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Adverse Maternal Metabolic Intrauterine Environment and Placental Epigenetics: Implications for Fetal Metabolic Programming

Corina Lesseur, MD, PhD¹ and Jia Chen, ScD^{1,2,3,4}

¹Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, New York, NY, USA

²Department of Pediatrics, Icahn School of Medicine at Mount Sinai, Box 1057, 1 Gustave Levy Place, New York, NY 10029, USA.

³Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, Box 1057, 1 Gustave Levy Place, New York, NY 10029, USA.

⁴Department of Medicine, Hematology, and Medical Oncology, Icahn School of Medicine at Mount Sinai, Box 1057, 1 Gustave Levy Place, New York, NY 10029, USA.

Abstract

Purpose of review: Herein we summarize existent epidemiological studies relating adverse maternal metabolic environments of maternal obesity and gestational diabetes and placental DNA methylation.

Recent findings: Multiple studies have evaluated associations between intrauterine exposure to gestational diabetes and/or maternal glucose levels and DNA methylation at candidate metabolic genes as well as in epigenome-wide studies. Some of the genomic regions more consistently associated include lipid-related genes (*LPL* and *PPARGCIA*), the major histocompatibility complex (MHC) and imprinted genes. Studies solely focused on maternal obesity influences on the placental epigenome are scarce.

Summary: Understanding the placental mechanisms involved in fetal metabolic programming could lead to discovery of placental biomarkers at birth that predict later-life metabolic risk. Moving forward is important to standardize methods utilized in epigenetics research; consistent methodology can help interpret disparate findings. Larger studies with longitudinal follow-up are needed to address future challenges in fetal programming research

Keywords

Epigenetics; Placenta; Maternal Obesity; Gestational Diabetes; Metabolic Programming

Corresponding Author: Jia Chen, ScD, Department of Environmental Medicine and Public Health, Icahn School of Medicine at Mount Sinai, One Gustave L. Levy Place, Box 1057, New York, NY 10029, Tel: 212-241-7592, Jia.Chen@mssm.edu.

Compliance with Ethical Standards

Conflict of Interest Corina Lesseur and Jia Chen declare that they have no conflicts of interest.

Human and Animal Rights and Informed Consent

This article does not contain any studies with human or animal subjects performed by any of the author

Introduction

Obesity (body mass index [BMI] $\geq 30 \text{ kg/m}^2$) is a chronic inflammatory disease and arguably the most important public health problem of modern society. Worldwide the prevalence of obesity has been alarmingly rising. Women, including those of reproductive age, do not escape this trend; in this group the prevalence of obesity increased from 29% in 1980 to 38% in 2013 [1]. In the United States, more than one in five pregnant women were obese before being pregnant in 2009 [2]. Pre-pregnancy obesity is a risk factor for other pregnancy-related maternal comorbidities [3, 4] including increased risk of gestational diabetes (GDM) [5], preeclampsia [6], miscarriage, and stillbirth [7] and caesarean (C-section) delivery [8]. Similarly, newborns born to obese mothers are more likely to be large for gestational age (LGA) [9], macrosomic [10], preterm [11] and have congenital malformations [12]. Moreover, pre-pregnancy obesity and GDM influence offspring susceptibility to disease beyond the perinatal period. Children and adults born from obese mothers are at increased risk of obesity and T2D later in life [4, 13, 14], which perpetuates the problem into what has been called the intergenerational cycle of obesity and diabetes [15, 16].

The Developmental Origins of Health and Disease Hypothesis (DOHaD) posits that suboptimal intrauterine conditions are associated with later-life disease risk. This paradigm was first derived in the 1980's by David Barker from epidemiological observations of higher cardiovascular disease risk (CVD) in individuals born with low birth weight (LBW) [17]. Subsequently, studies derived from the Dutch Famine Birth Cohort [18] reported associations between peri-conceptional exposure to famine and increased risk of reduced glucose tolerance [19] and obesity [20] in adulthood. Presently, a myriad of epidemiological studies in multiple populations have described associations between birth weight and later-life outcomes, particularly cardiovascular and metabolic disease [21]. Weight at birth is commonly used as a fetal growth proxy in DOHaD studies, particularly because of its amenability of measure, but also because it summarizes the intrauterine milieu provided by the maternal nutritional, hormonal and metabolic environment.

The placenta is the central regulator of the intrauterine environment that mediates maternal-fetal physiological interactions. The placenta is the first organ to develop and is in charge of multiple functions including anchoring the embryo, nutrient and waste delivery, immunological barrier and endocrinological organ [22–24]. Placental development is intimately related and is essential for fetal development, but is also interrelated to the maternal environment [25]. Thus, the placenta is the crucial link between maternal physiology and fetal development and consequently is the central organ to study in the context of DOHaD [25–27]. Adverse intrauterine metabolic environments such as that of famine, maternal obesity and/or gestational diabetes (GDM) can lead to permanent placental and fetal adaptations that could result in maladaptive later in life leading to increased metabolic disease risk.

The molecular mechanisms involved in these maladaptive changes have proven elusive and hard to characterize, especially in humans. Nevertheless, it is clear that intrinsic genetic

factors cannot explain all the latent effects because of their lack of environmental plasticity. Epigenetic mechanisms have been proposed as candidate molecular mediators in DOHaD because these integrate environmental signals into the control of gene expression. The principal mechanisms of epigenetic regulation are DNA methylation, histone modifications and non-coding RNAs [28]. The environmental responsiveness of epigenetic mechanisms is best exemplified in the elegant studies conducted in Agouti mice [29]; variability in DNA methylation upstream of the *Agouti* gene results in changes in body weight and fur color in otherwise genetically identical animals [30]. Moreover, DNA methylation at this locus can be influenced by changes in maternal nutrition and environmental exposures during early-life [31]. Maternal nutrition has also been associated with placental epigenetic marks in humans; demonstrated in a small intervention study dose-response choline diet during the third-trimester of pregnancy. Higher choline diet increased placental global and promoter methylation of cortisol-related genes (*CRH* and *NR3C1*). However, these effects seem to be specific to certain genomic regions as other genes were not affected by nutritional supplementation [32].

In this review, we summarize the existing evidence relating adverse metabolic environments of obesity and GDM to placental epigenetic variation in humans. We focused our attention on DNA methylation because this is the epigenetic mechanism most widely studied in epidemiological settings.

Candidate gene studies

Some of the genes evaluated in the epidemiological placental epigenetic programming literature come from associations between *in utero* famine exposure and candidate-gene methylation changes in blood DNA in the Dutch Famine Studies. This is the case of *IGF2*, *LEP*, *ABCA1*, *INSIGF*, *IL10*, *LEP*, *GNASAS* and *MEG3* [33, 34], which were initially investigated because these were known to be imprinted or related to metabolism. Although, all of these have been associated with perinatal famine exposure, only some have been evaluated in the context of GDM and maternal obesity in placental tissue. The results from these studies are summarized in Table 1.

Metabolic Genes—One of the most studied famine-associated genes is leptin (*LEP*), an important adipokine involved in energy metabolism. The first cross-sectional study that evaluated placental *LEP* methylation profiled CpG DNA methylation within the *LEP* promoter region in 48 term placentas from a French-Canadian population [35]. The authors did not observed absolute differences in *LEP* methylation between placentas exposed to impaired glucose tolerance (IGT, N=25) compared to normal glucose tolerance (NGT) (N=23) pregnancies. The authors reported significant correlations between *LEP* DNA methylation and maternal glucose levels measured two hours after a 75-g oral glucose tolerance test (2-h post-OGTT) performed at 24–28 weeks of gestation, only in women in the IGT group. Intriguingly, glucose levels showed a positive correlation with *LEP* methylation in the maternal side of the placenta and inverse correlation with *LEP* in the fetal side [35]. Importantly, this study by Bouchard et al also found a negative correlation between placental *LEP* methylation and gene expression, as well as a positive correlation between placental *LEP* mRNA and maternal serum leptin levels during the 3rd trimester. In

another study in a German population [36], the authors evaluated DNA methylation at multiple candidate genes in relation to GDM, including *LEP* promoter methylation and did not observe differences in chorionic villi samples from diet-treated GDM (N=27) or insulin-treated GDM (N=32) compared to non-GDM controls (N=41). Our group has also investigated *LEP* promoter methylation in relation to maternal obesity and GDM in 535 term placentas from the Rhode Island Birth Cohort Study [37]. We observed placental *LEP* hypermethylation in 47 GDM placentas (diagnosis abstracted from medical records) compared to non-GDM. Concomitantly, pre-pregnancy obesity was also associated with higher placental *LEP*. Using structural equation modeling, we found that GDM act as significant mediator of the effects of pre-pregnancy BMI in placental *LEP* methylation. Importantly, our analyses accounted for potential confounders, particularly infant factors (sex and rs2167270 genotype) which we had previously described to influence placental *LEP* methylation [38]. Overall, the summary of existent placental *LEP* methylation in relation to GDM and maternal glucose levels shows contrasting results, future studies should evaluate this issue further and in different populations.

In 2013, adiponectin (*ADIPOQ*) another important adipokine, was evaluated in a study involving 93 French-Canadian women, also conducted by the Bouchard group. Lower placental *ADIPOQ* promoter methylation was associated with higher maternal 2nd trimester glucose levels (2-h post-OGTT) and with higher insulin resistance index (2nd and 3rd trimesters). Importantly, lower *ADIPOQ* methylation was associated with higher maternal circulating adiponectin [39]. In the same year, this group also described results related to another metabolic gene, the *ABCA1* (member 1 of human transporter sub-family ABCA), previously related to famine exposure and implicated in high-density lipoproteins cholesterol (HDL-C) metabolism. The study reports positive correlations between *ABCA1* promoter DNA methylation in the maternal side of the placenta and maternal glucose levels (2 h post-OGTT) and circulating HDL-C. On the fetal side of the placenta, *ABCA1* methylation was negatively correlated to cord blood triglyceride levels, but not to maternal values. Prominently, placental *ABCA1* promoter methylation was inversely correlated to gene expression, suggesting a functional role for *ABCA1* methylation [40].

A later study also by Bouchard (2014)[41], described associations between placental DNA methylation at another lipid-related gene: the placental lipoprotein lipase (*LPL*). In an analysis including 27 GDM cases and 99 NGT controls, *LPL* DNA methylation was lower in placentas from infants exposed to a hyperglycemic environment. *LPL* methylation at CpG1 (promoter) and CpG3 (intron) methylation was negatively correlated to maternal glucose levels (2 h post-OGTT) and HDL-cholesterol, but not to gene expression. Interestingly, only methylation at the *LPL* intron 1, and not within the promoter, was negatively associated with gene expression. Notably, a more recent follow-up study [42], reported positive associations between placental *LPL* DNA methylation and birth weight z-scores, weight z-scores and fat mass at 5 years of age [42]. Interestingly, both at birth and at the 5-year follow up, children exposed to *in utero* to GDM, had lower weight z-scores compared to non-exposed counterparts, which contrasts previous epidemiological associations between GDM and risk of LGA and macrosomia [43] and higher BMI in children [44]. However, the authors comment that women in this study received well-monitored medical treatment, which could help explain these results. A similar decrease in the rate of LGA and macrosomic births was

recently reported in a large epidemiological study in the US, which shows a reduction in these adverse fetal outcomes even when GDM incidence rates keep increasing, also arguing for the efficacy of currently implemented GDM treatment protocols [45].

The PGC-1 α is transcriptional coactivator encoded by *PPARGC1A* gene and involved in energy homeostasis and metabolism. Two recent studies evaluated *PPARGC1A* methylation in human placentas. A small Chinese study of 58 Han Chinese ancestry women observed a positive association between glucose levels (2nd trimester) and placental promoter *PPARGC1A* methylation, observed in all placentas regardless of GDM-exposure status [46]. Following these associations, Côté et al also reported positive associations between *PPARGC1A* methylation and 2nd trimester fasting glucose and 2-h post-OGTT glucose, which were replicated in an independent cohort study also of French-Canadian ancestry (Gen3G). This study also surveyed other genes related to brown adipose tissue (BAT), and reported correlations between multiple measures of maternal glucose and *PRDM16* methylation, only *BMP7* methylation was significantly different between GDM-exposed and non-exposed placentas [47].

The glucocorticoid receptor gene (*NR3C1*) has been previously shown as an environmentally responsive and epigenetically regulated with effects in metabolism and behavior in both rodents [48] and humans [49]. Importantly, placental *NR3C1* hypermethylation is associated with LGA status [50]. Given the relation between GDM and LGA, it is not surprising that *NR3C1* methylation could also relate to GDM. Only one study [36] has reported on placental *NR3C1* methylation in relation to GDM, placentas exposed to both diet-treated GDM and insulin-treated GDM have significantly lower *NR3C1* methylation compared to controls, a counterintuitive result especially after considering the previous LGA findings. Thus, additional research is needed to clarify if indeed *NR3C1* methylation is related to GDM and/or LGA status.

Imprinted genes—Genomic imprinting results in parent-of-origin-specific gene expression because DNA methylation silences the other allele. Imprinting is particularly important in the placenta where many genes exhibit tissue-specific imprinting and are related to fetal growth [51]. Moreover, blood methylation marks at imprinted loci (*INSIGF*, *GNASAS* and *MEG3*) have been associated with famine exposure in the Dutch Famine Studies. Two studies have examined methylation at imprinted genes in the placenta in relation to maternal glucose levels. Recently, a study performed in French-Canadians, reported that *IGF1R* and *IGFBP3* methylation were significantly lower in placentas exposed to IGT and negatively correlated with glucose levels (2-h post-OGTT) [52]. A more recent and larger study, conducted in a Chinese population observed similar results; placental *IGF2* methylation was negatively associated with GDM status and infant macrosomia, correspondingly opposite results were described for *IGF2* mRNA [53]. Importantly, a German study reported that *MEST* is hypomethylated in placentas exposed to GDM [36]. *IGF1R*, *IGF2* and *MEST* are paternally-expressed genes, thus lower methylation levels is consistent with overgrowth outcomes related to intrauterine hyperglycemia exposure.

Global DNA methylation and Epigenome-Wide Studies

In Table 2, we list studies evaluating global DNA methylation and epigenome-wide association studies (EWAS) related to GDM, maternal glucose levels and/or maternal obesity and placental DNA methylation.

Two studies have evaluated global DNA methylation and adverse maternal metabolic environments [54, 55]. In a small pilot study (N=50), our group, assessed global DNA using a semiquantitative luminometric methylation assay in term placentas from a birth cohort in the New York metropolitan area. We observed lower DNA methylation in GDM placentas compared to non-GDM, whereas maternal obesity was associated with higher global DNA methylation compared to normal BMI, we also observed marginally significant hypomethylation in placentas exposed to pre-eclampsia [55]. A more recent and considerably larger study, conducted in Germany used placentas from 1,030 term pregnancies, measured global DNA methylation with liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI/MS/MS) and reported global hypermethylation in GDM placentas compared to non-GDM. Importantly, this study excluded pre-eclampsia cases from their analysis and adjusted for potential confounders including pre-pregnancy BMI[54].

The first placental epigenome-wide study (EWAS) considering the effects of GDM was a small study including 14 controls and 30 GDM-exposed placentas from French-Canadian women. The authors report 781 differentially methylated genes (P -value<0.01) that were enriched for pathways related to cardiovascular and metabolic disease. However, none of the CpG loci evaluated reached genome-wide significance [56]. In follow-up analyses, the authors attempted to replicate 10 of the top loci in a different French-Canadian population, including 20 GDM-exposed placentas and 60 non-GDM controls [40]. However, they were unable to reproduce the initial EWAS findings. Nevertheless, they observed associations between glucose levels and methylation at *BRD2*, *CACNA1D* and *LRP1B* in normoglycemic women[57].

A more recent study by Finer et al [58] used placental tissue from 48 South Asian origin women receiving antenatal care in a hospital in London (UK). Their findings report 1,708 placenta methylation variable positions (MVPs) (FDR-adjusted P -value<0.05), >5% of these changes were located within 1st exons, genes. These MVPs were enriched for pathways related to endocytosis, MAPK signaling and extracellular triggers to intracellular metabolic processes. Concomitantly, the authors performed a parallel EWAS using cord blood methylation and describe some shared MVPs. However, none of the CpG loci reported by the authors had been identified in previous placental epigenetic GDM studies.

Another EWAS study [59] conducted in Nanjing (China) included 36 GDM-exposed and 40 control placentas. The authors report more than 6,000 DMRs in 3,000 differentially methylated genes as significant. However, this analysis was not adjusted for multiple testing or maternal and infant covariates. Subsequent, technical validation with bisulfite pyrosequencing and qPCR was performed for 4 metabolic-related genes (*RBP4*, *GLUT3*, Resistin and *PPARα*). Methylation at *GLUT3*, Resistin and *PPARα* were validated by

bisulfite pyrosequencing, significant differences in placenta mRNA levels between GDM and controls were observed for *GLUT3* and Resistin.

In 2015, Binder et al conducted a GDM EWAS using 41 GDM-exposed and 41 controls placentas from the Harvard Epigenetic Birth Cohort (HEBC) [60]. Both DNA methylation and gene expression changes were evaluated using arrays. Using a multiple-step approach, the authors first identified differentially methylated CpG which were next prioritized using a bum-hunting approach. This strategy resulted in 4 regions to follow-up with pyrosequencing validation in an independent population in Rhode Island. One of these DMRs mapped to the major histocompatibility complex (MHC) region was marginally validated in RICHs. Moreover, differential transcriptome-wide analyses in a subset of placentas also highlighted multiple differentially expressed genes in the MHC. The MHC region has also been associated through genome-wide association studies to type 1 diabetes (T1D)[61], gestational diabetes in a Korean population [62] and to a multitude of other immune-related outcomes [63]. These findings should be followed-up in future studies that also integrate genetic variation to the DNA methylation analysis, especially since the MHC is the most polymorphic area of the genome [64].

Recently, a large EWAS of glucose levels (2h post-OGTT) and term placenta DNA methylation was performed in a population of 448 French-Canadians from the Genetics of Glucose regulation in Gestation and Growth Birth Cohort (Gen3G)[65]. Maternal glucose levels were associated with 7 CpG sites after FDR adjustment. Four of these CpGs mapped to *PDE4B* (phosphodiesterase 4B) gene, higher maternal glucose was associated with lower *PDE4B* methylation also associated with expression. Importantly, the authors attempted to replicate their findings in an independent birth cohort (ECO-2), however, none of the four loci evaluated replicated. The authors argued that CpGs within the *PDE4B* region had similar effect sizes for associations with maternal glucose and this might be due to decreased statistical power in the smaller replication dataset.

To our knowledge, the only EWAS study conducted to date on maternal obesity and placental methylation was recently published by Mitsuya et al [66] in a population from San Antonio, Texas. The authors report multiple differentially methylated and hydroxymethylated regions associated with maternal obesity. Similarly, they describe a 21% increase in methylated along with a 31% decrease in hydroxymethylated regions in placentas exposed to obesity compared to lean counterparts. Regions that showed differential methylation and hydroxymethylation were enriched for pathways related to pregnancy, immune response and cell adhesion-linked processes. Interestingly, the authors also evaluated placental metabolites and report a positive correlation between ascorbate and α -ketoglutarate.

To summarize the findings of existent studies, we compiled all the genes at which placental methylation changes have been associated with GDM, maternal glucose and maternal obesity status (Figure 1). These include candidate genes and published EWAS results. Methylation at these genes does not correspond to a specific gene region but to CpGs within or near these genes reported in these studies, we summarize the results at the gene level to maximize potential overlap. Only two genes have been associated in multiple studies. The

insulin-like growth factor 1 receptor (*IGF1R*) gene (15q26.13) was first reported to be associated with GDM in a EWAS[56] and later followed-up by the same group in a candidate gene study including a larger French-Canadian population[52]. The *VIPR1* gene (3p22.1) encodes for the vasoactive intestinal polypeptide receptor 1, was first associated with GDM in 2013 in French Canadians[56] (GDM 15% vs. NGT 20% methylation; $P=7.3 \times 10^{-5}$) and later associated with GDM in a US cohort study[60] ($\beta=0.06$, $P=6 \times 10^{-6}$), although with opposite effect direction between studies. However, some genomic regions show associations in multiple studies. For example, a region in chromosome 19 (1p13.3–19p.13) contains five genes (*SIRT6*, *ADAMTSL5*, *RESISTIN*, *LDLR* and *ZSWIM4*) associated in at least four studies. Moving forward future maternal obesity and GDM-related EWASs should provide summary statistics from association analyses; this is critical to enable future meta-analysis to identify consistent results and not isolated findings that appear in one study but not in others. Recent collaborative initiatives such as the Pregnancy and Childhood Epigenetics Consortium are starting to address this issue to move epigenetics research into larger sample sizes and reproducible results [67].

Conclusions and Future Perspectives

We have outlined the current state of the evidence pertaining to the effects of GDM and obesity to placental DNA methylation. We conclude: 1) Associations between DNA methylation at candidate genes and placental exposure to GDM or glucose levels have been inconsistent with a few exceptions of glucose and lipid-related genes, the MHC and imprinted genes. 2) Placental EWAS studies investigating GDM or maternal glucose have not followed consistent rigorous methodology specifically as pertains to multiple testing correction and replication of the findings, however more recent studies have started to recognize [68] and change[65] these practices. 3) Only a few studies have assessed the effects of maternal obesity independently on placenta epigenetic marks, thus there is a clear need for future focused studies; 4) Placental epigenetic research related to GDM and maternal obesity will benefit from the inclusion of multiple more diverse populations and birth cohorts; 5) There is also a clear need to study possible mediating effects of these epigenetics marks in relation to later-life adverse metabolic outcomes, to this end more longitudinal studies are needed.

The contribution of fetal metabolic programming to the rising incidence of chronic metabolic disease requires further studies to elucidate the mechanisms behind these phenomena. The epigenome is a likely mechanistic candidate, particularly when interrogated in functionally relevant tissues such as the placenta. Examining the impact of adverse maternal metabolic factors on placental epigenetic marks is particularly important given the current obesity epidemic and its possible consequences on later-life disease susceptibility. Placental epigenetic marks could help develop biomarkers at birth that prospectively predict metabolic risk which in turn can help design early prevention/intervention to reduce metabolic disease burden.

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Figure 1. Chromosome ideogram illustrating genes for which a CpG methylation change has been reported to be associated with GDM, maternal glucose or maternal obesity association studies (Table 1 and 2). Methylation changes locate within or near these genes. Data obtain from candidate gene studies (n=x), Binder et al 2015 (data from Table S2), Cardenas et al 2018 (data from Table 2), Finner et al 2015 (top 20 loci from supplemental file 2), Mitsuya et al 2017 (genes reported in article text), Ruchat et al 2013 (top 20 genes from Table S1) and Rung et al 2015 (genes reported in article text).

Candidate-gene studies of placental DNA methylation, gestational diabetes, maternal glucose levels and prepregnancy obesity

Table 1.

1 st Author, Year	Study Population Design	GDM/obesity measures	Platform, gene (s), quality control	Confounders	Main Findings
Bouchard et al 2010 Ref. 35	N=48 IGT N =23 NGT N =25 Saguenay (Québec, Canada) Cross-sectional E-21 birth cohort	Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28)	Sequenom Epi-TYPER system <i>LEP</i> promoter No methylation replicates.	Initial analysis did not found correlations between possible confounders (age, BMI, weight change, and insulin) and placental <i>LEP</i> methylation or expression. No multiple-testing adjustment.	IGT group: <i>LEP</i> methylation correlated with maternal glucose (fetal side: $\rho = -0.44$, $P = 0.05$; maternal side: $\rho = 0.53$, $P = 0.01$) and with $\downarrow$ <i>LEP</i> gene expression ($\rho = -0.30$, $P = 0.05$). <i>LEP</i> mRNA levels correlated with serum leptin ($r = 0.4$, $P = 0.01$)
El Hajj et al 2013 Ref. 36	N=221 N=65 Non-GDM, N=88 diet-treated GDM Insulin-treated GDM N=98 Moenchengladbach, Germany.	GDM diagnosis fasting glucose & Maternal glucose following 2h post-OGTT test	Bisulfite DNA Pyrosequencing <i>H19</i> , <i>MEG3</i> , <i>MEST</i> , <i>LIT1</i> , <i>NESPAS</i> , <i>PEG3</i> , <i>SNRPN</i> , <i>LEP</i> , <i>NDUFB6</i> , <i>NR3C1</i> , <i>PPARA</i> , <i>IL10</i> , <i>PC</i> , <i>OCT4</i> , <i>ALU</i> and <i>LINE1</i> Pyrosequencing software QC standards.	Multivariate parametric ANOVA models including the covariates maternal age, BMI, and birth weight were used to account for potential confounders. P values were multiple testing-corrected using the Holm method.	<i>MEST</i> , <i>NR3C1</i> and <i>ALU</i> repeats showed $\downarrow$ methylation in GDM groups (diet-treated D-GDM or insulin-treated I-GDM) compared to controls. Methylation levels by group: <i>MEST</i> (Non-GDM 45.5, D-GDM 39.3, I-GDM 38.3, $P = 1.06E^{-10}$), <i>NR3C1</i> (Non-GDM 4.5, D-GDM 3.0, I-GDM 3.0, $P = 2.23E^{-03}$), <i>ALU</i> (Non-GDM 23.0, D-GDM 22.2, I-GDM 22.4, $P = 0.015$).
Lesseur et al 2014 Ref. 37	N=535 GDM N=47 Obesity N= 135 74.1% Caucasian Providence (Rhode Island, USA) Birth Cohort	GDM diagnosis from medical reports Pre-pregnancy BMI calculated from self-reported height and weight Obesity BMI>kg/m ²	Bisulfite DNA Pyrosequencing <i>LEP</i> promoter. Three pyrosequencing replicates and pyrosequencing software QC standards.	Multivariable regression and structural equation models adjusted for potential confounders (rs2167270 genotype, infant sex, maternal age, and birthweight group). No multiple-testing adjustment.	Placental <i>LEP</i> methylation in infants exposed to GDM $\uparrow$ compared to non-GDM ($\beta = 1.89$, $P = 0.04$) <i>LEP</i> methylation marginally associated with pre-pregnancy obesity ($\beta = 1.891.17$, $P = 0.06$). GDM is a mediator of the effects of maternal obesity in <i>LEP</i> ($\beta = 0.81$, 95% CI 0.27–2.71)
Bouchard et al 2013 Ref. 39	N=98 IGT N=31 NGT N=67 French-Canadian Saguenay (Québec, Canada) Cross-sectional E-21 birth cohort	Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28)	Bisulfite DNA pyrosequencing <i>ADIPOQ</i> promoter Pyrosequencing software QC standards.	Confounders (age, BMI, pregnancy weight change, insulin, HOMA-IR, and time between birth and placenta sampling) also evaluated using correlations to main variable; those significantly correlated were added to the linear models. No multiple-testing adjustment.	$\downarrow$ placental <i>ADIPOQ</i> methylation (fetal side) correlated with $\uparrow$ 2 nd trimester maternal glucose ($r = -0.21$; $P < 0.05$). $\downarrow$ <i>ADIPOQ</i> methylation (maternal side) correlated with higher 2 nd trimester maternal insulin resistance index ($r = -0.27$; $P < 0.05$). $\downarrow$ <i>ADIPOQ</i> methylation associated with $\uparrow$ maternal plasma adiponectin throughout pregnancy ($r = -0.26$ $P < 0.05$).
Houde et al 2013 Ref. 40	N= 100 IGT N=26 NGT N=74	Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28)	Bisulfite DNA pyrosequencing <i>ABCA1</i> promoter.	Multivariable linear regression analyses remained unchanged after including potential	Placental <i>ABCA1</i> methylation (maternal side) correlated with maternal HDL-C ($r = -0.21$; $P < 0.04$) and glucose 2h post-OGTT, 2 nd trimester ($r = 0.25$; $P = 0.02$). Placental

1 st Author, Year	Study Population Design	GDM/obesity measures	Platform, gene (s), quality control	Confounders	Main Findings
	French-Canadian Saguenay (Quebec, Canada) E-21 birth cohort		Pyrosequencing software QC standards.	confounders (age, BMI, TG, smoking, gravidity, parity, mode of delivery, newborn sex, GDM history and pregnancy weight gain).	<i>ABCA1</i> methylation (fetal side) correlated with cord blood triglycerides ($r = -0.28$; $P = 0.01$). Placental <i>ABCA1</i> /DNA methylation associated with <i>ABCA1</i> mRNA levels ($r < -0.35$; $P = 0.05$)
Houde et al 2014 Ref. 41	N=126 GDM N = 27 NGT N = 99 French-Canadian Saguenay (Quebec, Canada) E-21 birth cohort	Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28) HDL-cholesterol levels 3 rd trimester (cord blood). Insulin resistance (HOMA-IR)	Bisulfite DNA pyrosequencing <i>LPL</i> proximal promoter region CpG1 and intron 1 (CpG2 and 3) Pyrosequencing software QC standards.	Models adjusted for possible confounders (when applicable): age, BMI, GDM history, delivery method, infant sex and birth weight. No multiple-testing adjustment.	<i>LPL</i> methylation lower in GDM-exposed placentas. <i>LPL</i> -CpG1 & CpG3, methylation negatively correlated with maternal glucose ($r = -0.22$, $P = 0.02$) and HDL-cholesterol levels ($r = -0.20$; $P = 0.03$). <i>LPL</i> -CpG2 methylation negatively related to gene expression ($r \leq -0.51$; $P < 0.001$) <i>LPL</i> -CpG2 correlated to cord blood lipid profile ($r = -0.20$; $P = 0.03$).
Gagne-Quellet et al 2017 Ref. 42	N=66 GDM N=24 NGT=42 French-Canadian Saguenay (Quebec, Canada) E-21 birth cohort	Maternal glucose 2h post-OGTT (2 nd trimester, week 24–28) Anthropometric and body composition (bioimpedance) in children (5 years)	Bisulfite DNA Pyrosequencing <i>LPL</i> proximal promoter region CpG1 and intron 1 (CpG2 and 3). Pyrosequencing software QC standards.	Analyses adjusted for gestational age at birth, child sex, maternal age, and pre-pregnancy BMI. No multiple-testing adjustment.	Placental <i>LPL</i> methylation correlated with birth weight z-scores ($r = 0.252$, $P = 0.04$), and with 5-year weight z-scores ($r = 0.314$, $P = 0.01$) and fat mass ($r = 0.275$, $P = 0.04$), and negatively correlated with lean mass ($r = 0.306$, $P = 0.02$).
Xie et al 2015 Ref. 46	N=58 GDM N=24 Non-GDM N=34 Han Chinese South China	Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28)	<i>PPARGC1A</i> Promoter Pyrosequencing software QC standards.	When appropriate, confounders (maternal age, pre-gestational BMI, gestational weight gain, cord blood HOMA-IR, and offspring birth weight, sex, parity and gestational age) were incorporated into the correlation analysis. No multiple-testing adjustment.	<i>PPARGC1A</i> at CpG site -260 correlated with fasting OGTT glucose ($r = 0.4$, $P = 0.002$). Placenta PGC-1 α protein level not correlate with <i>PPARGC1A</i> promoter methylation
Côté et al 2016 Ref. 47	2 French-Canadian populations E-21 birth cohort N=133; GDM 33 cases Gen3G Cohort n = 172 (Non-GDM)	GDM diagnoses and Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28). Fasting glucose and insulin levels (2 nd and 3 rd trimesters)	<i>PRDM16</i> , <i>BMP7</i> , <i>CTBP2</i> , and <i>PPARGC1a</i> bisulfite pyrosequencing and Infinium 450K array Pyrosequencing software QC standards.	Linear regression included covariates that $\uparrow$ GDM risk or influence fetal growth: gestational age, infant sex, birth weight, smoking during pregnancy, weight gain between 1st and 2nd trimester, and 2 h post-OGTT glucose, maternal age, and 1 st trimester BMI. No multiple-testing adjustment.	<i>PPARGC1</i> methylation associated with fasting glucose (2 nd trimester) $r = 0.1-0.2$ $P < 0.05$) <i>PPARGC1</i> methylation correlated with cord blood leptin ($r = 0.4$, $P < 0.05$) <i>PRDM16</i> methylation correlated with cord blood leptin ($r = -0.22$, $P < 0.05$) $\downarrow$ <i>BMP7</i> methylation GMD placentas ($P < 0.05$). Replication (Gen3G). <i>PPARGC1a</i> associated with 2h post-OGTT (cg27514608 and cg11270806, $r = 0.20$, $p = 0.008$), $\downarrow$ <i>PRDM16</i> methylation (cg04873098, $r = -0.14$, $P = 0.07$) <i>BMP7</i> cg18759209, $r = -0.16$, $p = 0.04$) associated with $\uparrow$ maternal fasting glucose (2 nd trimester)

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1 st Author, Year	Study Population Design	GDM/obesity measures	Platform, gene (s), quality control	Confounders	Main Findings
Desgagné et al 2014 Ref. 52	N= 140 IGT: N = 34; NGT: N= 106 French-Canadian E-21 Birth cohort	GDM diagnoses and levels from glucose from Maternal glucose following 2h post-OGTT test (2 nd trimester, wk 24–28)	<i>IGF1R</i> <i>IGFBP3</i> <i>INSR</i> <i>IGF1</i> Pyrosequencing software QC standards.	Analyses were adjusted for maternal age and BMI at first trimester and weight gain between first and third trimesters of pregnancy. No multiple-testing adjustment.	↓ <i>IGF1R</i> and <i>IGFBP3</i> methylation in IGT placentas (−4.3%; <i>P</i> = 0.021 and − 2.5%; <i>P</i> = 0.006) <i>IGF1R</i> and <i>IGFBP3</i> methylation correlated with 2-h post-OGTT glycaemia (<i>r</i> = −0.23; <i>P</i> = 0.010 and <i>r</i> = −0.20; <i>P</i> = 0.028, respectively). <i>IGF1R</i> mRNA associated with birth weight (<i>r</i> = 0.20; <i>P</i> = 0.032)

IGT=impaired glucose tolerance; NGT=normal glucose tolerance; GDM gestational diabetes mellitus; OGTT oral glucose tolerance test; wk= weeks; BMI=body mass index; 2h post-OGTT =two hours after a 75-g oral glucose tolerance test

Global DNA methylation and epigenome-wide studies of gestational diabetes, maternal glucose and obesity

Table 2.

1 st Author, year	Population	Exposure definition	Platform	Confounders, Validation & Replication	Main findings
Reichetzer et al 2016 Ref. 54	N=1030 GDM N=56 Berlin, Germany	GDM diagnoses from (Medical records)	Global DNA methylation LC-MS/MS	Confounders included in the models: maternal age, BMI at the beginning of pregnancy, a history of recurrent (2) miscarriages, ethnicity and family history of type 2 Diabetes. No multiple-testing adjustment.	GDM placentas ↑ global DNA methylation compared to non-GDM (3.22 ± 0.63 vs. 3.00 ± 0.46 %; $P=0.013$).
Nomura et al 2014 Ref. 55	N=50 GDM N=7 Obesity N=34 New York, NY, USA	GDM diagnosis based Maternal glucose following 2h post-OGTT (2nd trimester, wk 24–28) Obesity BMI >30 kg/m ²	Global DNA methylation Luminometric Methylation Assay (LUMA)	Confounders included in linear models: infant sex, maternal educational, welfare status, marital status and ethnicity. No multiple-testing adjustment.	Lower DNA methylation in GDM-exposed placentas compared to non-GDM (57.08 vs 52.73, $P=0.03$) ↑ DNA methylation in maternal obesity placentas compared to normal BMI (53.64 vs 56.17, $P=0.01$)
Ruchat et al 2013 Ref. 56	N=44 GDM N = 30 NGT N= 14 French-Canadian Saguenay (Quebec, Canada) E-21 birth cohort	Maternal glucose following 2h post-OGTT (2nd trimester, wk 24–28)	450K Infinium Methylation BeadChip Array	Pyrosequencing validation 16 CpG in placenta correlation with array ($r = 0.88$; $P < 0.001$). Adjustment for newborn gestational age and sex. No FDR or Bonferroni adjustment or replication.	781 DMGs ($P < 0.01$) DMGs enriched for cardiovascular and metabolic disease in pathways Overall methylation (DMRs) ↑ GDM-exposed placentas compared to NGT placentas (5.7 vs 3.2%.)
Finer et al 2015 Ref. 58	N=48 GDM N=27 NGT N=21 South Asian London, UK	GDM diagnosed with glucose following 2h post-OGTT (2nd trimester, wk 24–28)	450K Infinium Methylation BeadChip Array	FDR correction applied to moderated t-test (not adjusted for confounders) Cell-type heterogeneity corrected results using the reference-free approach in the supplemental.	1708 MVPs at adjusted $P < 0.05 > 5\%$. MVPs in 1st exons enriched for pathways involved in endocytosis, MAPK signaling and extracellular triggers to metabolic processes
Rong et al 2015 Ref. 59	GDM N= 36 Control N = 40 Nanjing, China	GDM diagnosed with glucose following 2h post-OGTT (2nd trimester, wk 24–28)	NimbleGen microarray Bisulfite DNA pyrosequencing	Bisulfite pyrosequencing used to validate array results for <i>RETN</i> , <i>GLUT3</i> , <i>PPARA</i> and <i>RBP4</i> Non-adjusted one-way ANOVA. No FDR or Bonferroni adjustment or replication.	6,641 DMRs (3,320 genes) DMGs enriched for pathways related to cell growth/ regulation, immune/inflammatory responses and nervous system development. Technical validation of 3 metabolic genes <i>GLUT3</i> , <i>Resistin</i> and <i>PPARA</i> . Differences in placenta mRNA between GDM and controls observed for <i>GLUT3</i> and <i>RETN</i> .
Binder et al 2015 Ref. 60	N=82 GDM N=41 Controls N=41 Harvard Epigenetic Birth Cohort (HEBC) Boston, MA USA & RICHs cohort Providence, RI, USA	GDM cases (GDM diagnosis excluded preexisting hypertension or diabetes) Controls (no GDM, no pregnancy-induced hypertension/preeclampsia, no previous hypertension or diabetes)	450K Infinium Methylation BeadChip Array Bisulfite DNA Pyrosequencing for validation	Site-specific methylation modeled adjusting for maternal age (years), pre-pregnancy BMI (kg/m ²), infant sex, maternal smoking, and independent surrogate variables to assess technical variation in the methylation data. FDR to correct P values for multiple testing. Selected results tested for replication in an independent cohort (RICHs)	78 DMRs ($P < 0.001$) Findings in the MHC region partially validated in an independent cohort (RICHs). MHC transcripts also different between GDM compared to non-GDM placentas.

1 st Author, year	Population	Exposure definition	Platform	Confounders, Validation & Replication	Main findings
Cardenas et al 2018 Ref. 65	N= 448 French-Canadian Canada Gen3G Birth Cohort Sherbrooke, Quebec	Maternal glucose following 2h post-OGTT test (2nd trimester, wk 24-28)	850K Infinium Methylation BeadChip EPIC Array Bisulfite DNA Pyrosequencing	Robust linear regression models adjusted for maternal age, BMI, parity, smoking during pregnancy, gestational age, sex and the first 10 principal components estimated from a reference-free cell-type heterogeneity method as proxy for cellular heterogeneity. P-values adjusted with FDR at 5% (q-value<0.05) Bisulfite DNA pyrosequencing for replication in another smaller population (N=65 to 108).	Seven CpG sites (q<0.05) associated with ↑ 1 mMol/L 2h-glucose postOGTT: <i>TNFRSF1B</i> (cg26189983) 1.22% ↑ methylation <i>PDE4B</i> (cg07734160, cg13866577, cg03442467, cg13349623) ↓ methylation (-0.58 to -1.16%) <i>BLM</i> (cg20254265) -0.63% ↓ methylation <i>LDLR</i> (cg08483713) -0.27% ↓ methylation. <i>PDE4B</i> methylation (cg03442467 gene body/TSS200) correlated with <i>PDE4B</i> ↑ expression (r=0.31; P=1.81×10 ⁻³), <i>TNFRSF1B</i> methylation (cg26189983) correlated with expression (r= -0.24; P=0.013). <i>LDLR</i> methylation (cg08483713 gene body) associated with ↑ expression (r=0.32, P=9.62×10 ⁻⁴)
Mitsuya et al 2017 Ref. 66	Lean N=21 Obese N=21 San Antonio Texas, USA	Lean: pre-pregnancy BMI <25 kg/m ² Obese: pre-pregnancy BMI >30 kg/m ² Normotensive with no other medical or pregnancy complications	NimbleGen 2.1M human DNA methylation array & bisulfite pyrosequencing Mass spectrometry placental metabolites	Fisher's for differentially methylated between lean and obese (P<0.05). No multiple-comparison or confounder adjustment Array findings for <i>CM7MI</i> hydroxymethylation and methylation validated with TET-assisted bisulfite (TAB)-pyrosequencing.	21% ↑ methylated but a 31% decrease in hydroxymethylated regions in obese vs lean groups 262 genes showed reciprocal differential methylation/hydroxymethylation were enriched for pregnancy, immune response and cell adhesion-linked processes

IGT=impaired glucose tolerance; NGT=normal glucose tolerance; GDM gestational diabetes mellitus; OGTT oral glucose tolerance test; wk= weeks; PE= preeclampsia; BMI=body mass index; 2h post-OGTT =two hours after a 75-g oral glucose tolerance test; DMG=differentially methylated regions; DMGs= differentially methylated CpGs