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# The Role of Placental Exosomes in Gestational Diabetes Mellitus

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Additional information is available at the end of the chapter

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## 1. Introduction

Gestational Diabetes Mellitus (GDM) affects ~5% of all pregnancies and parallels the global increase in obesity and type 2 diabetes. In the USA alone, GDM affects more than 135,000 pregnancies per year. Lifestyle changes that impact adversely on caloric balance are thought to be a contributing factor in this emerging pandemic [1, 2]. The current 'gold standard' for the diagnosis of GDM is the oral glucose tolerance test (OGTT) at 24–28 weeks of gestation [3, 4]. When GDM is diagnosed in the late second or early third trimester of pregnancy the 'pathology' is most likely well-established and the possibility to reverse or limit potential adverse effects on perinatal outcomes may be limited [5]. Early detection of predisposition to and/or onset of GDM, thus, is the first step in developing, evaluating and implementing efficacious treatment. If such early detection tests were available, they would represent a major advance and contribution to the discipline and afford the opportunity to evaluate alternate treatment and clinical management strategies to improve health outcomes for both mother and baby. Based upon recent technological developments and studies, we consider it realistic that a clinically useful antenatal screening test can be developed. Unlike diseases such as cancer where biomarkers need to be exquisitely specific, a useful antenatal screening test would ideally be highly sensitive, but not necessarily highly specific. The consequence of a false positive would be no worse than an erroneous triage to high-risk care.

Recent studies highlight the putative utility of tissue-specific nanovesicles (*e.g.* exosomes) in the diagnosis of disease onset and treatment monitoring [6-11]. To date there is a paucity of

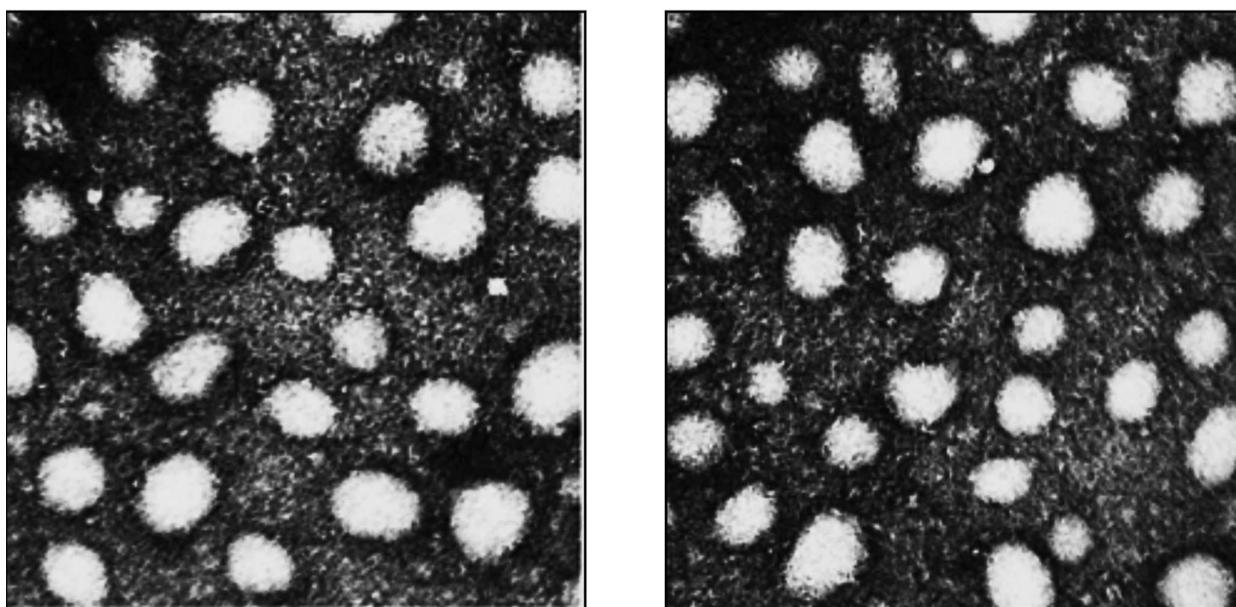
data defining changes in the release, role and diagnostic utility of placenta-derived nanovesicles (*e.g.* exosomes) in pregnancies complicated by GDM.

The aim of this brief commentary, thus, is to review the biogenesis, isolation and role of nanovesicles; and their release from the placenta. Placental exosomes may engage in paracellular interactions (*i.e.* local cell-to-cell communication between the cell constituents of the placenta and contiguous maternal tissues) and/or distal interactions (*i.e.* involving the release of placental exosomes into biological fluids and their transport to a remote site of action).

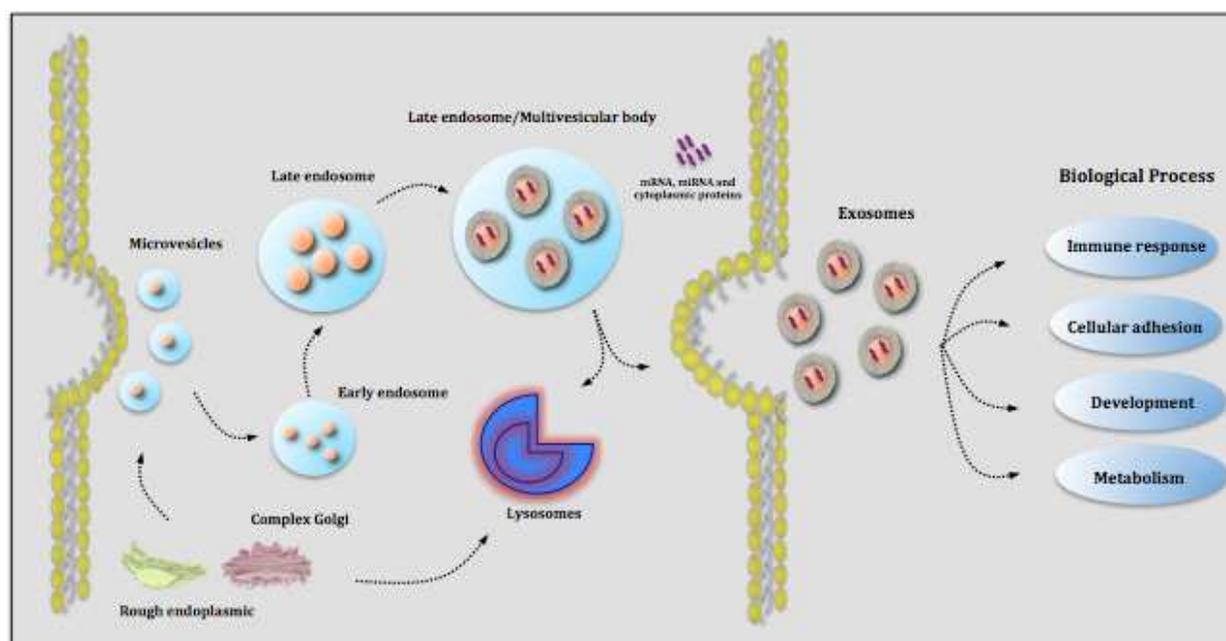
## 2. Exosome biogenesis and composition

### 2.1. Biogenesis

Exosomes are small [40-100 nm) membrane vesicles that are released following the exocytotic fusion of multi-vesicular bodies with the cell membrane (Figure 1). They are characterised by: a cup-shaped form: (a) a buoyant density of 1.13-1.19 g/ml [12, 13], (b) endosomal origin, and (c) the enrichment of late endosomal membrane markers including Tsg101, CD63, CD9 and CD81 [7, 14, 15]. While the process(es) of exosome formation remains to be fully elucidated, available data support an endosomal origin and formation by the inward budding of multi-vesicular bodies [16] (see Figure 2). Exosomes may also be directly transported from the Golgi complex to multi-vesicular bodies [14].



**Figure 1. Electron micrograph of circulating exosomes.** Exosomes are 40-100 nm membrane vesicles with a density ranging from 1.13-1.19 g/ml, characterised by a cup-shaped form and secreted by most cell types *in vivo* and *in vitro*. Villous chorionic explant-derived exosomes were isolated by ultracentrifugation and purified using a sucrose gradient. Scale bar = 100 nm.



**Figure 2. Schematic of exosome biogenesis and secretion.** Exosomes are generated in the endosomal structure participating the plasma membrane in this process, and secreted via constitutive endosomal pathways involving the Golgi complex from various cell types. The exosomes contain specific proteins and miRNA as a new form of exosome-mediated intercellular communication and with different biological function. In pathological pregnancies characterised by compromised placental perfusion and ischaemia, such as GDM, exosome secreted from the placenta can participate in an adaptive response of the mother and fetus and so interact with target tissue and modulate different biological processes, such as immune response, cellular adhesion, development and metabolism.

Exosomes have been identified in plasma under both normal and pathological conditions. The concentration of exosomal protein in plasma has been reported to increase in association with disease severity and/or progression, and in response to oxidative stress. Cell membrane budding and the deportation of cell membrane particles was originally considered as the elimination of cell debris and associated with apoptosis and/or necrosis. Recent data, however, suggest that the release of nanovesicles from cells may represent a normal mechanism for cell-to-cell communication [17]. Packaging of exosomal contents appears to be a direct process in which the ESCRT (endosomal sorting complex required for transport) systems play a significant role [18-20].

Exosomes are released from the placenta and the concentration of exosomes in maternal plasma increases during normal pregnancy [21-23]. *In vitro*, exosomes are released from both trophoblast cells and syncytium [24]. They contain placenta-specific proteins and miRNA and, as such, may be differentiated from maternally-derived exosomes [25]. The concentration of exosomes has been reported to increase in association with pre-eclampsia [22, 23, 26]. The role of exosomes in the development and progression of GDM has yet to be established.

## 2.2. Composition

The exosomal content is highly dependent on the origin cell and on pre-conditioning of the cell. One of the first exosomal proteomes characterised was from mesothelioma cells, in which

38 different proteins were identified [27]. Studies in cancer cells show the great variability of proteins expressed in exosomes [28-32]. In exosomes isolated from a human first trimester cell line (Sw7 1) Atay *et al.*, using an ion trap mass spectrometry approach, identified proteins implicated in a wide range of cellular processes including: cytoskeleton structure (adhesion, membrane transport, and fusion), ion channels, lysosomal degradation, molecular chaperones, amino-acid metabolism, carbohydrate metabolism, lipid metabolism, oxydo-reductase activity, protein synthesis and post-translational modifications, ubiquitin modifiers, signal transduction, transcription factors and regulators, DNA replication, chromatin structural and regulatory proteins, mRNA splicing, transcription/translation, post-translational protein modification enzymes, nuclear structural proteins, integrins, complement and coagulation, immune function, iron transport, and ER specific proteins. This study provides the first extensive analysis of the proteome of the exosome-derived trophoblast cells [7]. The data obtained in this study, highlights the extent of putative functional interactions that may be mediated by exosomes.

While the composition of exosomes appears to be cell-specific, a subset of common proteins has been identified. The lipid bilayer is composed of sphingomyelin [33, 34]. Among the most commonly used markers for characterisation of exosomes are tetraspanin proteins, including: CD63, CD81, CD9, and CD82. Other families of common proteins in all exosomes include chaperone proteins such as: Hsc70 and Hsp90; cytoskeletal proteins including actin, tubulin and myosin; transport proteins; and annexins [35]. Exosomes derived from antigen-presenting cells (APC) express MHC-I and MHC-II on their surface [36-38]. During exosome biogenesis, the phospholipid/protein ratio of exosomes may be regulated by the Golgi membranes [39].

Significantly, single cell types display the capacity to generate different subpopulations of exosomes. Laulagnier *et al.* 2005 demonstrated that RBL-2H3 cells (basophilic leukemia cell line) released two main subpopulations of exosomes that can be discriminated by protein and lipid contents. The first subpopulation contains phospholipids obtained mainly from granules and the second contains phospholipids from Golgi. In addition, proteins CD63, MHC-II, CD81-containing exosomes accounted for 47%, 32%, and 21%, respectively, of total exosomes [39, 40].

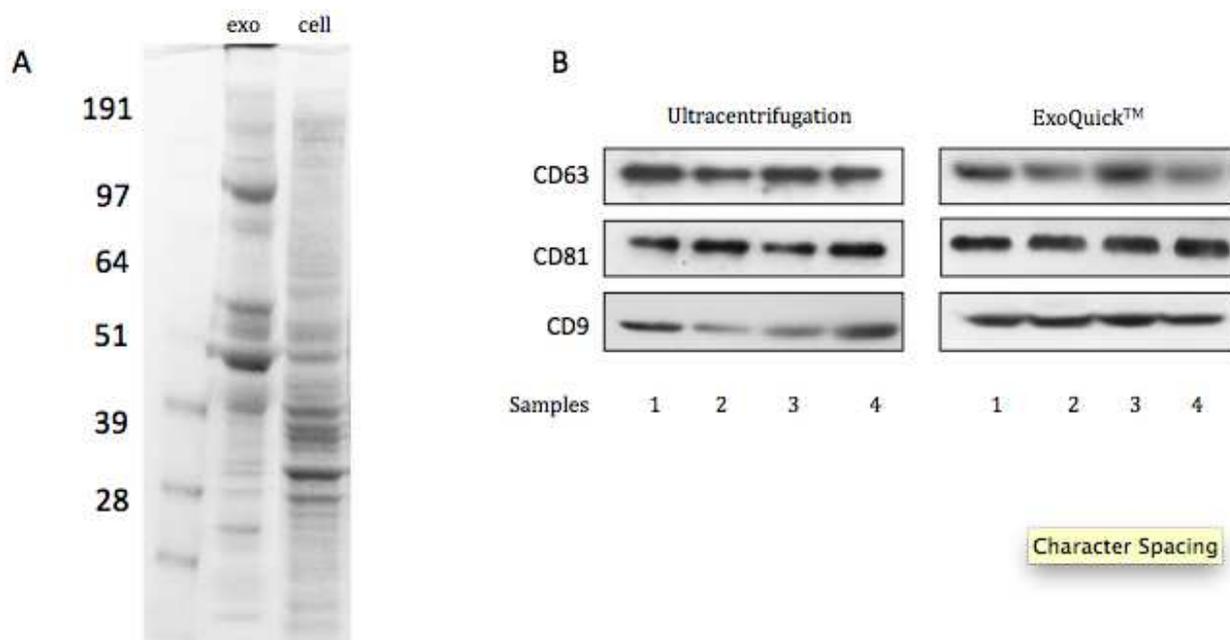
### 3. Isolation of exosomes

Exosome research is a burgeoning discipline with over 2000 articles published in the last 3 years. The putative role of exosomes spans from intracellular signaling to biomarkers of disease [41]. Germane to any study seeking to elucidate the physiological or pathophysiological role of exosomes is their specific isolation. Several methods for isolating exosomes have been developed and partially characterised. These isolation methods are primarily based on particle size and density. By definition, exosomes are nanovesicles with a diameter of 30-100 nm. Typically they display a density of 1.12 to 1.19 g/ml and express characteristic cell-surface markers.

The most common method of separation involves a series of differential centrifugation to remove intact cells and debris, and nuclei followed by size selective filtration (0.2  $\mu\text{m}$  pore

size) and sedimentation by ultracentrifugation (*e.g.* at 110,000 g for 1-2 hours [42, 43]. Exosomes may be further purified by differential sedimentation on sucrose gradients or sucrose-deuterium oxide (D<sub>2</sub>O) [ 7]. Alternative methods utilise size exclusion chromatography and density gradient centrifugation [44] or solid-phase immunoaffinity capture (*i.e.* anti-MHC-II Dynabeads) [38, 43, 45]. In the absence of specific, cell surface exosome markers, the veracity of immuno-affinity methods remains to be established.

More recently a commercial kit for the isolation of the exosomes has been released (ExoQuick™, System Biosciences). The isolation process involves a simple one-step precipitation [43, 46]. While the commercial kit provides significant advantage with respect to processing time, the resulting preparation may not be equivalent to that obtained by ultracentrifugation and differential segmentation. In our own laboratory, parallel preparations of exosomes using both methods reveal differences in the biophysical characteristics of the exosomes isolated. Exosome preparations isolated using the commercial kit were characterised by a great range in particle diameter (30-300 nm, as estimated by transmission electron microscopy (TEM) and have a higher protein content than similar preparations isolated using the ultracentrifugation method. In addition, analysis of protein patterns in SDS-PAGE electrophoresis and western blot against CD63, CD81 and CD9 show similar characteristics between exosomes from ultracentrifuge and ExoQuick™ methods, however, we had to dilute ExoQuick™ samples ~ 10 times to obtain comparable concentrations with ultracentrifuge methods in exosomes isolated from trophoblastic cells (see Figure 3). Differences in exosome protein and mRNA content and functional activity between different preparations remain to be established.



**Figure 3. Typical characteristics of exosomes isolated from trophoblast cells.** (A) Exosome protein pattern analysis. 10 ug of exosome proteins and trophoblast cell lysate were separated on 4-12% SDS-PAGE and stained with SimplyBlue™ SafeStain (Invitrogen). (B) Western blot characterisation of exosomes with antibodies against CD63, CD81 and CD9 for 4 different samples isolated with ultracentrifuge or ExoQuick™ methods.

## 4. The role for exosomes in cell-to-cell communication

Recently, evidence supporting a role for exosomes in cell-to-cell communication has been obtained [47, 48]. Exosome release may represent a significant, hitherto unappreciated, communication mechanism between cells, host cell and microbes [47].

For example, exosome function as a carrier of specific molecules such as mRNA and miRNA can interact with neighbouring cells or travel long distances in the bloodstream to reprogram the phenotype and regulate their function [40]. In the placenta, exosome-derived trophoblastic cells are able to reprogram monocytes to secrete specific cytokine profiles independent of cell-to-cell contact [8]. Placental-derived exosomes may also play a role in modulating immunological responses through the induction of lymphocyte apoptosis, [21, 44, 49].

### 4.1. Information encoding by exosomes

Exosomes have been reported to express a diverse range of cell surface receptors, proteins (including, heat shock proteins, cytoskeletal proteins, adhesion molecules, membrane transport and fusion proteins), mRNA and miRNA with the potential to affect the acute and long-term function of the cells with which they interact [50]. In addition, in the absence of energy production, normal membrane phospholipid asymmetry is lost and amniophospholipids translocate to the outer leaflet of the cell membrane and generate a fusogenic and pro-coagulant surface. Given that exosomes circulate in blood, these fusogenic moieties may be masked by, for example, annexin V.

*In vitro* effects of exposing cells to exosomal proteins has been reported and include: induction of differentiation of stem cells [51], suppression of activation of natural killer cells and macrophages [52, 53], and stimulation of cell migration [8, 54]. Putative roles of exosomes, thus, include cell differentiation, immunomodulation and migration [55]. Exosomes are not merely inert fragments of cell membrane but display capacity to affect cell function at remote loci and possibly be a source of disease biomarkers.

Exosomes also contain miRNA that may transfer to other cells and alter the expression of the transcriptome and ultimately cell phenotype. miRNAs are a class of small non-coding RNAs that function as translational repressors involved in a variety of physiological and pathological processes in animals [56, 57]. They act via binding to messenger RNA and, thus, prevent the translation of the encoded protein. Previous studies have reported that miRNAs are involved in the pathogenesis of diabetes and are required for pancreatic development and the regulation of glucose-stimulated insulin secretion [50, 58]. Moreover, differences in the expression of miRNA such as miR-146a, miR-21, miR-29a, miR-34a, miR-222, and miR-375 have been reported in pancreatic  $\beta$ -cells, liver, adipose tissue, and/or skeletal muscle of animal models of type 1 or type 2 diabetes [59]. Another study found that miR-20b, miR-21, miR-24, miR-15a, miR-126, miR-191, miR-197, miR-223, miR-320, and miR-486 were lower in prevalent type 2 diabetes [60].

## 4.2. Placental exosome release and effects

### 4.2.1. Placental exosome release

Exosomes are released by the placenta during pregnancy and their release may correlate with pregnancy outcome. The syncytiotrophoblasts and cytotrophoblasts are the most abundant cell types of the human placenta and sense and regulate oxygen and nutritional exchange between mother and fetus during the pregnancy [61, 62]. Pathologies of pregnancy including preeclampsia, intrauterine growth restriction (IUGR) and GDM are associated with placental dysfunction [4, 63, 64] and may display differential and specific exosome release profiles.

It has been established that the concentration of exosomes in maternal peripheral blood is greater than that observed in non-pregnant women [21]. In this study, exosomes of placental origin were specifically isolated from the maternal blood using anti-PLAP (anti-placental-type alkaline phosphatase) conjugated to agarose micro-beads. In peripheral blood mononuclear cells (PBMC), placental exosomes suppressed T signalling components such as CD3-zeta and JAK3, while inducing SOCS-2 [21]. These results are consistent with those of Taylor *et al.* [44, 65] who demonstrated the presence and composition of placenta-derived exosomes in maternal circulation along with their effects on T cell activation markers. Exosomes appear to play an essential role in preventing an excessive immune response and in the development of autoimmunity in human pregnancy.

Recently, it has been demonstrated that placental miRNAs circulate in the blood of pregnant women [66, 67]. For example, maternal plasma concentration of placental miRNA-141 increases with gestational age [66]. Placenta-specific miRNA-517A is released from chorionic villous trophoblasts into maternal circulation, where it may affect maternal tissues (*e.g.* maternal endothelium) during pregnancy [25]. There is a paucity of data, however characterising the release of exosome from endothelial cells during normal and pathological pregnancies. It will be important to determine if the placenta communicates with the maternal endothelium via microvesicles, and, if so, to elucidate the role and mechanism of action of exosome pathologies associated with endothelial dysfunction, such as GDM. Placenta-derived miRNAs, therefore, may be of utility as biomarkers of placental function and/or pregnancy outcome. It remains to be elucidated how much of this “free” miRNA and mRNA is actually contained within exosomes and thereby confers stability. Indeed, the exact mechanisms involved in the release of miRNA from the placenta remain to be established. A recent study, however, reported that miRNAs are selectively packaged into microvesicles and are actively secreted [68-70]. miRNAs are also released from the syncytiotrophoblast to the maternal circulation in the pregnancy packaged inside exosomes [25, 71].

### 4.2.2. Effects

There remains a paucity of data about the effect of placenta-derived exosomes on both fetus and mother. The available data, however, support a role for placental exosomes in mediating communication at the materno-fetal interface and, possibly, at the distal site within the mother. Recent data show that trophoblast-derived exosomes induce proinflammatory cytokines such IL-1  $\beta$  in human macrophages cells [8]. Furthermore, *in vitro* exposure of PBMC and dendritic

cells to exosomal proteins induce differentiation of stem cells; suppression of activation of natural killer cells and macrophages; and stimulation of cell migration [53, 72, 73]. Interestingly, protein analysis revealed that exosome release from trophoblast cells increases with low oxygen tension and their exosome promote the cell migration in extravillous cytotrophoblast (HTR-8) (Salomon et al. manuscript in preparation).

## 5. Exosomes and GDM

Exosomes released from the placenta of women with GDM may alter maternal physiology. Via a process of *exosomal placento-maternal transfection* a “payload” of receptors, proteins and/or oligonucleotides” that have been specifically pre-conditioned by the GDM placenta may be delivered to maternal response systems. Such mediators include: vascular, pancreatic and adipose tissues, and the innate immune response system. The extent and impact of placenta-derived exosomes on maternal physiology, however, remains to be elucidated.

In addition to a *placento-maternal transfection* pathway, trophoblasts or placental mesenchymal stem cells (MSCs) may induce paracellular effects in association with GDM. For example, in placental villi, exosomes released by perivascular MSCs may alter transport activity within the placental vascular endothelium (e.g. the glucose transport GLUT 3) and thus the delivery of energy substrates to the fetus.

In support of the role of exosomes in modulating glucose homeostasis, Deng *et al.*, reported that exosomes isolated from adipose tissue induce differentiation of monocytes into activated macrophages and promote insulin resistance in an obese mouse model [74]. Exosomes isolated from mouse insulinoma induce the secretion of inflammatory cytokines including IL-6 and TNF- $\alpha$  in splenocytes cultured from non-obese diabetic mice (NOD) [75]. In this regards, these cytokines as well as other inflammatory mediators play an important role in glucose tolerance and insulin sensitivity dysregulation in women with previous GDM.

### 5.1. The effects of hyperglycaemia and oxidative stress on exosome release

GDM is a state of hyperglycaemia and increased oxidative stress [76]. In addition, hyperglycaemia-induced oxidative stress makes an important contribution to the aetiology of GDM [77], with consequences for both mother and baby [78]. In support of an aetiological role of hypoglycaemia and attendant oxidative stress in poor pregnancy outcome, the HAPO study reported a strong and continuous association between maternal glucose concentrations and pregnancy outcome and confirmed a relationship between birth weight and maternal hyperglycaemia [79, 80].

Reactive oxygen species (ROS) include oxygen ions such as superoxide ions and hydrogen peroxide ( $H_2O_2$ ) that are generated continuously during cellular metabolism. GDM pregnancies are characterised by an overproduction of ROS and free radicals and impaired antioxidant capacity [81]. Oxidative stress and increased exosome release are common features of many pathologies including: cancer, kidney disease, hypertension, and preeclampsia. It remains to

be established whether or not exosome release in these circumstances is a paraprophenomenon of, or an adaptive response to, increased ROS formation and oxidative stress.

GDM is a syndrome that leads to fetoplacental vascular endothelial dysfunction involving higher nitric oxide (NO) concentrations and increases of oxidative state and vascular resistance [63, 82]. Exosomes – endothelial cell interactions may result in activation of NO synthesis via a number of mechanisms. Exosomes isolated from platelets obtained from patients with septic induced endothelial dysfunction through the NADPH oxidase-dependent release of superoxide and have been implicated in the induction of NO and peroxynitrite. NO synthase is also induced by miR-203. miRNA-203 has been identified in exosomes [83]. It remains to be established whether or not miRNA-203 is present in exosomes isolated from women with GDM.

In non-gestational tissues, the available evidence supports an active role for exosomes in regulating cellular redox status. For example, oxidative stress enhances exosome release from Jurkat and Raji cell lines and the resultant increase in NKG2D receptor bioactivity impairs cytotoxic response [84]. In 3T3-L1 adipocytes, oxidative stress increases microvesicle release [85]. Melanoma-derived exosomes induce ROS production in T cells compared to exosomes from normal cells, suppressing the immune response and improving carcinogenic invasion [86]. Exosomes isolated from mouse mast cells MC/9 exposed to oxidative stress alter the response of others cells to oxidative stress [87], increasing their resistance to oxidative stress and reducing cell death. Interestingly, the mRNA content of exosomes produced under oxidative stress conditions differ from those produced under normal conditions. These data are consistent with the observations of Atay *et al.* [7, 8], Luo *et al.*, [25] and Taylor *et al.* [83] who similarly report cell- and condition-specific variation in exosomal protein, mRNA and miRNA content.

## 5.2. Exosome biomarkers of GDM

In addition to their putative functional involvement in the pathophysiology of pregnancy, placental-derived exosomes may be of utility as diagnostic markers of GDM in asymptomatic women.

In 2011, the American Diabetes Association (ADA) and the International Association of Diabetes and Pregnancy Study Groups (IADPSG) revised recommendations regarding GDM. It is now recommended that patients at increased risk for type 2 diabetes be screened for diabetes using standard diagnostic criteria at their first prenatal visit (ADA 2012). Currently, GDM is diagnosed in the late second or early third trimester of pregnancy. Pathology is probably already established by this time and reversal of the potential adverse perinatal outcomes may be limited. The lack of a reliable early test for GDM has hampered the development of useful intervention therapies that may impact not only on the acute but also the long-term health outcomes [88-90]. Thus, there is a need to diagnose and predict GDM earlier so that appropriate management can be initiated and tailored to the needs of the patient in order to minimise perinatal complications and their sequelae.

Currently, the diagnosis of GDM is between 24-28 weeks of gestation by an oral glucose tolerance test. The aim of the treatment for GDM is to maintain the glucose level in euglycaemia

with dietary modifications or in some cases with insulin therapy, however, when it is diagnosed, the pathology is established and the clinical and obstetric management is limited [5, 91].

The quantitation of exosomes and/or exosome-specific content may be of diagnostic utility [14, 43, 83, 92, 93]. Exosomes are found in all body fluids tested to date including blood, urine, saliva and breast milk. They can be obtained by minimally invasive methods (blood) or non-invasive methods (using urine or saliva) [94]. Several studies have demonstrated the putative utility of exosomes as biomarkers, particularly in cancers, where exosomal protein is correlated with disease burden.

The measurement of exosomal miRNA in biofluids has proven of utility in cases of lung cancer, colorectal cancer, prostate cancer and diabetes [95-99].

Zhao *et al.* isolated miRNA from blood circulation at 16-19 gestational weeks. Interestingly, these authors found that the expression of miRNA-132, miRNA-29a and miRNA-22 were decreased in GDM women compared with normal pregnancies in similar gestational weeks [70]. Finally, there are few reports suggesting that mesenchymal stem cell and trophoblast cells-derived exosomes may serve as therapeutic agents for use in regenerative medicine to repair damaged tissue [100, 101].

Finally, in normal pregnancies, the placenta secretes significant amounts of macro- and microvesicles, including exosomes [22, 26]. We suggest that in pregnancies complicated by GDM, oxidative stress and hyperglycaemia increase the release of exosomes from the placenta into the maternal circulation during in the first trimester of pregnancy. The quantification and characterised of exosomes in the blood of these pre-symptomatic women, thus, may be of utility as an early biomarker of disease onset. Furthermore, we propose that during first trimester, pre-symptomatic women who subsequently develop GDM: have higher plasma concentrations of placental-derived exosomes; and a different exosomal protein and miRNA profile than women who experience a normoglycaemic pregnancy. These characteristics could potentially be used for diagnostic markers for exosome profiling to screen asymptomatic populations.

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