Review Article

The current and future role of laboratory medicine for diagnosing gestational diabetes mellitus

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Abstract: Gestational diabetes mellitus (GDM) is currently defined as any degree of glucose intolerance with onset or first recognition during pregnancy. Although this condition is typically diagnosed with evidence of abnormal plasma glucose values after glucose load, there is no international consensus regarding timing of screening and the optimal cut-off points. Some epigenetic alterations, such as DNA methylation and small non-coding RNAs, seem to play a key role in the pathogenesis of disorders of glucose metabolism, and it has also been hypothesized that epigenetic signatures could even find an important place in GDM diagnostics. In a foreseeable future, it is hence conceivable that the assessment of some predictive epigenetic biomarkers may allow early identification of women at enhanced risk of developing pregnancy complications, even before glucose metabolism is significantly impaired. Therefore, this article provides a brief update on screening/diagnostic approaches currently used in clinical practice, an overview of important studies in which epigenetic mechanisms have been associated with the pathogenesis of GDM and, finally, the emerging diagnostic contribution of these epigenetic biomarkers.

Keywords: Biomarkers; circular RNAs (circRNAs); diagnosis; epigenetics; gestational diabetes mellitus (GDM); methylation; microRNAs (miRNAs)

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Introduction

Gestational diabetes mellitus (GDM) is a complication of pregnancy, defined as the "diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation" (1). The prevalence of this condition approximates 14% of worldwide pregnancies, but varies widely from 1% to 28% depending on population characteristics (e.g., maternal age, socioeconomic status, race/ethnicity and body composition) and diagnostic criteria (2-4). Caucasian women are at lower risk of developing GDM compared to Native Americans, Hispanics, Asians, and African-American women (5), though the prevalence significantly increases in women with predisposing conditions or risk factors. In a large European multicenter study based on a cohort of women with body mass index (BMI) ≥29 kg/m^2, the prevalence of GDM was found to approximate 40% using the International Association of Diabetes and Pregnancy Study Groups (IADPSG)/World Health Organization (WHO) 2013 diagnostic criteria (6).

The pathophysiology of GDM is characterized by progressive development of insulin resistance, mainly triggered by placental production of diabetogenic hormones such as estrogen, progesterone, leptin, cortisol, placental lactogen and placental growth hormone (7). GDM develops in predisposed pregnant women in whom the adaptation β-cell hyper-functionality fails to compensate maternal insulin resistance (8). Risk factors for GDM include classical

risk factors for metabolic as well as for cardiovascular diseases (CVDs) (i.e., overweight/obesity, advanced age, gestational weight gain, westernized diet, a family history of insulin resistance and/or diabetes) (9,10), but also other non-typical risk factors, such as ABO blood group (11) or genetic (12,13) and autoimmune conditions (14).

Although GDM typically resolves with and after delivery, early diagnosis and consequent management (i.e., lifestyle changes followed by oral blood-glucose-lowering agents or insulin, if necessary) are essential for lowering the maternal risk of developing type 2 diabetes mellitus (T2DM) (15), fatty liver disease (16), metabolic syndrome (17) and CVD (18), along with other short-term and long-term complications for the offspring (i.e., high birth weight, congenital malformations, intrauterine growth restriction and preterm birth, respiratory distress syndrome, and so on) (19,20). In keeping with this evidence, the Hyperglycemia and Adverse Pregnancy Outcome Follow-up Study (HAPO FUS) has recently confirmed that GDM is independently associated with childhood impaired glucose tolerance (IGT) (21).

The current screening/diagnostic approach

Several guidelines have been developed during the past decades, which differ for timing of oral glucose tolerance test (OGTT), population tested, number of samples analyzed, glucose load and glucose thresholds (22) (*Table 1*). Although universal agreement has not been reached, the most followed guidelines are those recommending one-step 75 g OGTT strategy, following the IADPSG criteria (i.e., to be performed between 24–28 gestational weeks) (36). This approach has been proposed after the publication of the HAPO study, including more than 23,000 pregnant women, which demonstrate that that complications for mother and offspring increases in parallel with maternal glycaemia at 24–28 weeks of gestation (37). According to IADPSG criteria, one value exceeding the following established cutoffs is sufficient for diagnosing GDM: 5.1 mmol/L (92 mg/dL) for fasting plasma glucose, 10 mmol/L (180 mg/dL) after 1 hour and 8.5 mmol/L (153 mg/dL) after 2 hours from the glucose intake (38). Since it has been demonstrated that selective screening based on traditional risk factors for GDM has a relatively low sensitivity (39), the screening approach has been extended to the entire population of pregnant women not only by the American Diabetes Association (ADA), but also by other international scientific organizations such as the WHO, the International Diabetes Federation (IDF) and the International Federation of Gynecology and Obstetrics (FIGO).

Unlike other countries which actually follow the recommendations of ADA, WHO, IDF and FIGO, the Italian National Health System guidelines in 2011 has limited the screening to women at risk of GDM rather than endorsing an approach based on universal screening (40). According to this Italian guideline, high risk women (i.e., previous GDM, pre-pregnancy BMI ≥30 kg/m^2, FPG 100–125 mg/dL in the first trimester of pregnancy) should be screened between the 16th and 18th gestational weeks, which screening repeated between the 24th and 28th gestational weeks in the presence of normal glucose tolerance, whilst in women with medium risk (i.e., age ≥35 years, pre-pregnancy BMI 25–29.9 kg/m^2, family history of T2DM, previous macrosomia and of an ethnic group at GDM risk) screening is only recommended between the 24th and 28th gestational weeks (40). Recently published studies showed that this approach is characterized by low sensitivity for detecting GDM, thus emphasizing the real need of a substantially critical revision (41,42).

Another aspect that merits consideration is the preanalytical quality of the samples, which include reliable conditions of storage, sample management and use of suitable glycolysis inhibitors (43-46). Screening and diagnostic tests can be performed more or less accurately, thus potentially increasing the number of false negatives (47).

Epigenetics changes as potential biomarkers of GDM

Epigenetics plays a substantial role in the pathogenesis of several conditions, as well as in disorders of glucose metabolism (48-51). It has also been hypothesized that some epigenetic mechanisms, including DNA methylation, histone modifications and small non-coding RNAs, could fill the knowledge gap between environmental factors (i.e., diet, pollution, stress, smoke and others) and heritable genetic susceptibility (52).

DNA methylation is a reversible process consisting of addition of a methyl group to the fifth carbon position of a cytosine residue within cytosine-phosphate-guanine (CpG) dinucleotides (a process catalyzed by the enzyme DNA methyltransferase), thus inhibiting gene transcription (53). Both aberrant global methylation and DNA methylation of specific genes involved in insulin resistance, for example in response to nutritional and environmental factors, have been

Table 1 Different strategies for screening/diagnosis of GDM

Year	References	Number of steps	Oral glucose load	Number of abnormal values required	Glucose cut-offs
1964	O'Sullivan *et al.* (23)	1	100 g	≥2	Fasting ≥2 SD above the mean
					1 h ≥2 SD above the mean
					2 h ≥2 SD above the mean
					3 h ≥2 SD above the mean
1973	O'Sullivan *et al.* (24)	2	First-step: 50 g; second-step: 100 g	≥2	Fasting ≥5.0 mmol/L (90 mg/dL)
					1 h ≥9.2 mmol/L (165 mg/dL)
					2 h ≥8.1 mmol/L (145 mg/dL)
					3 h ≥7.0 mmol/L (125 mg/dL)
1979	NDDG (25)	1	100 g	≥2	Fasting ≥5.9 mmol/L (105 mg/dL)
					1 h ≥10.6 mmol/L (190 mg/dL)
					2 h ≥9.2 mmol/L (165 mg/dL)
					3 h ≥8.1 mmol/L (145 mg/dL)
1982	Carpenter *et al.* (26)	2	First-step: 50 g; second-step: 75 g	≥2	Fasting ≥5.3 mmol/L (95 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.6 mmol/L (155 mg/dL)
1996	EASD (27)	1	75 g	≥1	Fasting ≥6.0 mmol/L (108 mg/dL)
					2 h ≥9.0 mmol/L (162 mg/dL)
1999	WHO (28)	1	75 g	≥1	Fasting ≥7.0 mmol/L (126 mg/dL)
					2 h ≥7.8 mmol/L (140 mg/dL)
2007	Metzger *et al.* (29)	1	100 g	≥2	Fasting ≥5.3 mmol/L (95 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.6 mmol/L (155 mg/dL)
					3 h ≥7.8 mmol/L (140 mg/dL)
2000	ADA (30)	1	75/100 g	≥2	Fasting ≥5.3 mmol/L (95 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.6 mmol/L (155 mg/dL)
					3 h ≥7.8 mmol/L (140 mg/dL)
2010	IADPSG and ADA (31)	1	75 g	≥1	Fasting ≥5.1 mmol/L (92 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.5 mmol/L (153 mg/dL)
2013	WHO (32)	1	75 g	≥1	Fasting ≥5.1 mmol/L (92 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.5 mmol/L (153 mg/dL)
2015	NICE (33)	1	75 g	≥1	Fasting ≥5.6 mmol/L (100 mg/dL)
					2 h ≥7.8 mmol/L (140 mg/dL)

Table 1 *(continued)*

Table 1 *(continued)*

Year	References	Number of steps	Oral glucose load	Number of abnormal values required	Glucose cut-offs
2015	FIGO (34)	1	75 g	≥1	Fasting ≥5.1 mmol/L (92 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.5 mmol/L (153 mg/dL)
2018	ACOG (35)	2	First-step: 50 g; second-step: 100 g	≥2	Fasting ≥5.3 mmol/L (95 mg/dL)
					1 h ≥10.0 mmol/L (180 mg/dL)
					2 h ≥8.6 mmol/L (155 mg/dL)
					3 h ≥7.8 mmol/L (140 mg/dL)

ACOG, American College of Obstetricians and Gynecologists; GDM, gestational diabetes mellitus; IADPSG, International Association of Diabetes in Pregnancy Study Groups; NICE, National Institute for Health and Care Excellence; OGTT, oral glucose tolerance test; WHO, World Health Organization; EASD, European Association for the Study of Diabetes; NDDG, National Diabetes Data Group; ADA, American Diabetes Association; FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation.

linked to the pathogenesis of GDM (54). On the other hand, it has been suggested that also GDM may have an impact on epigenetic modifications in mother and offspring (55).

Several studies investigating the role of epigenetics in the pathogenesis of GDM have been originally carried out in the animal model (56-59). More recently, these epigenetic modifications have been also demonstrated in different human tissues, including placenta, cord blood, as well as in visceral and subcutaneous adipose tissues. Placenta DNA methylation of more than 385,000 CpG sites has been assessed by Rong *et al.* (60) in 36 GDM women and 40 controls. The authors identified both hyper- and hypo-methylated regions in GDM patients compared to healthy subjects, thus hypothesizing a role of this epigenetic modification in the pathophysiology of GDM. The differentially methylated genes, *IGF2*, *GCKR* and *KCNQ1*, are involved in pathways of cell growth, death regulation, immune and inflammatory response and nervous system development (60).

Although Nomura *et al.* failed to find an association between methylation status and GDM in an earlier study based on 50 placenta samples (61), Reichetzeder *et al.* (62) analyzed a larger number of placental tissues (n=1,030) by means of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), observing that placental global DNA hypermethylation was associated with GDM, independently from other risk factors. Deng *et al.* (63) conducted a global gene methylation and whole genome expression profiling in visceral omental adipose tissue of GDM and normal pregnancies. The authors found that 935 genes were commonly dysregulated in the GDM group compared to healthy pregnant women (63).

Since it has been shown that maternal peripheral blood reflects placental epigenetics changes (64), several studies have been carried out for evaluating methylation status in blood. In a study performed in peripheral blood cells of 63 South African women with GDM, no difference in global DNA methylation could be observed between women with or without GDM (65). In this study the analysis was performed in women between the 24–28 gestational weeks, and it is hence conceivable that differences in methylation would be more clearly evident when measured earlier. In keeping with this hypothesis, Enquobahrie *et al.* (66) studied GDM women before the 20th gestational week, and reported that 17 CpG sites were hypomethylated, whilst 10 CpG sites were found to be hypermethylated. Even more interestingly, a recent study identified a characteristic genome-wide DNA methylation profiling measured between the 12th and 16th gestational weeks in 11 GDM women compared to 11 matched controls (67). In particular, five genes (*COPS 8*, *PIK3R5*, *HAAO*, *CCDC124*, and *C5orf34*) displayed a significantly different methylation status (67). Kang *et al.* carried out a genome-wide DNA methylation profile on 8 women with GDM and 8 healthy controls, observing as many as 151 genes with different degree of methylation (68). Among these, genes codifying for pro-inflammatory cytokine interleukin-6 (IL-6) and for anti-inflammatory cytokine IL-10 were

identified. In addition to studying the global methylation profile, another approach involves the study analysis of methylation of single genes, though locus-specific DNA methylation methods are expensive and require large bioinformatics expertise (69). The gene codifying for leptin is among those most studied in GDM, since it is finely regulated by many epigenetic mechanisms (70). In two subsequent studies, Bouchard *et al.* (71,72) observed an association between hyperglycemia and alterations in placental DNA methylation of leptin and adiponectin genes in a cohort of mothers with impaired glucose metabolism. Kang *et al.* enrolled a Taiwanese population encompassing of 8 GDM and 24 controls, and found decreased methylation of IL-10 in blood of GDM, which was also found to be associated with increased serum values of IL-10 (73). More recently, Zhang *et al.* studied the methylation level of *HIF3A* promoter region in 20 GDM patients, showing that *HIF3A* expression is down-regulated in omental tissues (74). Notably, previous studies showed that *HIF3A* is involved in insulin resistance and glucose metabolism, thus providing a reasonable support to these findings (75).

Although translation of methylation study into clinical practice is challenging, microRNAs (miRNAs)—small non-coding RNAs (approximately 20 nucleotides in length) that regulate gene expression—can be seen as potentially useful circulating biomarkers for monitoring pregnancy and screening GDM. Studies performed using placenta tissues revealed that placenta has a specific miRNA expression pattern (76,77), and that this profile dynamically changes during pregnancy (78). A number of studies have explored differential expression of miRNAs at delivery in GDM pregnancies versus healthy controls to date (79-84), whilst other studies have investigated the potential clinical usefulness of miRNAs deregulation in assessing the risk of developing GDM though measurement of circulating miRNA levels in first or second-trimester in women with and without GDM (85-90).

In 2011, Zhao *et al.* (85) studied 24 GDM pregnant women (16–19 gestational week) and 24 healthy pregnant women, and identified three miRNAs (miR-132, miR-29a and miR-222) significantly down-regulated in GDM. In another study based on 28 women with GDM and 53 controls, down-regulation of miR-222, associated with low expression of miR-20a, was also confirmed in the study of Pheiffer and colleagues (89). Unlike these findings, Wander *et al.* (88) failed to find significant differences in plasma levels of miR-222 and miR-29a assayed in 36 GDM cases and 80 controls from the Omega prospective study. More interestingly, Wander *et al.* reported enhanced circulating values of miR-155 and miR-21 in GDM women, especially in overweight/obese pre-pregnancy or pregnant with male offspring (88). MiR-222 expression was also studied by Shi *et al.* (84) in omental adipose tissue of GDM patients and was found to be over-expressed. Moreover, miR-222 levels were positively correlated with maternal estradiol concentrations and negatively with estrogen receptor, thus reinforcing the idea of a role of this miR in the pathogenesis of insulin resistance.

These findings were confirmed in the recent study of Tagoma *et al.* (90), who found that miR-222 and several other miRNAs were over-expressed in maternal plasma of women with GDM. Other case-controls studies, mainly based on small sized populations (86,87,91-94) identified a number of other miRNAs that were deregulated in pregnant women with GDM.

Despite many studies could identify many miRNAs which can be potentially used as diagnostic biomarkers, the real utility of this approach in clinical practice has not been demonstrated so far, and larger well-performed studies are needed. Moreover, consensus is lacking on several pre-analytical and analytical aspects (i.e., biological matrix, sample handing procedure, sample storage, quantification technique, data normalization, etc.), which finally preclude results comparability. Important standardization or harmonization efforts shall hence be planned to overcome these current drawbacks (95,96).

Future perspectives

A new class of endogenous ncRNA biomarkers, named circular RNAs (circRNAs), has been investigated in different diseases during the past decade, including age-related pathologies such as cancer, CV disorders, neurodegenerative disease and diabetes (97,98). CircRNAs essentially originate from pre-miRNAs, are characterized by a covalently closed loop structure and regulate miRNAs expression through acting as miRNA sponges (99). These molecules carry some notable advantages compared to linear RNAs, being essentially more stable and expressed at high levels in paternal tissues (100).

Yan and colleagues (101) carried out next-generation sequencing (NGS) in placental villi of women with GDM and normal controls, and were capable of identifying as many as 48,270 circRNAs, 227 of which were found to be significantly up-regulated and 255 circRNAs

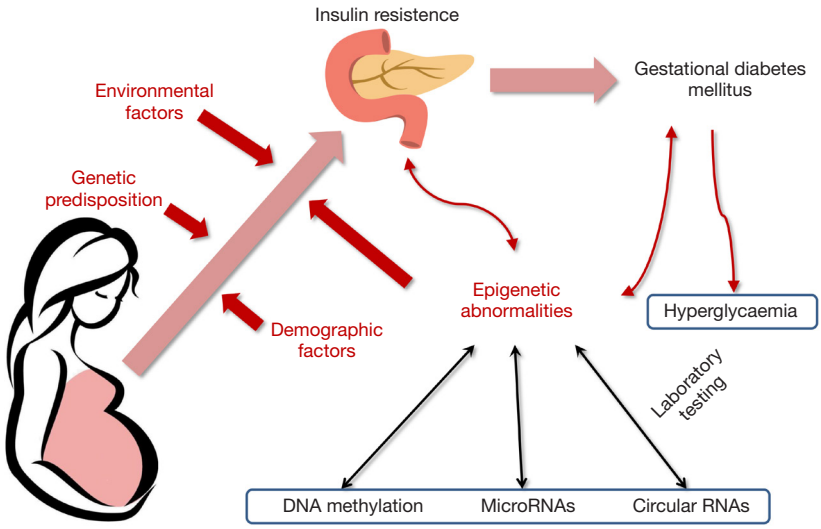

Figure 1 Multifactorial predisposition to GDM. GDM, gestational diabetes mellitus.

significantly down-regulated in the GDM cohort. They could hence hypothesize that these circRNAs may play some important roles in the development of GDM (101). Wu *et al.* carried out an interesting study measuring six circRNAs (hsa_circRNA_0054633, hsa_circRNA_103410, hsa_circRNA_063981, hsa_circRNA_102682, hsa_circRNA_0018508, and hsa_circRNA_406918) in serum samples of 40 healthy pregnant women, 40 women with GDM during the second trimester of pregnancy, 65 controls and 65 GDM cases during the third trimester of pregnancy, as well as in placental tissues and cord blood of 20 GDM cases and 20 controls (102). Notably, circRNA_0054633 was found to be highly expressed in blood during the second and third trimesters. The expression was also high in the placenta, low in the cord blood ($P<0.05$) and was highly correlated with glycosylated hemoglobin (HbA1c) values levels in maternal blood samples. The assessment of this circRNA displayed a notable diagnostic performance in the second and third trimesters of pregnancy, placenta, and cord blood [area under the curve (AUC) of 0.79, 0.66, 0.75, and 0.78, respectively; all $P<0.001$] (102). Since circRNA_0054633 is involved in cell cycle progression and molecular catabolism (103), it was finally hypothesized that it may be also involved in regulating the proliferation of pancreatic β cells (104).

Very recently, Wang *et al.* explored the differential expression of circRNAs in the placentas of 30 GDM and 15 normal pregnant women (105). Among the 8,321 circRNAs identified in human placenta, three were found to be over-expressed and 43 down-regulated in GDM patients. By performing functional analysis of differentially expressed circRNAs, the authors concluded that these circRNAs may be active players in the pathogenesis of GDM since they are involved in advanced glycation end products-receptor for advanced glycation end products (AGE-RAGE) signaling pathway (106).

Conclusions

Epigenetic testing, encompassing the assessment of DNA methylation, miRNAs and circRNAs, is an intriguing and promising perspective for prediction/early diagnosis of GDM, whereby epigenetic abnormalities not only emerge throughout the pathogenesis of GDM, but may also contribute to development and progression of the disease by means of a bidirectional interrelationship (*Figure 1*). Thereby, it is conceivable that the assessment of some predictive epigenetic biomarkers may allow—in a foreseeable future—the early identification of women at enhanced risk of developing pregnancy complications, even before glucose metabolism is significantly impaired.

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Footnote

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