocytes [140-142]. Apart from hormones and cytokines, the placenta also releases large quantities of extracellular vesicles (EVs) into the maternal circulation.

5. EVs in GDM- with a special focus on placenta-derived EVs

Interestingly, the EV biogenesis machinery in placental cells can interact with changes in the extracellular environment such as hypoxia and high glucose, which increase the release of sEVs from trophoblast and alter their protein content as well as bioactivity on target cells [71]. In this study, first-trimester primary trophoblast cells were incubated with 5mM and 25mM glucose, and 1%, 3% and 8% O₂, and identified that glucose increased the release of sEVs from trophoblast cells under all oxygen tensions. The sEVs released under high glucose increased the release of pro-inflammatory cytokines from endothelial cells indicating an interaction between oxygen and glucose sensing pathways and exosome release from placental cells [71]. Most importantly, longitudinal analysis of circulating sEVs in GDM patients identified that the total circulating and the placenta-derived sEVs increase across gestation, with higher levels in GDM compared to healthy pregnant women and altered pro-inflammatory effect on endothelial cells [59]. However, there are limited studies on the effect of EVs in maternal circulation or EVs derived from placenta on insulin sensitivity and insulin response in healthy pregnancy and GDM.

GDM is associated with altered protein and miRNA cargo of circulating sEVs [143, 144]. sEVs enriched from plasma collected at the time of the diagnosis (i.e., 22-28 weeks) from GDM women showed a differential expression of proteins such as calcium/calmodulin-dependent protein kinase II beta (CAMK2β) and Pappalysin-1 (PAPP-A) [143]. Interestingly, the proteins CAMK2β and PAPP-A are capable of influencing insulin signalling and glucose metabolic pathways in target cells [143]. Concomitantly, circulating EVs enriched from maternal plasma from early gestation (6-15 weeks) are enriched in miRNAs such as miR-122, miR-132, miR-136, miR-182, miR-210, miR-29, miR-342, and placenta specific miRNAs such as miR-520 and miR-1323) which can target the insulin signalling pathways [144]. The same study has shown that these miRNAs can target insulin signalling and associated pathways in target cells [144]. However, the contribution of placenta-derived EVs to the molecular cargo of total circulating EVs is not clearly identified. The placenta in GDM shows differential expression of certain miRNAs (such as hsa-miR-125a-3p,
hsa-miR-99b-5p, hsa-miR-197-3p, hsa-miR-22-3p, and hsa-miR-224-5p) in GDM compared to NGT controls, which is reflected in the circulating sEVs in GDM patients [74]. sEVs from GDM patients can alter the insulin-stimulated glucose uptake in skeletal muscle in vitro, indicating the effect of EVs in regulation of maternal insulin sensitivity [74]. Interestingly, mice which were continuously infused with sEVs enriched from GDM patients developed glucose intolerance and showed changes in the miRNA profile and insulin signalling in skeletal muscle tissues, most importantly, in the phosphorylation of IRS-1 and Akt, [91].

As previously mentioned, fewer studies have reported the effect of EVs on pancreatic insulin secretion in healthy pregnancy and GDM. A study by Kandzija et al., 2019 reported dipeptidyl peptidase-4 (DPPIV)-bound placental EVs are present in a higher concentration in the circulation of GDM women and identified to have higher DPPIV activity in vitro [145]. The increased activity of DPPIV can lead to cleavage of Glucose-dependent insulinoijotropic peptide (GIP) which decrease the pancreatic insulin secretion [146]. Also, our study in mice models infused with sEVs from GDM patients showed decreased glucose stimulated insulin secretion from islets compared to those receiving sEVs from normal healthy pregnancy [91]. These studies provide some insights into the role of EVs in the pathophysiology of GDM.

Increased maternal body mass index (BMI) is strongly associated with the development of GDM (reviewed in [147]). Circulating levels of sEVs increase with an increase in maternal BMI across gestation and sEVs enriched from plasma of obese women increased the release of pro-inflammatory cytokines from endothelial cells compared with sEVs from lean women [56]. This study provided a link between maternal BMI and the concentration and bioactivity of sEVs during pregnancy. Adipose tissue-derived sEVs in GDM are enriched in proteins that can alter cell metabolism in target tissues and can induce changes in the expression of genes associated with glycolysis and gluconeogenesis in placental cells in vitro [148]. Although this study shows that EVs can influence the metabolic pathways in placental cells, it is not clearly known if EVs can contribute to the pathophysiological placental changes in GDM or to its short- and long-term consequences. However, there is some evidence to show that EVs derived from fetoplacental endothelial cells in GDM are enriched in proteins involved in oxidative stress response and altered bioactivity on target cells [149, 150], but whether this contributes to placental
endothelial dysfunction requires further investigation. So far, circulating EVs, as well as EVs of different cellular origin, have been studied with regard to their concentration, content and effect on target cells. However, our knowledge in this area is limited and further studies are required to understand the roles of EVs in the regulation of maternal glucose homeostasis in normal pregnancy and GDM.

6. Conclusions

In summary, molecules and factors released from the placenta, including placental hormones, cytokines, proteins, miRNAs, and EVs, may influence various aspects of maternal physiology, including metabolic homeostasis (Figure 2). The potential impact of these biomolecules in mediating pathophysiological conditions such GDM paves way for utilizing them as prognostic and predictive biomarkers, as well as targets for therapeutic interventions. A better understanding of the precise molecular mechanisms through which they target the cell signalling pathways and physiological outcomes is fundamental to gaining in-depth knowledge about pregnancy associated metabolic disorders, and identifying methods to tackle them.

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Conflict of Interest

The authors declare no competing interests.

Data Availability

Data sharing not applicable to this article as no datasets were generated or analysed during the current study

References


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Table I summarizing studies that have analysed EVs of different cellular origin derived from different biological fluids

<table>
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<tr>
<th>Biological Fluid</th>
<th>Origin of EVs</th>
<th>Method to enrich EVs</th>
<th>Methods of characterisation of EVs</th>
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<td>Flow cytometry</td>
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<td></td>
<td>Density gradient centrifugation</td>
<td>NTA using quantum dots coupled with CD63\textsuperscript{+ve} and PLAP\textsuperscript{+ve} antibodies</td>
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<td></td>
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<td>Ultracentrifugation and size exclusion chromatography</td>
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<td></td>
<td>Commercial kit</td>
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<td>Immuno-isolation</td>
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<tr>
<td>Maternal Plasma</td>
<td>Placental EVs</td>
<td>Differential centrifugation</td>
<td>Flow cytometry</td>
<td>[46, 53, 56, 59, 80, 97, 145, 151,]</td>
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<td>Material</td>
<td>EVs isolated from</td>
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<td>Endothelial,</td>
<td>Differential centrifugation</td>
<td>Flow cytometry</td>
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<td>Total EVs</td>
<td>Ultracentrifugation and size exclusion chromatography</td>
<td>NTA using quantum dots coupled</td>
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<td>Fetal plasma</td>
<td>Total EVs</td>
<td>Differential centrifugation</td>
<td>NTA using quantum dots coupled</td>
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<td></td>
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<td>with CD63^ve and PLAP^ve</td>
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</table>
Maternal urine | Total EVs | Differential centrifugation | [63]
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**Figure Legends**

**Figure 1.** Biogenesis of different types of EVs, the structure and content of exosome and mechanism of their interaction with recipient cell. Exosome biogenesis begins with the inward invagination of the plasma membrane forming early endosomes. The invagination of the early endosomal membrane give rise to ILVs and the endosomes with ILVs are termed ‘MVBs’, which later fuse with the plasma membrane and are released as ‘exosomes’ into the extracellular space. The protein machinery involved in each step of the biogenesis process has been described. The exosomal membrane is loaded with adhesion molecules and other transmembrane receptors, and encapsulates several proteins (such as cytosolic and signal transduction proteins) and nucleic acids (such as mRNAs and miRNAs). At the recipient cells, exosome uptake occurs by receptor-ligand interaction followed by phagocytosis, endocytosis or direct fusion with the membrane. Other types of EVs such as microvesicles originate by direct budding from the plasma membrane and apoptotic bodies by apoptosis.

**Figure 2.** Multicellular crosstalk between the placenta and maternal organs via extracellular vesicles during pregnancy. Placental hormones, EVs, and pro-inflammatory cytokines such as TNFa and IL-6, secreted by the diabetic placenta can affect insulin secretion in the pancreas, which is regulated by glucose levels. In the pancreas, glucose enters the cell through the glucose transporter GLUT2. Specifically, glucose metabolism promotes an increase in the ATP/ADP ratio, which induces the closure of the ATP-sensitive potassium channel (K<sub>ATP</sub>), which then favors the entry of calcium Ca<sup>2+</sup> through the voltage-dependent calcium channel, and subsequently lead to the secretion of insulin. Likewise, several factors secreted by the placenta, as well as alterations in insulin secretion by the pancreas can affect insulin sensitivity by altering different stages of the signaling pathway in...
target tissues, such as skeletal muscle and adipose tissue, which can ultimately promote the development of insulin resistance. The initiation of insulin signaling requires an interaction between the activated receptor with activator molecules, such as IRS and SHC. Such interaction then triggers the activation of two main activation cascades: the ERK pathway responsible for the mitogenic effects of insulin and the Akt pathway, responsible for the metabolic effects of insulin. In the ERK pathway, growth factor binding protein 2 (Grb2) binds to the tyrosine phospho-residue of the activated insulin receptor, through the SH2 domain-containing adapter (SHC), which gives place to the downstream activation of Ras and Raf, which in turn results in the phosphorylation and activation of the MAP kinase Erk 1 and 2 isoforms. In the Akt pathway, the activated insulin receptor is recognized by the adapter protein members of the insulin receptor substrate family (IRS). In addition, phosphorylated IRS interacts with PI3K, which once activated, phosphorylates phosphatidylinositol (4,5) - bisphosphate (PIP2) thus leading to the formation of (3,4,5)-trisphosphate (PIP3). Furthermore, Akt has to be phosphorylated by protein kinase 3'-phosphoinositide-dependent protein kinase-1 (PDK1) so as to become activated. Once active, Akt leads to the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3), which activates glycogen synthase. Akt also directly activates the transcription factors mTOR and Forkhead (FOXO). Additionally, Akt plays an important role in the translocation of GLUT4 to the plasma membrane through its interaction with the AKT substrate of 160 kDa (AS160), as a result of this event; glucose is capable of entering the cell. EV cargo (miRNA, proteins and metabolites) reflects the metabolic state of the cell of origin and is responsible for the metabolic changes induced in target cells. The metabolic changes induced in GDM by EVs may be responsible for future metabolic consequences in the mother and in the regulation of glucose metabolism in the fetus. Characterizing the mechanisms by which EVs, cytokines and placental hormones are generated during GDM are critical for understanding how these factors can contribute to the pathogenesis of metabolic disorders induced during GDM.
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