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# Maternal Obesity and Gestational Diabetes are Associated with Placental Leptin DNA Methylation

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## Abstract

**OBJECTIVE—**In this study, we aimed to investigate relationships between maternal prepregnancy obesity and gestational diabetes mellitus and placental leptin DNA methylation.

**STUDY DESIGN—**This study comprises data on 535 mother-infant dyads enrolled in the Rhode Island Child Health Study (RICHS), a prospective cohort study of healthy term pregnancies. Prepregnancy body mass index was calculated from self-reported anthropometric measures and gestational diabetes mellitus diagnoses gathered from inpatient medical records. DNA methylation of the leptin promoter region was assessed in placental tissue collected at birth using quantitative bisulfite pyrosequencing.

**RESULTS—**In multivariable regression analysis adjusted for confounders, infants exposed to gestational diabetes mellitus had higher placental leptin methylation ($\beta$=1.89, $P$=0.04), as did those demonstrating prepregnancy obesity ($\beta$=1.17, $P$=0.06). Using a structural equations model (SEM), we observed that gestational diabetes mellitus is a mediator of the effects of prepregnancy obesity on placental leptin DNA methylation ($\beta$ =0.81, 95% CI: 0.27, 2.71).

**CONCLUSIONS—**Our results suggest that maternal metabolic status before and during pregnancy can alter placental DNA methylation profile at birth and potentially contribute to metabolic programming of obesity and related conditions.

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**Keywords**

leptin; DNA methylation; maternal obesity; gestational diabetes mellitus; placenta

## 1. INTRODUCTION

Maternal obesity and gestational diabetes mellitus (GDM) constitute two common, often comorbid pregnancy complications.[1] In line with the developmental origins of health and disease (DOHaD) hypothesis[2], increasing evidence suggests that these conditions modify the intrauterine environment and augment the offspring's risk of obesity and diabetes in adult life.[3, 4] Epigenetic marks have been proposed as a mechanism for this developmental programming because they respond to environmental stimuli but they are also mitotically stable.[5] Due to the high tissue-specificity of epigenetic mechanisms[5], though, it is critical to appropriately focus studies in relevant tissues. The placenta, a metabolically active organ that regulates the intrauterine environment and is crucial for fetal growth and development, is such a tissue.[6, 7]

Leptin is an adipokine central for energy homeostasis that functions as a satiety signal. During pregnancy, leptin is produced by the placenta where it has pleiotropic functions, including regulating growth and nutrient exchange.[8] Leptin gene (*LEP*) expression is inversely correlated with promoter DNA methylation[9–13] and has been proposed as mediator of metabolic programming[14]. In male rodents, *in utero* exposure to a low protein diet is associated with *LEP* promoter hypomethylation in adipose tissue, changes in body composition and increased food intake[15, 16]. In humans, *in utero* famine exposure has been associated with *LEP* promoter hypermethylation in blood of adult men compared to their non-exposed siblings[17]. In humans and rodents, maternal over-nutrition produces similar adverse metabolic offspring phenotypes to under-nutrition[14]. Hence, in this study, we sought to investigate associations between maternal prepregnancy obesity and GDM and placental *LEP* DNA methylation in a birth cohort of healthy newborns.

## 2. MATERIALS AND METHODS

### 2.1 Study Population

Study participants are part of the Rhode Island Child Health Study (RICHS), which enrolls mother-infant dyads following delivery at Women and Infants Hospital of Rhode Island.[18] Term infants born small for gestational age (SGA, <10th percentile), or large for gestational age (LGA, >90th percentile), based on birth weight percentiles[19] are selected, and infants appropriate for gestational age (AGA, 10th and 90th percentile) matched on sex, gestational age (±3 days), and maternal age (±2 years) to SGA and LGA participants are enrolled[20]. Only singleton, viable infants without congenital or chromosomal abnormalities were recruited. Additional exclusion criteria include maternal age <18 years and life-threatening conditions. Post-recruitment infants were re-classified into birth weight groups using sex-specific growth charts.[21] In this analysis, we examined the first 535 RICHS participants enrolled between September 2009 and October 2012 with placental *LEP* methylation information. A structured chart review served to collect information from

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inpatient medical record from delivery, and mothers completed an interviewer-administered questionnaire. Self-report of weight and height obtained during the interview served to calculate maternal prepregnancy body mass index (BMI). GDM status was obtained from medical charts. All subjects provided written informed consent. Protocols were approved by the Institutional Review Boards for Women and Infants Hospital of Rhode Island and Dartmouth College and carried out in accordance with the Declaration of Helsinki.

### 2.2 *LEP* DNA methylation analysis and genotyping

Placental samples were collected from all subjects within two hours following delivery. Twelve fragments of placental parenchyma, three from each quadrant, were obtained two centimeters (cm) from the umbilical cord and free of maternal decidua. Collected tissue was immediately placed in RNAlater solution (Life Technologies, Grand Island, NY, USA) and stored at 4°C. After at least 72 hours, tissue segments from each placental region were blotted dry, snap frozen in liquid nitrogen, homogenized by pulverization using a stainless steel cup and piston unit (Cellcrusher, Cork, Ireland) and stored at −80°C until needed. DNA was extracted from homogenized placental samples using the DNAeasy Blood & Tissue Kit (Qiagen, Inc, Valencia, CA, USA) and quantified using the ND 2000 spectrophotometer (Thermo Fisher Scientific Inc., Watham, MA, USA). DNA (500 ng) was sodium bisulfite-modified using the EZ DNA methylation Kit (Zymo Research, Irvine, CA, USA). For DNA methylation detection, bisulfite pyrosequencing was employed. Bisulfite PCR conditions, primer sequences (Integrated DNA Technologies, Inc, Coralville, IA) and pyrosequencing assays are detailed Supplementary Table 1. We measured DNA methylation at 23 CpGs in the *LEP* promoter using the PyroMark MD (Qiagen) and genotyped the SNP rs2167270 (+19 G>A) in the region. Genotype calls were made by comparing peak heights; triplicate wells were called independently and compared for quality control. All procedures were performed following manufacturer's protocols.

### 2.3 Statistical analysis

Pairwise Pearson correlations were used to compare continuous *LEP* methylation between the 23 CpGs loci analyzed. Self-reported gestational weight gain (GWG) data was combined with prepregnancy BMI to construct a categorical variable following the Institute of Medicine cutoffs.[22] Bivariate analyses were performed using Student's t-test, one-way ANOVA or Pearson's correlation, as appropriate. $\chi 2$ tests were used to assess frequency distributions. Multivariable analyses were completed using linear regression models, with continuous *LEP* methylation as the outcome and maternal and infant characteristic as predictor variables. A structural equation model (SEM) was used to assess mediation effects between predictors using Mplus, version 7.11 (Muthén & Muthén, Los Angeles, CA). A bootstrap method[23] was used to estimate the mediational effect. All other analyses were conducted in R 3.0.1. The multivariable regression and SEM were adjusted for potential confounders: rs2167270 genotype, infant sex, maternal age, birth weight group. Confounders included in the models were significantly associated with methylation in the bivariate analysis and also associated with methylation at a $P = 0.1$ level in a fully adjusted multivariable linear model (data not shown) or are part of the RICHS study matching criteria (maternal age and birth weight group). All tests were two-sided and statistical significance was determined at $P<0.05$.

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# 3. RESULTS

## 3.1 Study Population

The study population characteristics are summarized in Table 1. In accordance with the study design, all infants were born at term with overrepresentation of LGA and SGA and even distribution by sex. The majority of infants were born to Caucasian mothers (74.1%) that ranged between 18 and 40 years (yrs.) of age (mean=30 yrs.). The prevalence of maternal prepregnancy obesity and GDM in this study was 26% (n=135) and 10% (n=47), respectively. In addition, amongst study participants with medical chart diagnosis of GDM 61% were prepregnancy obese. There were no significant differences between the sample of participants analyzed in this study and the larger RICHS cohort in terms of maternal age, prepregnancy maternal obesity, GDM, infant sex or birth weight group.

## 3.2 Placental *LEP* DNA methylation

There was a high degree of inter-correlation of DNA methylation at each of the 23 CpGs (mean r = 0.7), thus we used the mean across the region. Mean *LEP* methylation was normally distributed and ranged from 9 to 45%. Genotypes frequencies at rs2167270 were in Hardy-Weinberg equilibrium, with 15% of the infants homozygous for the variant allele (A), 44% heterozygous and 41% and homozygous for the dominant allele (G).

## 3.3 Infant and maternal predictors of placental *LEP* DNA methylation

The results of the bivariate analyses between *LEP* methylation and maternal and infant characteristics are presented in Table 2. As previously reported[10], placental *LEP* methylation extent was higher in infants with the A/A genotype and in males. Strikingly, we did not observe associations with infant birth weight. We observed higher methylation in placentas from infants born to prepregnancy obese mothers ($P$=0.03) and from those diagnosed with GDM ($P$=0.01). Subsequently, we constructed a multivariable linear regression model to predict *LEP* methylation adjusted for all significant covariates from the bivariate analyses and the study matching criteria (Table 3). Consistently, we observed associations between placental *LEP* methylation and infant sex and genotype. In addition, placentas from infants exposed to GDM had 1.89% higher methylation compared to those from the non-GDM group. In contrast, in the multivariable model, maternal prepregnancy obesity was no longer a significant predictor of *LEP* methylation ($\beta$=1.17, $P$=0.06). However, obesity was strongly associated with GDM ($P$<0.001, $\chi 2$ test). This attenuation pattern suggested that the initial association between obesity and placental *LEP* methylation may be mediated through GDM. We did not observe associations with GWG or any other maternal characteristic.

## 3.4 GDM mediates prepregnancy obesity effects on placental *LEP* methylation

To build a formal path from maternal obesity to *LEP* methylation that accounts for the effects of GDM, we constructed a SEM (Figure 1) adjusted for the variables used in Table 3. This model has a good fit according to the fitness indices (e.g., Tucker Lewis Index=1). In this model, we found a significant association in the path from prepregnancy obesity to GDM (OR=2.7, $P$<0.001) and from GDM to increased placental *LEP* methylation ($\beta$=2.51,

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*P*=0.04), suggesting that placentas from infants born to a GDM mother have 2.5% higher methylation levels than those born to a non-GDM mother. Moreover, using a bootstrap method, we observed that GDM is a significant mediator between prepregnancy obesity and *LEP* methylation (β=0.81, 95% CI: 0.27, 2.71). In the SEM, the direct effect of prepregnancy obesity on *LEP* methylation is non-trivial (β=0.59, *P*=0.41) although is not significant, implying that GDM is likely a partial mediator. As expected infant sex and genotype also contributed independently to *LEP* methylation.

## 4. DISCUSSION

This is the first study that has associated *in utero* exposure to preconception maternal obesity and GDM with placental *LEP* DNA methylation. Our findings suggest that methylation is higher in infants born to prepregnancy obese mothers and that this association is mediated by GDM. These findings are in line with other epidemiological studies that described associations between periconceptional parental obesity and cord blood DNA methylation patterns at imprinted[24] and non-imprinted loci[25], suggesting that metabolic exposures can influence offspring epigenetic signatures and possibly later-life disease risk.

The placenta is a substantial source of leptin.[8] *LEP* mRNA is expressed on the maternal and fetal sides, while the leptin receptor (*LEPR*) is expressed predominantly on the maternal side, suggesting that placental and maternal serum leptin could regulate placental production of the hormone.[8] In obese pregnancies, downregulation of *LEPR* mRNA in the syncytiotrophoblast without increased leptin protein levels[26], suggests the existence of placental leptin resistance; analogous to hypothalamic resistance encountered in obesity.[8] Furthermore, during pregnancy obese women exhibit overall higher serum leptin, but non-obese women display higher increases of leptin production per BMI unit[27], suggesting differential placental leptin production. A recent study supports these findings, maternal obesity was associated with a lipotoxic placental environment characterized by widespread of changes in placental gene expression, including reduced *LEP* gene expression compared to placentas from non-obese controls.[28] Hence, we can hypothesize that our observation of higher *LEP* promoter DNA methylation in placentas from obese pregnancies could result in lower placental leptin production due to resistance mechanisms in response to basal hyperleptinemia. This could serve as a placental adaptive response to control fetal growth in cases of positive energy balance such as prepregnancy obesity.[8] However, we cannot directly investigate this hypothesis, and future research should address this issue.

We observed higher *LEP* methylation in GDM placentas compared to the non-GDM group. In contrast, a previous report[9] did not find differences in placental *LEP* DNA methylation in infants from mothers with normal (n=25) compared to impaired glucose tolerance (IGT) (n=22). However, they observed a negative correlation between glucose levels (at 24–28 weeks) and *LEP* methylation on the fetal side of the placenta and, intriguingly, a positive correlation between glucose and maternal side *LEP* methylation. A more recent study[29], measured *LEP* methylation in chorionic villus samples at birth from 100 newborns, and observed lower methylation in the GDM group (n=59), but these results did not withstand adjustment for confounders including BMI and infant sex. We have consistently shown[10, 30] that sex is an important predictor of placental *LEP* methylation. Given the sexual

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dimorphism displayed by leptin[8], the differential effect of *LEP* methylation on expression by sex[10], and sexually dimorphic placental biology[31], future research should take infant sex into account. With regards to placental *LEP* gene expression in GDM, the literature shows mixed results[32]; a number of studies report higher expression and others no differences in expression.[26, 32] These differences might result from inter-study variability and low sample size. Interestingly, a study in tissue explants found significantly higher leptin release from placenta, amnion and choriodecidua obtained from normal pregnancies compared to GDM[33], result consistent with our findings.

During normal gestation, placental leptin production induces a hyperleptinemic state compensated by hypothalamic leptin resistance, necessary to increase food intake.[8] Additionally, around mid-gestation progressive insulin resistance occurs mediated in part by increased adiposity and placental hormones.[34] In GDM, pregnancy-induced insulin resistance usually occurs over the chronic insulin resistance state frequently related to prepregnancy obesity.[1] Moreover, maternal prepregnancy overweight status has been associated with overweight and abdominal obesity in offspring, with stronger associations when GDM is also present.[35] Given the metabolic similarities within the endocrine milieu of obesity and GDM, it is possible that these states produce similar effects on placental *LEP* methylation. Our results support this hypothesis and given the temporality and comorbidity between these conditions, it is plausible that GDM acts as a partial mediator of the effects of maternal obesity on epigenetic control of placental *LEP* DNA methylation.

Previously, we observed an association between prepregnancy obesity and lower *LEP* DNA methylation in maternal blood, possibly reflecting hyperleptinemia and increased adiposity.[30] Additionally, *LEP* methylation in maternal blood correlated to cord blood and consequently methylation was lower in cord blood of infants born to obese mothers. This study complements those findings and supports the known inter-tissue variability of DNA methylation.[5] Interestingly, these findings show that maternal obesity can produce contrasting patterns of *LEP* promoter methylation between fetal tissues. As a key endocrine organ, placental methylation patterns could reflect adaptive responses to adverse metabolic intrauterine environment of prepregnancy obesity and GDM. Importantly, we have also demonstrated a link between increased placental *LEP* methylation and membership in a neurobehavioral profile characterized by lethargy and hypotonicity[10], similar to the behavioral phenotypes of *Lep* deficient ob/ob mice. This association was only observed in male infants and could be partially explained by differential relation between *LEP* methylation and gene expression between sexes[10]. However, sex differences in offspring outcomes have been observed before in human and animal studies of developmental programming[36] and could result from differential fetal and placental adaptations to the early-life environment. It remains to be determined whether placental *LEP* methylation could program obesity risk during childhood or later in life and help explain the relation between adverse nutritional *in utero* environments and offspring disease risk.[37]

Our study has several strengths, including a large sample of placentas from healthy infants and reliable measurements of *LEP* DNA methylation. However, this study is limited in our ability to define mechanisms behind these associations and we only studied one gene in two complex polygenic phenotypes. Also, in this study we used the average DNA methylation

value across a region of 23 CpG sites. This could add a variance component that is not accounted in our analyses. Additionally, maternal BMI was derived from self-reported data that might lead to misclassification, although is likely under-reported and would bias our results to the null. GDM diagnoses were collected from medical chart records and we could not reliably obtain data on laboratory testing for this condition.

In summary, we established an association between prepregnancy obesity and placental *LEP* DNA methylation mediated by GDM in the largest study to date of healthy term infants. We confirmed previously observed associations between infant sex and genotype and placental *LEP* methylation[10]. These data suggest that placental epigenetic alteration of *LEP* may be one mechanism through which maternal phenotypes can program offspring health.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Abbreviations

| | |
|---|---|
| ***LEP*** | leptin gene |
| **GDM** | gestational diabetes mellitus |
| **AGA** | adequate for gestational age |
| **LGA** | large for gestational age |
| **SGA** | small for gestational age |
| **GWG** | gestational weight gain |
| **SEM** | structural equation model |
| **SNP** | single nucleotide polymorphism |

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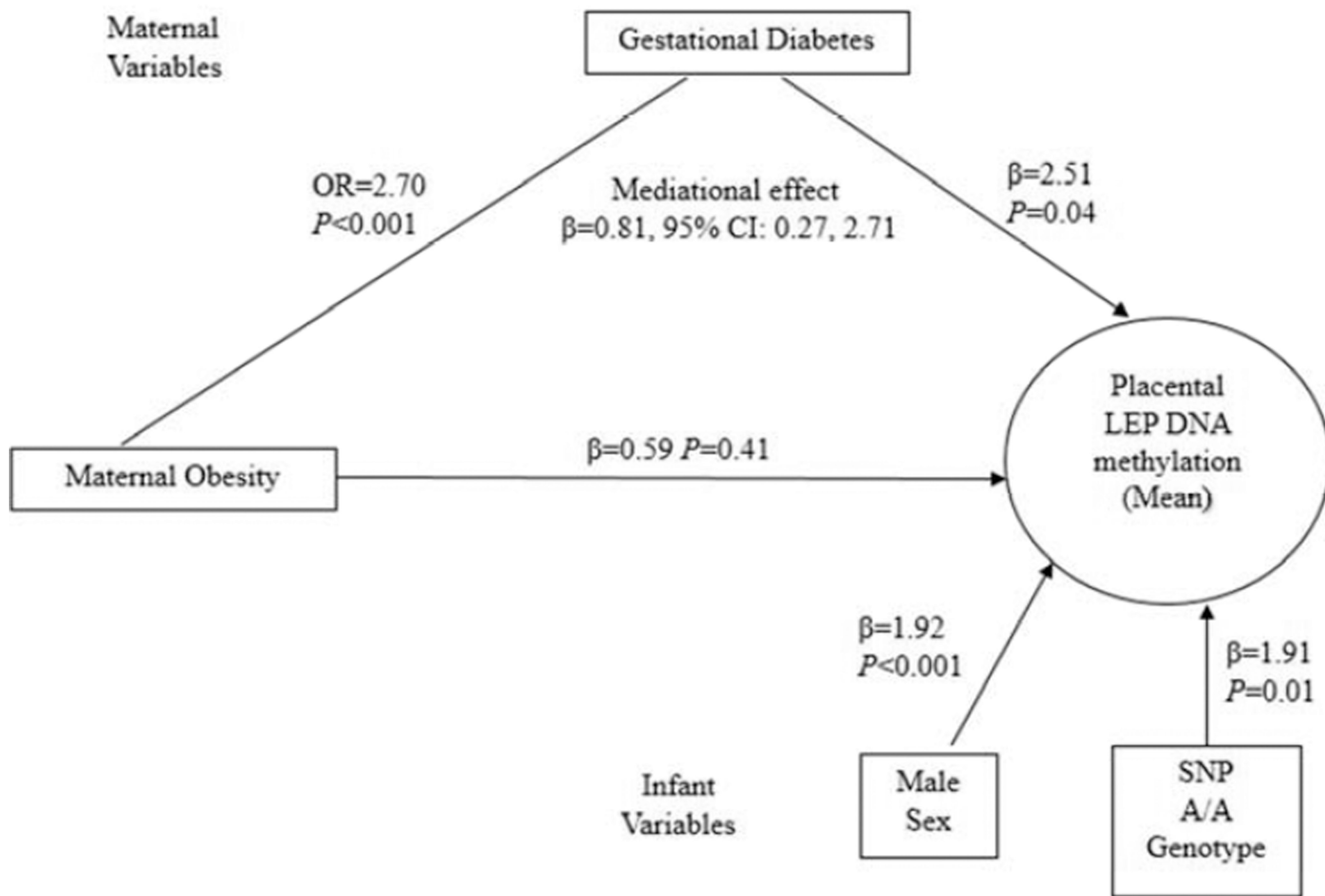


**Figure 1.**

Structural equation model relating maternal prepregnancy obesity to placental *LEP* DNA methylation through gestational diabetes in RICHS participants (n=473). Model is adjusted for variables in the figure, birth weight group and maternal age (continuous). Odds ratios (OR) are provided from logistic regression when the outcome (in this case gestational diabetes) was dichotomous, and the betas provided individual paths are standardized partial regression coefficients that can be interpreted similar to betas for linear regression.

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**Table 1**

Study population characteristics n % mean SD missing data

| | n | % | mean | SD | missing data |
|---|---|---|---|---|---|
| **Infant characteristics** | | | | | |
| Gestational age | 535 | | 39.0 | 1.0 | |
| Birth weight | 535 | | 3486.6 | 696.6 | |
| AGA | 281 | 52.5 | | | |
| LGA | 140 | 26.2 | | | |
| SGA | 114 | 21.3 | | | |
| Sex | | | | | |
| Male | 270 | 50.5 | | | |
| Female | 265 | 49.5 | | | |
| Delivery Method | | | | | |
| C-Section | 273 | 51 | | | |
| Vaginal | 262 | 49 | | | |
| Genotype (rs2167270) | | | | | |
| G/G,G/A | 457 | 85.4 | | | |
| A/A | 78 | 14.6 | | | |
| **Maternal characteristics** | | | | | |
| Age | 535 | | 29.6 | 5.6 | |
| BMI (Kg/$m^2$) | 529 | | 26.8 | 7.1 | 6 |
| Prepregnancy obesity | | | | | 6 |
| No (BMI <30) | 394 | 74.5 | | | |
| Yes (BMI 30) | 135 | 25.5 | | | |
| Gestational diabetes mellitus | | | | | 56 |
| No | 432 | 90.2 | | | |
| Yes | 47 | 9.8 | | | |
| Gestational weight gain | | | | | 9 |
| Inadequate | 101 | 19.2 | | | |
| Adequate | 135 | 25.7 | | | |
| Excessive | 290 | 55.1 | | | |

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| | n | % | mean | SD | missing data |
|---|---|---|---|---|---|
| **Infant characteristics** | | | | | |
| Ethnicity | | | | | 3 |
| Other | 138 | 25.9 | | | |
| White | 394 | 74.1 | | | |
| Tobacco use during pregnancy | | | | | 7 |
| No | 502 | 95.1 | | | |
| Yes | 26 | 4.9 | | | |
| History diabetes type I | | | | | 59 |
| No | 471 | 98.9 | | | |
| Yes | 5 | 1.1 | | | |
| History diabetes type II | | | | | 59 |
| No | 472 | 99.2 | | | |
| Yes | 4 | 0.8 | | | |
| Pregnancy hypertension | | | | | 10 |
| No | 490 | 93.3 | | | |
| Yes | 35 | 6.7 | | | |

SD, standard deviation; AGA, adequate for gestational age, LGA, large for gestational age, SGA, small for gestational age

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**Table 2**

Bivariate analysis of infant and maternal variables and placental *LEP* methylation

| **Infant** | **n** | **Mean *LEP*** | **SD** | ***P*** | **Maternal** | **n** | **Mean *LEP*** | **SD** | ***P*** |
|---|---|---|---|---|---|---|---|---|---|
| Birtd weight group | | | | 0.80 | Prepregnancy obesity | | | | 0.03 |
| AGA | 281 | 24.1 | 6.0 | | No | 394 | 23.6 | 5.9 | |
| LGA | 140 | 23.9 | 5.8 | | Yes | 135 | 24.9 | 6.2 | |
| SGA | 114 | 23.7 | 6.3 | | Gestational diabetes mellitus | | | | 0.01 |
| Sex | | | | <0.0001 | No | 432 | 23.7 | 5.8 | |
| Female | 265 | 22.8 | 5.5 | | Yes | 47 | 26.2 | 6.4 | |
| Male | 270 | 25.1 | 6.3 | | Ethnicity | | | | 0.51 |
| Genotype | | | | 0.006 | Other | 138 | 24.3 | 6.5 | |
| Any G | 457 | 23.7 | 6.0 | | White | 394 | 23.9 | 5.9 | |
| A/A | 78 | 25.7 | 5.8 | | Tobacco use during pregnancy | | | | 0.51 |
| | | | | | No | 502 | 24.0 | 6.1 | |
| | | | | | Yes | 26 | 24.6 | 4.9 | |
| | | | | | Gestational weight gain | | | | 0.46 |
| | | | | | Low | 101 | 24.2 | 6.7 | |
| | | | | | Adequate | 135 | 23.3 | 5.3 | |
| | | | | | High | 290 | 24.0 | 6.0 | |
| | | | | | Pregnancy hypertension | | | | 0.76 |
| | | | | | No | 490 | 24.0 | 6.0 | |
| | | | | | Yes | 35 | 24.3 | 6.4 | |
| | | | | | History of type II diabetes | | | | 0.40 |
| | | | | | No | 472 | 23.9 | 5.9 | |
| | | | | | Yes | 4 | 21.5 | 4.9 | |
| | | | | | History of type I diabetes | | | | 0.95 |
| | | | | | No | 471 | 23.9 | 5.9 | |
| | | | | | Yes | 5 | 24.1 | 4.7 | |
| | | | | | | | r | | *P* |
| | | | | | Maternal age | | −0.05 | | 0.26 |

SD, standard deviation; AGA, adequate for gestational age, LGA, large for gestational age, SGA, small for gestational age

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## Table 3

Multivariable linear regression model† of placental *LEP* methylation predictors

| | (n=473) | | |
|---|---|---|---|
| | **Estimate** | **Standard Error** | ***P*** |
| Prepregnancy obesity | | | |
| No | Reference | | |
| Yes | 1.17 | 0.62 | 0.06 |
| Gestational diabetes mellitus | | | |
| No | Reference | | |
| Yes | 1.89 | 0.92 | 0.04 |
| Maternal age | –0.07 | 0.05 | 0.15 |
| Infant sex | | | |
| Female | Reference | | |
| Male | 2.28 | 0.53 | <0.0001 |
| Infant genotype (rs2167270) | | | |
| G/G and G/A | Reference | | |
| A/A | 2.17 | 0.73 | 0.003 |
| Birth weight group | | | |
| AGA | Reference | | |
| LGA | –0.70 | 0.61 | 0.25 |
| SGA | –0.25 | 0.70 | 0.73 |

†Model is adjusted for all variables in the table

AGA, adequate for gestational age; LGA, large for gestational age; SGA, small for gestational age.