**EXOSOMES AND THEIR POSSIBLE APPLICATIONS IN THE MANAGEMENT OF GESTATIONAL DIABETES**

Rafał Sibiak\(^1,2\), Michał Jaworski\(^1\), Saoirse Barrett\(^4,5\), Rut Bryl\(^4\), Paweł Gutaj\(^2\), Ewa Wender-Ożegowska\(^2\)

**Abstract**
Gestational diabetes mellitus (GDM) is thought to be the most common metabolic gestational complication. Its prevalence has been continuously increasing in recent decades along with the rising epidemic of obesity in modern societies. GDM is associated with an increased risk of fetal growth abnormalities, birth traumas, and several neonatal complications. Widely available screening tools, fasting glucose measurements, combined with oral glucose tolerance test results, contribute to the reduction of the risk of those complications. Nevertheless, we are still looking for novel reliable early markers of GDM. It has been established that high 1\(^{st}\)-trimester exosome concentrations could predispose the development of GDM in later pregnancy. Exosomes can be easily isolated from various tissues and body fluids in pregnant patients. Due to this, extracellular vesicle concentration assessment appears as a new promising tool in the prediction of GDM at the preclinical stage of the disease. Furthermore, it has been found that women already diagnosed with GDM have significantly higher exosome concentrations compared with healthy individuals. These findings could help to elucidate the molecular pathogenesis of GDM. Exosomes are loaded with various molecules especially proteins, lipids, mRNAs, and microRNAs. Altered expression of numerous microRNAs and enzymes such as dipeptidyl peptidase-IV in exosomes isolated from patients with GDM may suggest their direct contribution to the mechanisms of glucose intolerance. This knowledge could be used in the development of new therapeutic strategies in patients with GDM. Nevertheless, it should be emphasized that these are only preliminary results that require further investigations.

**Running title:** Exosomes in gestational diabetes

**Keywords:** diabetes, exosomes, extracellular vesicles, gestational diabetes, glucose intolerance
Introduction

Gestational diabetes mellitus (GDM) is a common gestational complication, which is diagnosed when maternal hyperglycemia and glucose intolerance occur for the first time during pregnancy [1–3]. Due to various existing diagnostic criteria, the global prevalence of GDM is quite hard to estimate. Nonetheless, based on the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria, about 15% of all pregnancies are complicated with gestational diabetes, while the frequency of GDM among obese European women reaches about 39% [4–6]. The most current guidelines recommend performing an oral glucose tolerance test (OGTT) at 24–28 weeks of pregnancy, which is accepted as the gold standard in the GDM diagnostics [5,7,8]. The etiology of disturbances in glucose metabolism during pregnancy is quite complex. However, some parameters such as maternal obesity, maternal age, family history of diabetes, and GDM diagnosed in a previous pregnancy, are all treated as independent risk factors of GDM [9,10]. Gestational diabetes is characterized not only by altered d-glucose metabolism but also by endothelial dysfunction observed in the mother as well as in the developing fetus [11]. In both vasculatures of the placenta (micro and macro), endothelial cell activation and dysfunction are detected [12]. Peripheral insulin resistance is physiologically the most pronounced in the second and third trimester of pregnancy, but in cases when β-cell adaptation is inadequate, it is probably the most important causative factor of prolonged hyperglycemia. These changes lead to β-cell dysfunction, which is partially responsible for the effects of glucotoxicity [12,13]. It is therefore essential to diagnose GDM as early in pregnancy as possible, in the group without risk factors, and set up an effective therapy that protects against its complications, such as fetal growth abnormalities (fetal growth restriction and macrosomia), birth traumas, and neonatal hypoglycemia [10,14]. Moreover, GDM increases the long-term risk of developing metabolic syndrome, type 2 diabetes mellitus (T2D), and cardiovascular diseases in both patients and their offspring [15].

Exosomes are produced via a process of exocytosis. They are a specific type of extracellular vesicle called nanovesicles with a diameter of about 40-120 nm and are spherical or cup shaped. Many cell types release exosomes into the extracellular space [9,13,14]. The contents of exosomes include mRNA, microRNA, non-coding RNA, free-cell DNA; which take part in communication between cells, and other substances like proteins or lipids. Exosome-associated DNA and microRNAs detected in maternal circulation are important targets of studies focused on the etiology of pregnancy complications including gestational diabetes [12–14]. Patients with GDM show an increased level of placenta-derived exosomes in the maternal blood from about 6 weeks of gestation when the placenta begins its secretion [9,11,16]. The secretion of exosomes is thought to be under the control of, among other things, low oxygen tension or increased glucose concentrations [14].

Scope and Methodology

This review was compiled in order to provide a concise source of information about current knowledge on the potential role of extracellular exosomes in the pathophysiology of gestational diabetes and its possible utility in clinical practice.

PubMed and Scopus databases were searched for relevant references from the first records until August 2020, using the following MeSH terms: “exosomes gestational diabetes”, “extracellular vesicles gestational diabetes.”

Exosomes in gestational diabetes

Sources, methods of isolation and preparation

Exosomes can be isolated from various tissues and body fluids of women with GDM [17,18]. For example, one of the methods is that the dose of serum samples is thawed on ice and diluted in filtered PBS. The serum is then centrifuged two times to obtain clean supernatant and resuspended in filtered PBS. The material obtained for transmission electron microscopy (TEM) is applied to a formvar carbon-coated nickel grid. After 60 minutes of incubation at room temperature and three washing steps with filtered PBS, the sample is placed in 2% paraformaldehyde for 10 minutes, then in a solution of primary antibody, mouse monoclonal IgG2a placental alkaline phosphatase (PLAP) antibody diluted in filtered PBS. Later, 12 nm of Colloidal Gold-AfinediPure goat anti-mouse IgG secondary antibody diluted in filtered PBS is applied for 60 minutes at room temperature and the samples are fixed with glutaraldehyde. This method uses uranyl acetate and methylcellulose for contrast. In the end, the excess liquid should be removed, and the grid must dry at room temperature. Finally, the samples can be viewed in a TEM. This whole process allowed for analysis of the expression of 17 miRNAs suspected to be linked with the pathogenesis of GDM [17].

Other studies used alternative methods and protocols of isolation and exosomes preparation. The main differences are in centrifugation, used substances, or dehydration [19]. Beside TEM, flow cytometry [20], immunofluorescent nanoparticle tracking analysis (NTA) in fluorescent mode [18], or western blotting were used [21,22].

To measure total and placental exosome concentrations, placental alkaline phosphatase (PLAP) ELISA and CD63 were applied. PLAP was used as a placental-specific marker [18]. Because of the expression of specific CD we can recognize exosome source cells: leukocytes (CD45+), platelets (CD45−/CD41a+/CD31+), endothelial cells (CD41a−/CD45−/CD31+) and adipocytes (CD45−/CD31−/
CD36+) [20]. Exosomes can also be identified by endosomal membrane markers, including Tsg101, CD63, CD9, and CD81 [9,19] and tissue-specific substances for example sphingosine kinase 2 for liver or adiponectin, resistin, and tumor necrosis factor-α (TNF-α) for adipocytes [23].

Each method of isolation and preparation has its own advantages and disadvantages. Thus, the choice of method made should take into account the purpose of the study [23,24], or the source of the analyzed material [25]. Due to the many steps in the preparation and isolation protocols, there is no clear consensus or standard procedure for this process [24]. It may lead to significant methodological discrepancies between studies and resultant bias.

Clinical applications of exosomes concentrations measurements

**Prediction of gestational diabetes**

There are strict diagnostic criteria of gestational diabetes which are based on the results of fasting glucose levels and oral glucose tolerance test. Nevertheless, some patients with the correct results of 1st-trimester screening are more susceptible to developing diabetes in later pregnancy [5]. It seems crucial to ask, “why does it happen?” and “how does using different laboratory parameters and diagnostic tools in advance predict the occurrence of diabetes?”. Results of 1st-trimester measurements of placental exosomes concentrations in maternal plasma revealed that mothers who developed gestational diabetes in further stages of pregnancy had a significantly higher number of extracellular vesicles compared with euglycemic controls [9,26]. Authors matched pregnant women for age, BMI, and parity, however, did not analyze their family history of hyperglycemia. Interestingly, the measurement of exosome concentrations in samples of gingival crevicular fluid could also potentially help identify patients from groups with a higher risk of developing gestational diabetes. Testing of samples obtained at 11-14 weeks of pregnancy show a notable difference in extracellular vesicle concentrations between the groups of healthy controls and patients who developed GDM later in pregnancy at the pre-clinical stage of the disease [27]. With the results of those tests, physicians could encourage patients to make modifications to their diet prior to the manifestation of the first noticeable signs of GDM and its complications. This testing could also reduce the number of patients requiring insulin therapy. Nonetheless, before the potential implementation of these observations in clinical practice, an additional accurate validation with the calculation of sensitivity and specificity of these findings is required. Furthermore, some authors found that a number of placental exosomes in maternal plasma is rising in correlation with gestational age, which generates the next challenges of choosing optimal time points of measurements and establishing reliable cut off points [9,26].

**Possible contribution to the pathogenesis of gestational diabetes and its maternal and fetal complications**

Some molecules detected in extracellular vesicles are thought to play a role in the pathogenesis of gestational diabetes. To prove this hypothesis, exosomes were isolated from plasma in healthy pregnant patients, women with GDM and non-pregnant healthy controls, and these samples were transferred to non-pregnant mice. The injection of vesicles from women with GDM invoked a state of glucose intolerance, insulin resistance, and decreased islet glucose-stimulated insulin secretion in the mice compared to those treated with exosomes from the healthy controls [18]. However, Salomon et al. did not find any correlation between the basal glycemia, OGTT results, and the total number of detected exosomes [9]. Basing on the limited data, it is worth highlighting that the majority of recently published studies indicate the existence of higher concentrations of maternal exosomes in patients diagnosed with GDM (diagnosed according to the criteria of the Australasian Diabetes in Pregnancy Society ~ 28 weeks of pregnancy, and World Health Organization’s 1999 diagnostic criteria, between 24 and 28 weeks of gestation) in comparison to healthy controls [9,28]. Conversely to those observations, Franzago et al. reported that they did not observe any differences in the number of total or subtypes of isolated exosomes, at the third trimester, independently of the results of GDM screening [20]. Interestingly, the rise in the number of isolated vesicles was markedly linked with an increased neonatal birth weight in mothers with GDM [28]. It was suggested that a higher incidence of fetal macrosomia among diabetic mothers could be connected to an upregulated exosomal load of S100A9 protein [29]. Furthermore, there was a significant positive correlation between the increased number of isolated placental exosomes and placental weight in patients with GDM. This kind of relationship was not observed in a group of healthy pregnant women [9]. Surprisingly, the number of isolated vesicles was markedly inversely correlated with the values of the umbilical arterial pulsatility index measured during the Doppler ultrasound examination in both groups of participants [9].

GDM is described as a state of increased synthesis of pro-inflammatory cytokines. Elucidation of the possible mechanism of increased synthesis of cytokines could unveil new potential therapeutic targets for novel diabetes treatment strategies even at the pre-symptomatic stages of the disease. It was found that human umbilical vein endothelial
cells cultures treated with exosomes isolated from diabetic mothers produced abundant amounts of cytokines: IL-4, IL-6, IL-8, IFN-γ and TNF-α - significantly higher than those co-cultured with exosomes from healthy controls [9]. These outcomes could suggest that molecules carried in exosomes have a key role in maintaining a sustainable balance between proportions of excreted cytokines.

The proteomics analysis of exosomes isolated from blood samples in patients with GDM revealed remarkable changes in the expression pattern of 78 proteins which are mainly associated with the regulation of metabolic pathways [30]. Furthermore, the content of exosomes isolated from maternal adipose tissue supported the hypothesis that they are a probable etiologic factor of GDM. Vesicles derived from adipose tissue have a capacity to increase the expression of genes involved in the regulation of glycolysis and gluconeogenesis in placental tissue [28]. Soumyalekshmi et al. found that exosomes isolated from diabetic patients could influence glucose homeostasis in patients with normal insulin sensitivity. They discovered that those nanovesicles have a property to decrease insulin-stimulated migration and glucose uptake in skeletal muscle cells collected from healthy participants [31]. However, the exact pathophysiological mechanism of GDM is still not fully elucidated. The molecular aspects of this condition are somehow related to mechanisms of T2D - the relative deficiency in insulin secretion and increased insulin resistance. Supporting these opinions, it was noted that exosomes isolated from samples of maternal plasma from peripheral blood and uterine vein presented an expression of dipeptidyl peptidase IV (DPPIV). In comparison to normal pregnancies, the expression of DPPIV in exosomes isolated from plasma of patients with GDM was increased 8.58-fold [32]. DPPIV is an enzyme known for its capacity to inactivate the biological functions of incretins, such as glucagon-like peptide-1 (GLP-1), and as a consequence reduce the release of insulin and promote the state of hyperglycemia in T2D. These findings open a perspective on the application of the inhibitors of DPPIV, such as vildagliptin, in the causative treatment of GDM.

It has been found that placental exosomes isolated from the urine of patients with GDM carried significantly different amounts of several various microRNAs (miR-222-3p, miR-16-5p, miR-516-5p, miR-517-3p and miR-518-5p) compared with healthy controls [33]. Additionally, the early pregnancy serum expression of microRNAs (miR-122-5p; miR-132-3p; miR-1323; miR-136-5p; miR-182-3p; miR-210-3p; miR-29a-3p; miR-29b-3p; miR-342-3p, and miR-520h) was significantly increased in GDM patients compared with controls [17]. Analyzed particles were identified as potential regulators of the expression of genes involved in insulin signaling, glucose transport, and synthesis of fatty acids, and as a consequence may contribute to the increased risk of developing gestational diabetes [33,34]. Moreover, based on the results of the studies performed on the population of human umbilical vein endothelial cells (HUVECs) using exosomes obtained from HUVECs in patients with GDM and normal controls, we could speculate that those particles could significantly contribute to the explanation of the mechanisms of fetoplacental endothelial dysfunction [21].

Future perspectives

It is likely that after appropriate validation, the measurement of fractions of total and placental exosomes in maternal plasma, urine, or gingival crevicular fluid could be regarded as a reliable 1st-trimester laboratory marker in the prediction of the risk of developing gestational diabetes in the no-risk group later in pregnancy. Possible application of these findings in clinical practice could be limited by the total costs of isolation of exosomes and their following preparation. The common implementation of exosomal measurements in various medical conditions will contribute to the reduction of unitary costs and could encourage laboratories to perform those analyses. Notably, the most important factor(s) in the light of these considerations is quite challenging to grasp – we need to know how to effectively proceed once reaching a potential result of “high risk of developing gestational diabetes”. It must be assessed to what extent the early implementation of proper diet or drugs could reduce that relative risk. We do already know about a few demographic and anthropometric risk factors of GDM. It could be beneficial for the patients if we could predict the probability of developing GDM in a group of patients at no perceived risk, for instance those with normal body mass index and negative family history. Finally, the potential added value of this procedure will depend mainly on the conscientiousness of the patients and their willingness to adhere to medical recommendations. Furthermore, it was established that extracellular vesicles are loaded with an enormous number and variety of molecules. There is a high probability that some of them may also be involved in the pathogenesis of GDM. This knowledge can be taken into consideration during the implementation of such new treatment strategies.

Conclusions

Using advanced laboratory techniques, exosomes can be relatively easily isolated from various tissues and body fluids. There are relatively few studies focusing on exosomes for the purpose of testing for gestational diabetes. Nevertheless, we can note the initial conclusions and highlight future perspectives. The utility of measurement of exosomes concentrations in every pregnant woman as a 1st-trimester screening tool for GDM seems promising but
requires further validation. Moving forward, studies with a higher number of participants may greatly contribute to the understanding of the molecular aspects of GDM and prompt us towards novel therapeutic targets.

Ethical approval

The conducted research is not related to either human or animal use.

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Corresponding author

Ewa Wender-Ożegowska, Department of Reproduction, Chair of Obstetrics, Gynecology, and Gynecologic Oncology, Poznań University of Medical Sciences, 33 Polna St, 60-535 Poznan, Poland, e-mail: ewawozegow@ump.edu.pl.

Conflict of interest statement

The authors declare they have no conflict of interest.
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