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New approaches in the diagnosis and prognosis of gestational diabetes mellitus

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Abstract. – Gestational diabetes mellitus (GDM) is the most common pregnancy metabolic disorder in which a person with no history of hyperglycemia exhibits any degree of impaired glucose tolerance during gestation. GDM can be resolved on its own after birth, but mothers with GDM are more at risk for future problems, such as type 2 diabetes, obesity, and cardiovascular disease. In addition, GDM can cause macrosomia in infants and obesity or even the risk of diabetes in childhood. Standard diagnostic tests for GDM are the oral glucose tolerance test (OGTT) and glucose challenge test (GCT), which is a mandatory test at 28-28 weeks of pregnancy in most countries. Disorders in various molecular mechanisms, such as hepatocyte growth factor (HGF), mechanistic target of rapamycin (mTOR), and nuclear factor-kappaB (NF-κB) signaling pathways are involved in GDM. Therefore, a better understanding of these mechanisms can help find new therapeutic and diagnostic strategies accordingly. In this review, we first deal with molecular mechanisms involved in GDM occurrence and then summarized the studies that hired this knowledge for early diagnosis and prognosis of GDM. Finally, we present the latest achievements in the diagnosis of GDM based on exosomes, microRNAs, glycosylated hemoglobin, and inflammatory factors detection in maternal circulation.

Key Words:
Gestational diabetes mellitus, Exosome, miRNA, HbA1c, Inflammatory factors.

Introduction

Gestational diabetes (GDM) is a pregnancy disorder characterized by increased blood sugar during pregnancy because of the decrease in the sensitivity of maternal cells to insulin[1]. The mass of pancreatic β cells increases in normal pregnancies to compensate for this decrease in insulin sensitivity. Some complex metabolic changes can cause GDM by inhibiting this increase in β-cell mass, which makes insulin insufficient to regulate blood glucose. Although GDM usually resolves after birth, some mothers may develop type 2 diabetes, obesity, and cardiovascular disease in later years. Furthermore, because excess maternal blood glucose passes through the placenta and is stored as fetal body fat, these infants are predominantly macrosomia (birth weight of more than 4 kg), which causes birth complications and risk of neonatal infection[1]. There are also pieces of evidence showing that children who had GDM mothers are more prevalently susceptible to childhood obesity and diabetes[2]. Risk factors for GDM are not well understood, but women with advanced gestational age, obesity or overweight, and a family history of diabetes or previous history of GDM are high-risk groups. Therefore, the incidence of GDM has increased with the increase in female obesity rates and mild cases can be resolved by modifying diet and exercise, but in severe cases requires medical interventions, such as insulin injections.

Traditional diagnostic tests for GDM are fasting glucose concentration tests followed by an oral glucose tolerance test (OGTT) or a glucose challenge test (GCT), which is widely performed at 24-28 weeks of pregnancy for all women worldwide. Diagnostic criteria of GDM include one or more of the following results: fasting plasma glucose of 92-125 mg/dL followed by an OGTT (75 g oral glucose load) and plasma glucose of >180 mg/dL after 1 hour or greater than 153-199 mg/dL after 2 hours[3].

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Also, these tests and many others are used to determine GDM and various diagnostic criteria have been defined to cover all cases, yet many efforts have been made by scientists to find safer and newer methods for the prognosis and early diagnosis of GDM. This review attempts to summarize the most achievements of these efforts and to outline what the future of GDM diagnosis will be based on.

Molecular Mechanisms Involved in GDM

Dysfunction of pancreatic β-cell and resistance of target cells to insulin are the main causes of hyperglycemia in all types of diabetes. GDM can be caused by several complex events and physiological changes necessary to provide nutrients to the developing fetus and the hormones needed to maintain the pregnancy. Hormones produced by the placenta, such as estrogen, cortisol, and human placental lactogen (hPL), have an insulin-blocking effect to increase circulating glucose in the mother by inducing insulin resistance. This extra glucose is not only needed for the growing fetus but also provides the energy needed for the metabolic changes of the placenta[4].

In the second and third trimesters of pregnancy, the sensitivity of maternal cells to insulin decreases, and blood glucose increases relatively, so compensatory insulin secretion increases. These events help maintain glucose homeostasis by shifting maternal metabolism to produce energy from lipids instead of carbohydrates, thus saving glucose for fetal consumption[5]. The increase in insulin secretion is the result of the growth of pancreatic β-cell mass, which returns to a normal level within 10 days after birth through the increase of β-cell apoptosis. This growth in β-cell mass is critical for normal glucose tolerance during pregnancy, and if disrupted, β-cell compensation may fail, leading to GDM[6]. An *in-vivo* study[7] showed that hepatocyte growth factor (HGF) as a mitogenic, anti-apoptotic, and insulinotropic agent for β-cells plays an important role in the expansion of β-cell mass. The authors showed that loss of c-Met (HGF receptor) in the pancreas reduces maternal β-cell proliferation, leading to reduced β-cell mass expansion, hypo-insulinemia, hyperglycemia, and glucose intolerance in pregnant mice. Another study by Liu et al[8] investigated the molecular mechanism involved in the effect of high blood glucose on apoptosis and inflammatory response in pancreatic β-cells in GDM. The results of this study showed that the activation of TGFβ-activated kinase 1 (TAK1) was increased in these cells as a result of miR-143-3p downregulation. Activated TAK1 can ultimately activate the inflammatory transcription factor nuclear factor-kappaB (NF-κB). This activated NF-κB then promotes transcription of genes associated with pathways controlling inflammatory responses, cell proliferation, and apoptosis, and impairs β-cell survival and function[8]. Reduction of Peroxisome proliferator-activated receptor alpha (PPARα) expression in GDM is another mechanism to reduce β-cells mass by activating the inflammatory pathways caused by NF-κB[9].

In addition to the change of factors in maternal circulation, abnormal expression of some placental factors can also lead to GDM. The mechanistic target of rapamycin (mTOR) pathway is responsible for the availability of nutrients and growth factors that regulate cell growth in the placenta during pregnancy. Overactivation of the mTOR pathway in trophoblasts (a cell population in the placenta) is associated with the development of GDM[10]. Up-regulation of the placental mTOR signaling pathway along with the activation of placental insulin-like growth factor-I (IGF-I) can also increase the expression of amino acid transporters in women with GDM, which is the reason for the birth of macrosomic infants[11]. There is evidence[12] suggesting that maternal overweight leads to the upregulation of placental mTOR activity and nutrient transport, resulting in the overgrowth of the fetus.

Leptin is another GDM-related factor that produces both mother adipose tissue and placenta trophoblastic cells. Leptin is a peptide hormone that is primarily produced by fat cells and plays an important role in the regulation of energy homeostasis, glucose metabolism, and body weight. It acts on the hypothalamus to suppress appetite and increase energy expenditure, thus maintaining body weight. During pregnancy, there is an increase in maternal fat mass, resulting in higher leptin production. However, despite the increase in leptin levels, pregnant women with GDM often resist its effects due to a decrease in the sensitivity of target tissues to this hormone[13]. This ultimately leads to an inability to adequately regulate energy balance and appetite suppression, as well as the secretion of compensatory doses of hormones. The abnormal expression of leptin by the placenta also causes an increase in the size of the placenta and fetus and, finally, macrosomia.

Leptin is an inducer of protein synthesis, a key event that causes GDM. Also, leptin can facilitate the transport of nutrients to the fetus in cases of GDM by increasing the expression of the glycerol transporter aquaporin-9. Research[14,15] has shown that plasma leptin levels are significantly higher in GDM women compared to non-GDM women. High placental leptin production can be one of the reasons for this difference. Another reason is the high maternal fat mass in obese women, which secretes more leptin, and this amount of plasma leptin can eventually cause insulin resistance associated with GDM in obese mothers[13].

Another hypothesis regarding the relationship between leptin and diabetic hyperglycemia during pregnancy suggests that high glucose levels affect the epigenetic profile of the leptin gene by inducing DNA methylation. This hypothesis suggests that leptin promoter's DNA methylation can regulate placental leptin gene expression and is associated with GDM incidence[16,17]. Thus, leptin may have crucial influences on the development and prognosis of GDM.

Overall, insulin resistance during pregnancy, which causes the mother's body to need more insulin to maintain normal blood sugar levels, and the inefficiency of the pancreas to produce this excess insulin, pregnancy hormonal changes which can interfere with insulin signaling and contribute to insulin resistance, inflammatory factors that play a role in impairing insulin signaling and promoting insulin resistance are the most important mechanisms of the development of GDM.

New Approaches for Diagnosis and Prognosis of Gestational Diabetes Mellitus

Prognosis and early diagnosis of GDM in the first weeks of pregnancy are not possible without finding new biomarkers that appear in the mother's bloodstream at the beginning of pregnancy. In this regard, many studies in the literature have attempted to find new biomarkers to improve the current diagnostic practices performed in the clinic for the diagnosis and screening of GDM.

Exosomes, miRNAs, glycosylated hemoglobin, and inflammatory factors are some of the candidates that have been considered in many papers as potential biomarkers for GDM prognosis and early detection. In this section, we collected some of the most valuable findings of these studies.

Exosome

Using circulating exosomes for early diagnosis of GDM has many advantages, as they are safe and accessible particles secret from both placenta and mother cells and reveal valuable information about various factors within these cells. Exosomes contain cytoplasmic proteins, inflammatory factors such as cytokines, membrane proteins such as receptors, lipids, and various coding and non-coding RNAs (ncRNAs) derived from their cell of origin. Studying the content of circulating exosomes in GDM cases can not only help decipher the underlying mechanisms of GDM complications but is also considered a potential source of new biomarkers for early diagnosis of GDM. The concentration of circulating exosomes, especially placenta-derived exosomes, increases during pregnancy. Interestingly, this increase is significantly higher in GDM cases compared to normal pregnancies[18]. In this regard, Rice et al[19] designed a study in which primary first-trimester trophoblast cells were isolated from the placenta and incubated with D-glucose (25 mM). They showed that glucose significantly increased the release of exosomes from trophoblast cells, especially under hypoxic conditions.

The protein content of exosomes in GDM is mainly associated with metabolic pathways, energy-releasing pathways, and glucose and insulin-related proteins. These proteins also can include growth factors, cytokines, enzymes, and signaling molecules. The protein content of placental-derived exosomes in GDM is mainly associated with energy production, inflammation, and metabolism[20].

In addition, another study by Bernea et al[21] compared the protein content of exosomes isolated from maternal blood samples of women with GDM and the control group. The proteomics analysis revealed 78 significantly altered proteins, related to complement and coagulation cascades, platelet activation, prothrombotic factors and cholesterol metabolism[21].

One of these proteins is pregnancy-associated plasma protein-A (PAPP-A) secret, specifically in the placenta, and has lower concentrations in women who develop GDM and gestational hypertension, which is caused by changes in insulin sensitivity[22]. Calcium-calmodulin (CaM)-dependent protein kinase II (CaMKII) is another insulin-related protein that reduces insulin-stimulated glucose uptake through the Akt and ERK signaling pathways[23]. Jayabalan et al[20] showed that these two proteins' exosomal concentration has

the potential to be GDM biomarkers as PAPP-A downregulates and CaMKIIβ upregulates in the exosomes isolated from GDM pregnancies. Another study[24] conducted by this team compared the total protein content of exosomes derived from maternal adipose tissue in normal and GDM pregnancies. They showed that proteins related to mitochondrial function and signaling pathways targeting SIRT, oxidative phosphorylation (OXPHOS), and EIF2 were differentially expressed in exosomes derived from adipose tissue of GDM and normal pregnant women, which may play a role in the pathophysiology of GDM. They also found that proteins that target mTOR, eIF4, and p70S6K, as well as the SIRT and EIF2 signaling pathways, are differentially expressed in GDM exosomes. These findings[24] indicate that adipose tissue-derived exosomes of GDM women carry a specific set of proteins associated with glucose and gluconeogenesis metabolism.

The ncRNA content of exosomes is one of the most interesting topics for scientists to obtain new GDM biomarkers. Many microRNAs (miRNAs) have a higher abundance in placental exosomes of GDM pregnant women than in normal pregnant women, such as miR-518 family, miR-525-5p, and miR-520c-3p[25]. In a cross-sectional study[26], a total of 764 miRNAs were evaluated to investigate the diagnostic value of aberrant expression of placental and circulating exosomal miRNAs for GDM. The results showed that there are 114 up-regulated miRNAs and 43 down-regulated miRNAs in the placental tissue of GDM patients, whose expression patterns were analyzed in peripheral blood exosomes. Among them, decreased expression of circulating exosomal miRNA-125b was significantly associated with GDM. Also, the expression of miRNA-144 in GDM plasma exosomes increased significantly during the third trimester. In conclusion, this study[26] introduced these two miRNAs as potential novel biomarkers of GDM, which are also involved in the pathogenesis of GDM. Gao et al[27] isolated placental mesenchymal stem cell-derived exosomes from GDM pregnant women and measured miR-130b-3p content in these exosomes. The team found that this miRNA was highly expressed in exosomes derived from GDM-MSCs-derived exosomes.

Huang et al[28] used umbilical cord blood to isolate the exosomes from GDM patients and revealed that the expression of circular RNA circ_0074673 was upregulated in these pregnant women compared to normal pregnant women. They also showed that the knockdown of circ_0074673 can reduce the advance of GDM by sponging miR-1200.

To obtain a sample available in large quantities, with non-invasive collection methods, Herrera-Van Oostdam et al[29] purified placental exosomes from the urine of patients with GDM to evaluate their miRNA content as a potential biomarker[29]. Placental exosomes were isolated from urine in the first, second, and third trimesters of pregnancy and the concentration of chromosome 19 miRNA cluster (C19MC), including miR-516b-5p, miR-517-5p, and miR-518a-3p, as well as miR-222-3p and miR-16-5p were determined. The results of this study showed that miR-16-5p was detectable in the second trimester only in exosomes of GDM patients, while it was undetectable in healthy pregnant women. C19MC miRNAs were also differentially expressed in women with GDM compared to controls, which was detected with an increase in the second trimester and then a decrease in the third trimester. The team[29] suggests that urinary exosomes could be a noted source for biomarker screening strategies in the second trimester, and in this respect, miR-16-5p, miR-518-5p, and miR-517-3p have shown an acceptable diagnostic value. Overall, exosomes have attracted much attention as newly recognized participants in the development of GDM with great potential to become a biomarker, and studies in this direction are increasing.

MiRNAs

Nowadays, many studies have been done to determine the selective expression of miRNA in maternal circulation and its relationship with GDM conditions. Advances in current skills in nucleic acid amplification, sequencing, and analysis have made it possible to identify circulating miRNAs released from the mother or placenta in GDM conditions, to achieve novel biomarkers for diagnosis of this disturbance. To protect miRNAs against ribonucleases, in addition to encapsulating them in exosomes, they can bind to protein complexes to prevent their digestion in body fluids. Therefore, evaluating circulating miRNAs can be considered a separate approach from the isolation of miRNAs from exosomes.

A meta-analysis[30] investigated the diagnostic potential of circulating miRNAs in detecting GDM patients. By analyzing the results of twelve studies in this regard, the team suggested that miRNAs have a good capacity to become new

biomarkers for GDM, and their sensitivity for diagnosing GDM is 74.5%, while their specificity is 84.1%. To obtain an early diagnosis of GDM, Yoffe et al[31] investigated the frequency of circulating miRNAs in the plasma of pregnant women during their first trimester. They used several types of multivariable machine learning models to assess the prognostic value of differential miRNA expression in GDM *vs.* healthy pregnant women. They suggested that two upregulated circulating miRNAs (miR-223 and miR-23a) have the potential to be good GDM biomarkers in the first trimester. Other circulating miRNAs that have prognostic value in pregnant women at risk or with GDM and are overexpressed before 20 weeks of gestation are miR-17-5p, miR-210-3p, miR-16-5p, miR-342-3p, and miR-20a-5p, while miR-222 is downregulated. Circulating levels of miR-21-3p and miR-155-5p increase even faster and are detectable at 16 weeks of gestation, which is associated with a higher chance of GDM[32]. To detect which miRNAs can predict the occurrence of GDM in the second trimester of gestation, Légaré et al[33] examined maternal circulating miRNAs in the first trimester (before the 16th week) of pregnancy and followed the incidence rate of insulin resistance in these cases at the 24-29 weeks. They showed that 18 of the first-trimester miRNAs (most belonging to the C19MC) could predict insulin resistance in the late second trimester of pregnancy. Another study[34] systematically evaluated sixteen articles from GDM patients in 12 countries. They reported that among the 135 unique miRNAs described in these papers which were related to GDM, 8 miRNAs (miR-29a-3p, miR-20a-5p, miR-195-5p, miR-16-5p, miR-17-5p, miR-222-3p, miR-342-3p, and miR-210-3p) in two or more studies described. Among these, the upregulation of miR-16-5p was described in 4 studies, and the upregulation of miR-20a-5p, miR-17-5p, and miR-342-3p was described in 3 studies, which seem to be better candidates for more evaluations. The results of other studies in this field are summarized in Table I.

Table I. miRNAs that have diagnostic value in gestational diabetes mellitus.

MiRNA	Detection time in circulation	Circulation level compare to normal pregnancy	Other findings	Detection method	Reference
MiR-330-3p	Third trimester	Increased	Associated with insulin-secretory defects	RT-PCR	35
MiR-16-5p, -29a-3p and -134-5p	Before 20 weeks of gestation	Increased	—	RT-PCR	36
MicroRNA-16-5p, -17-5p and -20a-5p	24-28 weeks of gestation	Increased	Correlated with insulin resistance	RT-PCR	37
MiRNA-340	24-32 weeks of gestation	Decreased	Insulin can epigenetically induce miRNA-340 expression	RT-PCR	38
MiR-20a-5p and miR-222-3p	13-31 weeks of gestation	Decreased	MiR-20a-5p controls various metabolic pathways, including insulin signaling	MiScript® miRNA PCR arrays	39
MiR-222	38-39 weeks of gestation	Increased	MiR-222 is a potential regulator of estrogen receptor α expression in estrogen-induced insulin resistance in GDM	AFFX miRNA expression chips, and RT-PCR	40
MiR-223	Second or third trimester	Increased	Angiopoietin-Like Protein 8 (ANGPTL8) also increased in GDM cases	RT-PCR	41

Overall, miRNAs can be used as potential biomarkers for predicting the risk of GDM and its complications. Additionally, miRNAs can also be used to monitor the response to treatment and predict the long-term outcomes of GDM. However, further research is needed to validate these findings and develop miRNA-based diagnostic and prognostic tools for GDM.

Glycated Hemoglobin

Glycosylated hemoglobin A1c (HbA1c), which results from the spontaneous binding of glucose to the β-chain of hemoglobin, is increased in various types of diabetes in the circulation, indicating a high concentration of glucose during the two to three previous months. The use of HbA1c as a diagnostic factor for diabetes has many advantages, such as no need for overnight fasting, availability for measurement at any time of the day, less inter-individual variability, more reproducibility, and better analytical stability than blood glucose. It also has more advantages for diagnosing GDM because it is more convenient for pregnant women than taking the 75-100 g of glucose required for glucose tolerance tests. Today, HbA1c is mainly used to screen for type 2 diabetes, but its potential to diagnose GDM has been explored in the literature. A paper[42] published in 2020 systematically reviewed all studies from 2000 to 2019 on the performance of HbA1c in the early diagnosis of GDM. This article included studies that measured HbA1c at 20 weeks of gestation or less with no history of previous diabetes and examined HbA1c levels below 6.5%. By examining the results of all 10 studies included in this systematic review, they reported a positive relationship between higher HbA1c values in early pregnancy below 6.5% and the development of GDM. The risk of developing GDM increases with HbA1c ≥5.7%, and values ≥6.0% identify almost all patients who subsequently develop GDM. The findings also showed that HbA1c above 5.9% in early pregnancy was associated with an at least two-fold increase in GDM-related risks, such as preeclampsia, congenital anomalies, shoulder dystocia, and perinatal death. After identifying patients with HbA1c within these ranges, these articles recommend an early 75 g oral glucose tolerance test for early diagnosis of GDM. After that, in another related[43] study, the accuracy of HbA1c in the diagnosis of GDM was evaluated in comparison with 100 g OGTT. They included 114 pregnant women at 24 weeks or more of gestation with an abnormal GCT of 50 g and measured HbA1c simultaneously with a 100 g OGTT. The results showed that although HbA1c is higher in women with GDM than women without GDM, HbA1c values cannot replace 100 g OGTT to diagnose GDM. They suggested that using two cut-off values (<4.5% and >5.8%), only women with HbA1c values between 4.5-5.8% needed further OGTT to confirm the diagnosis of GDM. Likewise, a prospective study[44] was conducted on 700 Iranian pregnant women from March 2018 to March 2020 to evaluate the use of HbA1c in the early diagnosis of GDM. The results obtained from this study similarly showed that the first-trimester HbA1c cannot replace OGTT for the detection of GDM cases due to its inadequate specificity and sensitivity, but women with higher HbA1c in the first trimester had a higher risk of developing GDM.

To determine the relationship between HbA1c levels and fetal abnormalities in women with GDM, Al-Shwyiat and Radwan[45] reported monthly HbA1c in 157 pregnant women with GDM. Fetal abnormalities, in the order of more occurrence, included cardiovascular, skeletal-muscular, genitourinary system, digestive system, face, and central nervous system, which were recorded in 8.6% of the studied women with GDM. The results of this study showed that the value of HbA1c ≥6.5 in the first trimester significantly increases the relative risk and probability of these abnormalities[45].

Furthermore, a study by Muhuza et al[46] evaluated the association between HbA1c level and pregnancy adverse outcomes among 2,048 women with GDM. The results showed that there is a solid association between HbA1c and these adverse outcomes at the time of GDM diagnosis (24 to 28 weeks). Pregnant women with HbA1c greater than 5.5% had a higher risk of adverse events compared with pregnant women with HbA1c between 5.1-5.4% and especially less than 5.0%. Based on these findings, the team suggests that HbA1c may be an adverse pregnancy outcome-predicting biomarker in GDM women[46].

Inflammatory Factors

As we mentioned earlier, increased inflammatory factors, such as leptin and tumor necrosis factor alpha (TNF-α), contribute greatly to insulin resistance and unwanted consequences of GDM. Evaluating the amount of these circulating factors also has the potential to predict and diagnose GDM. In this section, we summarize the results of studies that have addressed this issue.

Adipokines are a group of bioactive molecules secreted by adipose tissue, which play a crucial

role in regulating various physiological processes, including metabolism, inflammation, and immunity. Some adipokines, such as adiponectin and leptin, are cell signaling proteins secreted by adipocytes that stimulate several inflammatory processes, and their potential in early detection of GDM has been reported. To determine the predictive value of adiponectin, leptin, and CMPF (3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid) circulation levels and the adipokines/leptin ratio in the early diagnosis of GDM, Florian et al[47] prospectively studied 68 Caucasian pregnant women between 11 and 13 weeks of gestation. Of this population, 21 individuals developed GDM in whom leptin levels were significantly higher (above the cutoff value of 16 ng/ml) than the rest, with a sensitivity of 100% and a specificity of 48.9% for predicting GDM. They also reported that the ratio of adipokine/leptin was significantly lower in GDM cases, while adiponectin and CMPF levels were not associated with GDM. This study showed that the levels of leptin and the ratio of adipokines/leptin, along with the advanced maternal age, are predictive factors for the development of GDM in the first trimester of pregnancy.

Two other candidate adipokines for the diagnosis and prognosis of GDM are nesfatin-1 and vaspin. Nesfatin-1 is a food intake regulator adipokine that is secreted from several regions of the hypothalamus and affects feelings of hunger and satiety. Nesfatin-1 plays a key role in glucose homeostasis and is involved in the pathogenesis of type 2 diabetes[48]. Vaspin is mainly expressed in adipose tissue, but its expression is also observed in the placenta, and its concentration gradually increases during pregnancy and reaches a maximum at the end of pregnancy[49]. To determine the predictive value of serum levels of these adipokines, Mierzyński et al[50] measured their concentrations in 153 women with GDM. The results showed that serum levels of both nesfatin-1 (5.15±3.51 *vs.* 6.69±4.21 ng/mL) and vaspin (0.49±0.24 *vs.* 0.83±0.27 ng/mL) were significantly inferior in the GDM group compared to the healthy pregnant women with similar BMI. The analysis showed that serum vaspin levels could predict approximately 40% of GDM cases, while serum nesfatin-1 levels could predict only 13%. Nevertheless, this group suggests that serum levels of nesfatin-1 and vaspin have the potential to be novel predictive biomarkers for the early detection of GDM.

Interleukin-6 (IL-6) is a cytokine produced by adipose and endothelial cells and affects glucose metabolism by increasing insulin secretion from pancreatic beta cells[51]. IL-6 has been well-studied as another inflammatory factor that has the potential to be a marker for the diagnosis of GDM. Amirian et al[52] addressed this issue and systematically evaluated the results of articles between 2009 and 2020 to determine the value of IL-6 as a potential GDM biomarker[52]. Among the 24 articles they evaluated, 16 reported an association between elevated IL-6 serum levels and GDM, while 8 studies reported no association. Overall, they concluded that the evaluation of IL-6 serum level can be considered as a diagnostic biomarker for GDM.

New Detection Methods for GDM Diagnosis

Although all the diagnostic and prognostic biomarkers introduced in this article have a bright future in the early diagnosis of GDM, the use of these biological products for this purpose requires the development of methods that can easily detect them in the bloodstream. Today, the discovery of new biomarkers simultaneous with detection techniques is growing to increase the possibility of using these markers in the clinic. Considering this issue, in this section, we take a look at the new developments in the detection methods of exosomes, miRNAs, and HbA1c that have been reported in recent articles[53-55].

As we mentioned earlier, the detection of placental exosomes, which are detectable in the maternal circulation as early as the sixth week of pregnancy, is one of the most useful methods for diagnosing GDM. Placental-type alkaline phosphatase (PLAP) is a reliable protein marker for exosomes of placental origin, and many studies[53-55] have used PLAP antibodies to isolate these exosomes by flow cytometry or fluorescence-based nanoparticle tracking analysis. Nanoparticle tracking analysis separates particles around 100 nm in size, and the combination of this method with a fluorescent dye makes it detectable by flow cytometry. A PLAP antibody and flow cytometer-based method for isolating placental exosomes is the single extracellular vesicle (SEV) analysis. Li et al[56] used this technique to analyze the distribution pattern of placental exosomes in maternal circulation by PLAP antibody. In this method, after the isolation of all plasma exosomes, these exosomes are labeled by PLAP antibody, and after washing the unlabeled exosomes, the labeled exosomes are collected by ultracentrifugation for

identification with the nano flow cytometer. Since the sensitivity of the nano flow cytometer is significantly higher than the ELISA, the SEV analysis method provides a more sensitive tool for the detection of circulating protein markers, such as PLAP. Therefore, this method can provide a more accurate detection of placental exosomes.

However, there is a need to improve antibody-free PLAP-based exosome isolation methods to advance the finding of placental exosome-based biomarkers of GDM. In this regard, Lai et al[57] developed an antibody-free method for PLAP quantification with a mass spectrometry-based proteomics method. In this workflow, which was introduced as Multiple Reaction Monitoring High Resolution (MRMHR), a tandem mass spectrometer was used to refine peptides that uniquely correspond to PLAP. The resulting data were processed using Skyline software for the TripleTOF 5600 system. This new and unique placental exosome isolation method could pave the way for exosome-based diagnostic methods for GDM.

Another advanced method in this regard was introduced by Gebara et al[58], who studied in depth the characteristics of exosomes in term amniotic fluid using several orthogonal techniques, including chip-based platform, cytofluorimetric bead-based multiplex assay, and super-resolution microscopy analysis. In this study, exosomes were purified from amniotic fluid by sequential centrifugation and size exclusion chromatography and characterized based on their size using nanoparticle tracking analysis. Then, they used a specific kit (MACSPlex) capable of semi-quantitative fluorescent analysis of 39 different exosome surface markers. In addition, super-resolution microscopy was used to analyze the size and expression of some of these markers at a single-molecule level in separated exosomes. With these advanced techniques, they identified the multiple origins of each exosome of amniotic fluid, such as placental tissues, fetal urine, and stem cells[58]. Using such a technique in the early stages of pregnancy can be a useful way to track and identify exosome-based GDM markers in the maternal bloodstream.

Advances in the development of sensitive technologies based on the amplification and identification of miRNAs in blood have made it possible to find the association of these nucleic acids with human diseases. Techniques such as TaqMan array microfluidic cards[59], next-generation sequencing (NGS)[60], liquid biopsy[61], and microarray[62] are expanding day by day and have made it possible to detect circulating GDM-related miRNAs more easily. However, the use of these methods is limited to research laboratories, and making them available for easy use in diagnostic laboratories and clinics in the future requires more effort.

HbA1c is another promising biomarker of GDM, as we mentioned before. There are also some advanced methods for the accurate detection of HbA1c in blood serum, such as Fluorescent-Based Lateral Flow Immunoassay (LFIAs) for HbA1c detection[63]. Whereas Roche COBAS is a well-known method that has been performed in many HbA1c studies[64-66] in GDM, the lateral flow immunoassays (LFIAs) is a novel rapid test for diabetes screening, which has the potential to get the attention of GDM in future studies.

There are also many other new methods that need more studies to consider for GDM diagnosis in the future. Some of these methods are Non-Invasive Prenatal Testing (NIPT), Continuous Glucose Monitoring (CGM), and Artificial Intelligence (AI) technology. CGM is a new approach that involves the use of a small sensor that is placed under the skin to measure glucose levels continuously. This approach provides real-time data on glucose levels, which can help in the diagnosis and management of GDM[67]. NIPT is a new approach that involves analyzing fetal DNA in maternal blood samples to detect genetic abnormalities and other conditions, including GDM[68]. AI technology is being used to develop predictive models for GDM. These models use data from electronic health records, medical imaging, and other sources to predict the risk of developing GDM and its complications[69]. Despite that, our knowledge of these techniques is limited due to the small number of studies and the high technology and devices required to use these methods, but they could be the future of GDM diagnosis.

Conclusions

GDM is a recognized pregnancy problem with high levels of glucose in the mother's circulation, which can be detected by OGTT and GCT tests at 24-28 weeks of pregnancy. During pregnancy, the sensitivity of the mother's cells to insulin decreases so that more glucose is available to the fetus. However, insulin production in normal pregnancy is compensated to some extent by increased proliferation of pancreatic beta cells. GDM occurs due to the inability of maternal pancreatic β cells to secrete excess insulin, which can be due to mu-

tations in HGF, a critical growth factor for β-cells and its receptor c-Met, or increased inflammatory factors, such as NF-κB, which cause the disorder in the survival and proliferation of β cells. Since early diagnosis and prognosis of GDM, with tests that are more convenient for pregnant women, are necessary to prevent subsequent adverse consequences, many studies have attempted to find novel biomarkers in maternal circulation during the first trimester of pregnancy. Exosomes are one of these factors that are secreted into the blood by placental and maternal cells. PAPP-A and PLAP are some placenta-derived exosome markers that can help identify the origin of these exosomes. Circulating miRNAs are likewise promising makers for early diagnosis of GDM. Despite this, miRNAs, such as C19MC, miR-17-5p, miR-20a-5p, miR-21-3p, miR-23a, miR-155-5p, miR-210-3p, miR-223, and miR-342-3p have been detected in the first trimester of pregnancy in pregnant women who developed GDM, but more studies are needed to find the best miRNA marker in this regard. HbA1c and inflammatory factors are the other potential biomarkers for early diagnosis of GDM. However, early diagnosis of GDM based on novel biomarkers need the achievement of advanced detection method due to the low rate of these markers in circulation in the beginning weeks of pregnancy. Techniques, such as SEV analysis, nanoparticle tracking analysis, mass spectrometry-based proteomics, and chip-based platforms have been developed to detect exosomes, especially placental exosomes, in maternal circulation. In addition, some miRNA detection techniques, such as microfluidic cards, NGS, liquid biopsy, and microarray, have been developed for more accurate detection of miRNAs in the bloodstream. However, the transfer of these methods to the clinic to help in the fast and easy diagnosis of GDM still requires a lot of effort to achieve.

Ethics Approval and Informed Consent
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ZG, QS, WG, GZ, YS, YT, MW , YG, WW and JC participated the conceptualization, writing—original draft preparation, designing preparing table, reviewing and editing. All authors read and approved final manuscript.

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