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IGF2/H19 methylation at birth and risk of overweight and obesity in children

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Abstract

Objective—To determine if aberrant DNA methylation at differentially methylated regions (DMRs) regulating *Insulin-like Growth Factor 2* (*IGF2*) expression in umbilical cord blood (UCB) is associated with overweight or obesity in a multiethnic cohort.

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The authors declare no conflicts of interest.

Study design—UCB leukocytes of 204 infants born between 2005 and 2009 in Durham, NC were analyzed for DNA methylation at two *IGF2* DMRs using Pyrosequencing. Anthropometric and feeding data were collected at age one year. Methylation differences were compared between children >85th percentile of CDC weight-for-age (WFA) and those ≤85th percentile of WFA at one year using generalized linear models, adjusting for post-natal caloric intake, maternal cigarette smoking and race/ethnicity.

Results—The methylation percentages at the *H19* imprint center DMR was higher in infants with WFA>85th percentile (62.7%, 95%CI=59.9–65.5%) compared with infants with WFA≤85th percentile (59.3%, 95%CI=58.2–60.3), (p=0.02). At the intragenic *IGF2* DMR, methylation levels were comparable between infants with WFA≤85th and those with WFA>85th percentile.

Conclusions—Our findings suggest that *IGF2* plasticity may be mechanistically important in early childhood overweight or obese status. If confirmed in larger studies, findings suggest aberrant DNA methylation at sequences regulating imprinted genes may be useful identifiers of children at risk for early obesity.

Keywords

Obesity; Breast Feeding; Child; DNA Methylation; Epigenetics; Genetic; Insulin-Like Growth Factor II

The prevalence of obesity in children under the age of five years has more than doubled since the 1990s, affecting one in five children in the US, with minority populations disproportionately affected (1, 2). Early childhood obesity and excessive infant weight gain have been associated with higher blood pressure (3) and wheezing (4) in childhood, and obesity and metabolic and cardiovascular diseases in adulthood (reviewed in Ong) (5). Childhood obesity may be an early adaptive response hypothesized to be largely driven by epigenetic mechanisms that guide expression of genes involved in energy balance, culminating in gene expression profiles that predispose to overweight and obesity.

A commonly studied epigenetic mechanism is DNA methylation, in part because of its stability in conditions in which human specimens are collected. Animal evidence from the last decade indicates that DNA methylation alterations at susceptible loci link the early environment to obesity in later life. The monoallelic expression of imprinted genes-a class of genes that is over-selected for growth effectors (11) is regulated (and dysregulated) by DNA methylation at differentially methylated regions (DMRs). Aberrant methylation at these DMRs has been associated with aberrant changes in gene expression. Because imprinted genes occur in clusters throughout the genome (12) and their regulation may be networked (11), a single DMR can regulate the expression of several genes; suggesting aberrant methylation at a single DMR can affect the expression of several of these growth effectors. The most studied imprinted gene is *insulin-like growth factor 2* (*IGF2*). *IGF2* is a paternally expressed imprinted gene that encodes a potent mitogenic growth factor that plays a critical role in placental and fetal development. Aberrant DNA methylation at the *IGF2* DMRs has been associated with increased gene expression, and presumably, circulating IGF2 levels and risk of overweight status, obesity and overgrowth disorders (13).

Numerous epidemiological studies have reported a small but consistently lower risk of rapid growth and obesity in breastfed children, Though epigenetic mechanisms have been proposed (23), the mechanism by which breastfeeding confers a lower risk of childhood obesity remains unknown. Because breastfeeding varies by race/ethnicity, we evaluated whether aberrant DNA methylation at two *IGF2* DMRs at birth increases the risk of overweight and obesity status in early childhood, and this association may vary by ethnic group and breastfeeding.

METHODS

Study participants were children born to women who sought obstetric care at Duke Obstetrics and Durham Regional Hospital between 2005 and 2009, as part of the Newborn Epigenetics STudy (NEST). NEST is a prospective, perinatal epidemiologic study aimed at determining how the *in utero* environment influences epigenetic profiles and phenotypes in children. Procedures for participant enrolment have been detailed elsewhere (24). Briefly, between 2005 and 2009 women who attended prenatal care at Duke's Maternal Fetal Medicine and one affiliated clinic, and intended to use Duke or Durham Regional Hospitals for their obstetrics care were enrolled into the study. Inclusion criteria were age ≥18 years and English speaking. We excluded women who planned to give offspring up for adoption and HIV-positive women because the effect of antiretrovirals on the methylation profile is still unknown. Most women (> 50%) were from Durham County, North Carolina, though the catchment area also included contiguous counties.

Between 2005 and 2009, 940 (85%) of the 1101 women approached consented to participate and were enrolled in NEST. Of these, methylation analyses were performed on cord blood leukocyte DNA of the first 438 (46%) neonates. The final sample includes the first 204 offspring who had methylation data and had reached one year of age by August, 2010, whose mothers completed a follow-up questionnaire. Follow-up is on-going. The distribution of factors that may affect overweight status or obesity including maternal age (p=0.54), education (p=0.94), race/ethnicity (p=0.43) and sex of infant (p=0.80) were comparable between the 940 infant-mother pairs enrolled and 438 with DNA methylation data. These factors were also comparable among the first 204 infants in whom follow-up data have been collected to date and the 428 in whom methylation analyses were conducted. The majority of questionnaires (78%) were completed by mailed survey, 16% were interviewer-administered during a pediatric office visit, and 6% were telephone-administered.

To characterize the *in utero* environment, pregnant women completed a questionnaire soliciting information on sociodemographic characteristics, maternal lifestyle factors, and morbidity, at recruitment. Women self-reported maternal pre-pregnancy anthropometric measurements, race, level of education and cigarette smoking status. Self-reported height and usual pre-pregnancy weight were used to calculate maternal BMI. At delivery, medical records were abstracted to obtain maternal age at delivery and parturition data including morbidity during pregnancy, mode of delivery, infection in labor, gestational age at birth, and infant data including birth-weight, head circumference and Apgar score. Approximately one year after the child's birth, a 1-year questionnaire was completed to obtain data on anthropometric measures, temperament, and use of childcare. We also estimated the infant's caloric intake from a single 24-hour dietary recall, using the University of Minnesota's Nutrition Data System for Research (2008). Although a single 24-hour recall cannot adequately reflect total energy intake over the first year of life, we used this information as an indicator to adjust for, in the investigation of *IGF2* DMR methylation at birth, and overweight and obesity in early childhood.

To estimate breastfeeding status, we used additional dietary information collected in the 1-year questionnaire, which asked mothers to report—for each of the first 12 months of life—whether their child was fed breast milk, cow's milk formula, and/or soy milk. Specifically, mothers were asked “How did you feed your baby during his/her first year? (Please go month by month)”; for each month, mothers responded either “yes” or “no” individually to breast milk, formula, and soy milk. Questionnaires were completed between 12 and 29 months of age. The median age when the first follow-up questionnaire was mailed was 14 months, and the median time to returning the questionnaire was one month.

To verify the accuracy of the infant weights reported by mothers in the questionnaire, anthropometric measurements at age one were abstracted from the medical records of 72 infants who had the data available within one month of receipt of their 1-year questionnaire. The Pearson correlation coefficient between anthropometric measurements abstracted from the medical record and weights reported by mothers was 0.94 ($p<0.0001$), suggesting that mothers accurately reported their offspring's weight.

At delivery, cord blood specimens were collected in EDTA-treated tubes within minutes of delivery. Specimens were processed to obtain plasma and buffy coat for DNA extraction (Qiagen; Valencia, CA); samples were stored at −80°C until processed. DNA was extracted using Puregene reagents according to the manufacturer's protocol (Qiagen; Valencia, CA).

DNA methylation from leukocytes of umbilical cord blood samples is generally used as a surrogate measure of genomic stability in the study of prenatal exposures and epigenetic response to these exposures, as they contribute to the long term health in humans (25–30). Genomic DNA was modified by treatment with sodium bisulfite using a high throughput methods as previously described (31). Bisulfite treatment of denatured DNA converts unmethylated cytosines to uracils, and leaves methylated cytosines unchanged. Pyrosequencing was performed using a Biotage Pyromark MD pyrosequencing instrument (Qiagen; Valencia, CA). We evaluated two regions, including three CpG sites comprising the intragenic *IGF2* DMR, upstream of exon 3 (chr11p15.5, site 1: 2,109,519; site 2: 2,109516; and site 3: 2,109,500; NCBI Human Genome Build 37.1) and four CpG sites, hereafter referred to as the *H19* DMR, within a sequence motif that binds the CTCF zinc finger protein within the *IGF2*/*H19* imprint center (chr11p15.5, site 1: 1,964,261; site 2: 1,964,259; site 3: 1,964,257; and site 4: 1,964,254; NCBI Human Genome Build 37.1). Using the same CpGs evaluated here as estimates of DMR methylation, hypermethylation at the intergenic DMR upstream of *H19* (32, 33), (referred to as the *H19* DMR), and CpG hypomethylation at the intragenic *DMR* upstream of *IGF2* exon 3 (referred to as the *IGF2* DMR) (33), have been associated with increased *IGF2* transcriptional activity and loss of imprinting, and had been found dysregulated in multiple obesity-related cancer types (32, 34–40). These sequences have been described extensively (41) and are available at NCBI. Pyrosequencing assays were designed using PSQ Assay Design Software. Bisulfite conversion efficiency for each specimen was confirmed to be >95.5% through evaluation of non-CpG cytosines within the region sequenced. Average methylation at each CpG site was calculated from duplicate runs. Methylation data were 98% complete. The study protocol was approved by the Duke University Institutional Review Board (IRB), University of Texas MD Anderson Cancer Center IRB, and National Cancer Institute IRB.

Obesity and overweight status in early childhood are often estimated by weight adjusted for length. In the absence of length measurements, we estimated rapid early growth by approximately one year of age for each infant, we used children's weight at the most recent doctor's visit (reported by mothers) and age in months at date of return of the 1-year questionnaire, to compute sex-specific weight-for-age (WFA) percentile rank, based on the Centers for Disease Control (CDC) expected sex-specific smoothed for WFA (42). Because WFA was not normally distributed, infants were dichotomized into overweight or obese status, if WFA was >85th percentile, or non-obese or overweight if WFA was ≤85thpercentile, although a cutoff at the WFA >95th percentile was also considered. Also considered was weight gain, defined as the difference between birth weight and weight at follow-up interview, with cutoffs at > 85th and >95th percentile, as well as weight for length in the subset of infants in whom length data were available at age ~one year. To test for potential bias associated with the length of time to return the questionnaire in infants who were breastfed and those who were not, we used t-tests to evaluate the association between

time to return the questionnaire and both breastfeeding status (p=0.4) and overweight or obesity status (p=0.5).

For each of the three CpGs at the *IGF2* DMR and four CpGs at the *H19* DMR, the distribution of methylation percentages obtained from Pyrosequencing data were evaluated for normality using Kolmogorov-Smirnov tests and we found no evidence to suggest the values were not normally distributed. To determine whether a single mean could be used to estimate methylation fractions, we computed correlation coefficients among CpG dinucleotide percents for each DMR. The correlation coefficient for the four CpG dinucleotides at the intergenic *H19* D.MR ranged from 93% to 96%, and the correlation coefficients among the methylation fractions at the three CpG dinucluiotides at the *IGF2* DMR ranged from 81% to 83%. Based on these high correlations, we used a single (average) mean for each DMR, to compare DMR methylation fractions between infants WFA>85th and WFA≤ 85th percentile, in early childhood. Despite these high correlations among CpGs at each DMRs, we also repeated these analyses using mixed linear models to allow for unstructured model entry of individual CpGs.

We examined two measures of breastfeeding status. First, we dichotomized all participants into “never” (“no” to breast milk for all 12 months) versus “ever” breastfed (“yes” to breast milk for any of the 12 months). We also evaluated the effect of exclusive breastfeeding in the first 3 months by categorizing children in three: “never breastfed” (“no” breast milk for all 3 months), “mixed breastfeeding and formula” (“yes” to both breast milk and formula in any of the first 3 months), and “exclusively breastfed” (“yes” to breast milk for all 3 months and “no” to formula and soy milk for all 3 months). Self-reported maternal race/ethnicity was used as a proxy for the race/ethnicity of the infant.

Statistical analyses

Chi-square tests were used to compare maternal and offspring characteristics in relation to overweight status or obesity (dichotomized at WFA>85th percentile of the CDC growth charts). Least squares mean (lsmeans) DNA methylation percentages at the *H19* and at the *IGF2* DMRs were then compared between children with WFA>85th percentile and those with WFA≤85th percentile, using generalized linear models. Because early obesity has been shown to differ by race/ethnicity and breastfeeding status in the general population, we also explored potential effect modification of the associations between methylation percentages and obesity in early childhood, using stratified analyses, and also by computing and including in final models, cross product terms for methylation fraction and race/ethnicity and for methylation fraction and breastfeeding. A cross- product term with a p-value <0.10 was considered statistically significant. Factors found significantly associated with overweight status or obesity in Table I were evaluated for potential confounding in these models and those significantly associated were retained. Factors considered for potential confounding were maternal pre-pregnancy body mass index (BMI), prenatal morbidity, mode of delivery, race/ethnicity, education, cigarette smoking, birth weight, sex and caloric intake. To minimize residual confounding by caloric input, birth weight, and maternal BMI, these factors were entered into statistical models as continuous variables. Maternal morbidity (any vs. none), mode of delivery (vaginal vs. cesarean section), race/ethnicity (African and American, Whites and Other), sex (male vs. female), cigarette smoking (never smoked, stopped during pregnancy or quit before knowledge of pregnancy), education (up to some college vs. college or higher) were entered as categorical variables.

These analyses were repeated using multiple logistic regression models examining the association between aberrant DNA methylation and obesity in early childhood, stratifying by race/ethnicity and breastfeeding status, while adjusting for the same potential confounding factors. For these models, methylation fractions were dichotomized into

aberrant and normal categories using a cutoff of ≥75th percentile for the *H19* DMR, because hypermethylation at this DMR has most frequently been associated with *IGF2* deregulation. Aberrant methylation for the *IGF2* DMR was defined as ≤25th percentile, as hypomethylation at this DMR has been associated with *IGF2* deregulation (33). All statistical analyses were conducted using SAS 9.2 (SAS Institute, Cary, NC).

RESULTS

Table I summarizes the distribution of socio-demographic characteristics, anthropometric measures and lifestyle factors of the mother-infant pairs. Thirteen percent of infants were categorized as overweight or obese, based on the WFA cutoff of > 85th percentile. Compared with children with WFA≤85th percentile, those with WFA>85th percentile were more likely to be female (p=0.10), African American (p=0.10), had a larger birth weight (p=0.13), a larger weight gain (p<0.01), and a higher caloric intake during the first year of life (p<0.002). Obese and overweight children were, however, comparable with non-obese children with respect to maternal age at delivery, self-reported health status and chronic morbidity, as well as gestational age at birth, educational level, maternal BMI before pregnancy, infant breastfeeding and use of childcare outside the home.

Of the 204, 66% (n=133) reported ever breastfeeding their offspring during the first year of life; these proportions were comparable in overweight or obese (69%) and non-obese or overweight children (66%). During the follow-up period, 8.4% (n=17) infants had been diagnosed with conditions that included eczema, seizure disorders, cerebral palsy, and tonsillitis. All but two of the 17 children were breastfed, and only one obese or overweight. A combination of breast- and formula-feeding was reported among 27% of non-obese and 15% of obese or overweight children, and exclusive breastfeeding for at least three months was reported by 54% of obese or overweight compared with 40% of non-obese children (p=0.34). Never breastfeeding was comparable in obese or overweight (31%) and non-obese children (34%). In infants who were ever breastfed, the average duration of breastfeeding was 6.1 months (sd=4.5 months) (p=0.95).

Table II summarizes unadjusted CpG-specific and average DNA methylation levels at the *H19* and *IGF2* DMRs at birth, by the child's obesity and overweight status at approximately age one year. Among all infants, the average methylation fraction was 61% at the *H19* DMR (sd=8%; interquartile range 56%–63%) and 47% at the *IGF2* DMR (sd=7%; interquartile range 43%–51%). Unadjusted average methylation fraction at the *H19* DMR, was 3.4% higher in overweight and obese children compared with children who were neither obese nor overweight (p=0.03). This methylation difference was similar at all CpGs evaluated. Adjusting for maternal cigarette smoking, race/ethnicity and postnatal caloric intake did not materially alter this methylation difference (2.9% higher in obese or overweight children). Although birth weight and age at follow-up may be causally related to early obesity because intrauterine growth restriction may result in `catch up' growth during the early post-natal period, additionally adjusting for these factors as well as maternal pre-pregnancy body mass index, age at follow-up, mode of delivery, education, and sex, did not alter these findings (data not shown).

We explored the possibility that DNA methylation differences may vary by race and by ever-breastfeeding status by repeating the analyses, stratified by these factors (Table III). We found no differences in methylation fractions of overweight or obese children (59.6%, 95%CI=56.2–63.1%) vs. children who were neither overweight nor obese (59.2, 95%CI=57.9–60.5%) who were ever breastfed (p=0.82). However, among infants who were never breastfed, we noted an 8.4% (p=0.01) methylation difference between overweight and obese (68.3, 95%=62.3–74.2%) and children who were neither overweight nor obese (59.8,

95%CI=57.6–61.9). However, the cross-product terms for breastfeeding and *H19* methylation in the model adjusted for cigarette smoking and postnatal caloric intake, were not statistically significant (p=0.26), although further adjusting for race/ethnicity reduced the cross-product term p-value (p=0.20). Repeating these analyses using linear mixed models to allow simultaneous entry of individual CpG dinucleotide methylation fractions into statistical models, did not alter our findings (data not shown). Neither modeling these differences as continuous in linear regression models dichotomized at WFA>85th percentile, nor WFA>95th percentile materially altered our findings although estimates were less stable. Repeating these analyses with weight gain dichotomized at >85th percentile as the outcome, further adjusted for sex and age of offspring, revealed a 2.7% (p=0.19) higher methylation fraction at birth, in children with higher weight gain at age one year (data not shown). In the subset of children with length data at age one year, methylation fraction differences of a similar magnitude were also observed between children with weight-for-length >85th percentile and those with weight-for-length≤85th percentile (p=0.19). Following race/ethnicity stratification, we also found that methylation fractions of African American children with WFA≤85th percentile were comparable to those with WFA>85th percentile (p=0.46); although Caucasian children with WFA>85th percentile had somewhat higher methylation fractions at birth than those with WFA≤85th percentile (p=0.09). However, the cross-product term for methylation fraction and race/ethnicity was associated with a p-value=0.58. We found no significant differences in *IGF2* DMR methylation fractions between overweight or obese children, during the first year of life.

Because continuous data such as methylation fraction are sensitive to extreme values, we further explored the relationship among breast feeding and race, in the association between aberrant methylation and subsequent obesity or overweight status by repeating these analyses using logistic regression models. Hypermethylation was defined as DNA methylation fractions >75th percentile, and normal methylation otherwise, at the *H19* locus (Table IV). We found that among all children, after adjusting for caloric intake and cigarette smoking, the odds ratio (OR) for overweight or obese status associated with hypermethylation at the *H19* DMR was 3.7 (95% CI= 1.4–9.7) (data not shown); further adjusting for maternal BMI before pregnancy, education, birth weight and sex, did not alter this association. However, further adjustment for race/ethnicity reduced this association somewhat (OR=3.1, 95% CI= 1.1–8.3) (Table IV). Race/ethnicity-stratified analyses suggested the association between *H19* DMR hypermethylation and obesity or overweight status may not be more apparent in the 108 Caucasians (OR=4.4, 95%CI=1.0–20.4) than in the 85 African Americans (OR=2.3, 95%CI=0.6–9.1). In contrast, breastfeeding-stratified analyses after adjusting for postnatal caloric intake, race, and maternal smoking suggested the association between hypermethylation at the *H19* DMR and overweight or obesity status in early life was most pronounced in children who never breastfed (OR=22.3, 95%CI=2.1–239.8) compared with those who ever-breastfed (OR= 1.3, 95% CI=0.3,4.7). Intriguingly, the cross-product term for breastfeeding and the *H19* DMR methylation fraction was statistically significant (p=0.05). Further adjusting for race/ethnicity reduced the p-value for the cross-product term to 0.03 (Table IV). At the *IGF2* DMR, we found no DNA methylation differences between overweight or obese children vs. children who are neither obese nor overweight.

DISCUSSION

We found that children who were overweight or obese at age one year had higher methylation percentages at the *H19* DMR at birth compared with those who were neither overweight nor obese. Methylation differences of strikingly similar magnitude have been previously reported in relation to gene expression and several phenotypic differences (32, 34, 37, 48, 49). DNA methylation differences of a similar magnitude were found between

Dutch famine survivors compared with their same-sex siblings (15), and among Gambians conceived in the nutritionally challenging rainy season compared with the dry season (50). Our findings are consistent with the interpretation that the plasticity of *IGF2* may be mechanistically important in early childhood overweight status and obesity.

Although it has been hypothesized that epigenetic mechanisms may drive obesity in early childhood (23), our study offers empirical evidence linking obesity and overweight status to methylation patterns at a well known DMR regulating *IGF2*. DNA hypermethylation at the *H19* DMR has been previously associated with deregulation of paternally-expressed *IGF2*. Through mechanisms that are still unclear, *IGF2* dysregulation relaxes imprint controls, resulting in aberrant biallelic expression of a gene that is otherwise monoallelically expressed from the paternally-derived allele, thereby increasing transcription activity and, presumably, *IGF2* protein levels. Indeed higher circulating IGF2 protein levels have been associated with obesity in adults (14). If confirmed in larger studies, and hypothesized co-regulation of imprinted genes is fully characterized, these findings would support the hypothesis that aberrant DNA methylation at regulatory sequences of imprinted genes may be useful biosensors or markers to identify newborns exposed to an intra-uterine environment that increase risk of obesity in early childhood.

Some of our findings suggested that the magnitude of methylation differences between overweight or obese children and those whose weight was within normal range, was modified by breast feeding status. Even though cause-and-effect cannot be established in this epidemiologic study, these findings raise the possibility that lack of breastfeeding, a modifiable postnatal behavior, may interact with a prenatally acquired aberrant DNA methylation profile, to increase the risk of obesity or overweight status in early life. This possibility warrants further investigation as the potential public health implications could be sizable. However, the mechanisms are still unknown. The myriad of differences between breastfeeding and formula feeding have made it difficult to elucidate the reasons for possible interaction of breastfeeding and *H19* DMR hypermethylation. It is possible that infants with *H19* DMR hypermethylation at birth also are more likely to have metabolic dysregulation, which, together with infant formula, may increase the risk of obesity or overweight status. Also, breast milk contains not only *IGF2*, but also *IGF1* and IGF binding proteins (51). The early protein hypothesis posits that the higher levels of protein in infant formula exceed metabolic requirements, and that the metabolic products of excess protein may stimulate secretion of excess insulin and *IGF1*, leading to increased weight gain in early life (52). The results of the European Childhood Obesity Project support the early protein hypothesis; the study, which randomized infants to higher or lower protein formula, revealed a lower prevalence of obesity at age two in the lower protein group (53).

These findings, however, do not exclude the possibility that the effect of the interaction between *H19* DMR methylation and breastfeeding we observed may not be epigenetically driven. Breastfeeding is also associated with other psychosocial factors that are not adequately captured by socioeconomic status (as measured by educational level), raising the possibility of confounding by these unmeasured factors. For example, breastfed infants take smaller and more frequent meals than non-breastfed infants, which may influence later eating habits (54). In addition, day-to-day variability in the taste and smell of human milk, as opposed to the consistency of formula, may program infants to make more varied food choices later in life (55). Finally, breastfeeding may enhance emotional bonding between mother and child, establishing a psychological well-being that subsequently could influence health, in general. Disentangling these effects will require larger studies.

Although our study assessed for confounding maternal education, cigarette smoking, mode of delivery, birth weight, pre-pregnancy BMI, and race, there may still be residual

confounding by maternal nutrition during pregnancy, differential postnatal morbidity, and breast feeding that could influence methylation patterns and infant growth patterns. Only one of the 17 children with postnatal morbidity was overweight or obese. Nonetheless, if confirmed in larger studies where maternal nutrition and breastfeeding are assessed at shorter intervals, our observation that the growth trajectory of infants with DNA hypermethylation of the *H19* DMR at birth depends on breastfeeding status offers possibilities for early public health interventions on childhood obesity, and a means by which such benefits can be monitored. Evidence from animal studies involving *Agouti* mice demonstrated that hypomethylation induced by *in utero* or neonatal exposure to bisphenol A was negated by maternal dietary supplementation with methyl group donor nutrients (9). Such findings suggest that stable methylation alterations are potentially reversible with nutrition, presumably restoring normal gene function and offering, prospects for public health intervention.

Reasons for the lack of association between the intragenic *IGF2* DMR methylation profile and obesity in our study are unclear. Adults exposed to severe caloric restriction periconceptionally during the Dutch Hunger Winter had decreased methylation relative to unexposed same-sex siblings at this DMR (15). In general, adult survivors of the Dutch Famine have a higher risk of obesity and obesity-related chronic disease (56).

A strength of our study is the analysis of multiple, previously evaluated CpG dinucleotides at two DMRs regulating a well-characterized imprinted gene that encodes a potent growth factor that also has been associated with obesity in children (57–59) and adults (60), albeit inconsistently (61) (62). A potential limitation is that we did not measure and control for the potential confounding effect of white blood cell(WBC) counts as their relative abundance in specimen may influence methylation percentages. However, adjusting for infection during parturition (a major cause of variation in WBC), did not alter our findings. Furthermore, measuring DNA methylation obtained from leukocytes of unfractionated umbilical cord blood raises the possibility that DNA methylation percentages may be dependent on the predominant cell population in the specimen. However, we have previously shown that at the DMRs under evaluation, the methylation profiles were similar between polymorphonuclear and mononuclear cells (41), suggesting our findings may not have been unduly influenced by differences in blood composition. In addition, although we did not evaluate the temporal stability of methylation marks at the DMRs under study, several other studies have shown the temporal stability of methylation at one of these DMRs (63) (15), albeit at older ages.

Although findings from our exploratory race-stratified analyses were intriguing, disentangling potential epigenetic effects of race/ethnicity from those of breast-feeding will require larger studies. A small sample size also limited our ability to identify factors associated with varying degrees of childhood overweight and obesity status, including WFA>95th or 99th percentiles. Our use of WFA unadjusted for length, to estimate obesity, makes comparisons with many childhood obesity studies difficult. However, repeating these analyses using weight gain as an outcome, and weight for length in a subgroup of 72 infants in whom height data were recorded within one month of questionnaire administration, revealed DNA methylation differences of a strikingly similar magnitude, albeit less stable. Further, mothers' inability to recall infant feeding practices over the first year of life point to a need for a cautious interpretation of our findings, although in an earlier study, maternal recall did not appreciably modify the magnitude of the odds ratio for growth in infancy (64). We did not collect information on maternal diet during gestation and breastfeeding, nor did we assess the varying nutrient and hormone composition of breast milk. We also did not inquire about reasons for not breast feeding; some which also may vary by breastfeeding status and race. Because the effect of these factors on the methylation profile at the *IGF2/*

H19 region is unknown, we cannot predict possible changes in the direction of the association between methylation profiles and obesity or overweight status according to variations in breast milk composition. Although paternal height has been associated with childhood obesity (65), most mothers in our study were unable to report paternal height. Finally, we did not conduct sub-analyses by weight-for-gestational-age at birth; however, based on the 12 small-for-gestational-age and 18 large-for-gestational-age infants in our sample, we found no association between weight for gestational age and breastfeeding status (p=0.2) or methylation fraction (p=0.6–0.7), suggesting that infants' size at birth did not unduly influence the associations found.

Because methylation patterns can be evaluated at birth, our findings offer the possibility to identify individuals at higher risk of obesity before obesity becomes clinically evident, targeting interventions at mothers of at-risk infants. Should suggested breastfeeding differences be replicated in larger studies, this insight may offer new avenues for public health interventions aimed at decreasing or preventing early obesity.

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List of Abbreviations

DMR	differentially methylated regions
IGF2	Insulin-like Growth Factor 2

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Table 1

Characteristics of mothers and children, by percentile of child's weight at age 1-year

Characteristic	≤85th percentile weight for age (n=172)	>85th percentile weight for age (n=26)	p-value*
Maternal race			
African-American	64(37.7%)	15(57.7%)	
Caucasian	97(57.1%)	11(42.3%)	0.10
All others	9(5.3%)	0	
Missing	2	0	
Maternal level of education			
Up to some college	88(51.2%)	16(61.5%)	0.32
College graduate or graduate school	84(48.8%)	10(38.5%)	
Maternal BMI			
Mean (kg/m^2)	26.4	27.2	0.66
Range (kg/m^2)	16.3–57.0	18.9–43.9	
BMI < 25	94(58.0%)	11(44.0%)	
BMI 25–30	31(19.1%)	7(28.0%)	0.40
BMI ≥30	37(22.8%)	7(28.0%)	
Missing	10	1	
Maternal self-reported health status			
Excellent	40(23.3%)	6(23.1%)	
Very good or good	118(68.6%)	19(73.1%)	0.74
Fair or poor	14(8.1%)	1(3.9%)	
Maternal smoking			
Never smoked	107(62.2%)	12(46.2%)	
Smoked during pregnancy	51(29.7%)	10(38.5%)	0.24
Quit before knowledge of pregnancy	14(8.1%)	4(15.4%)	
Mean maternal age at delivery (years)			
Range (years)	30	29	0.32
Gestational age at birth	18–49	18–42	
Mean (weeks)	38	39	0.24
Range (weeks)	26–42	34–41	
<35 weeks	13(7.6%)	1(3.9%)	0.49
≥35 weeks	159(92.4%)	25(96.2%)	
Infant sex			
Male	96(55.8%)	10(38.5%)	0.10
Female	76(44.2%)	16(61.5%)	
Birth weight			
Mean (grams)	3135.6	3347.6	0.13
Range (grams)	760.0–5160.0	2070.0–4510.0	
Use of any childcare			
No	87(51.8%)	13(50.0%)	0.87
Yes	81(48.2%)	13(50.0%)	

Characteristic	≤85th percentile weight for age (n=172)	>85th percentile weight for age (n=26)	p-value*
Missing	4	0	
Weight gain (pounds)	14.5	19.6	<0.01
Range	8.7–22.1	9.9–28.9	
Mean daily caloric intake (Kcal)	1061.2	1369.8	0.002
Range (Kcal)	187.9–2938.3	304.6–3998.9	
Missing			
Breastfeeding: first 12 months of life			0.83†
Never breastfed	58(33.9%)	8(30.8%)	
Ever breastfed	113(66.1)	18(69.2%)	
Breastfeeding: first 3 months of life			
Never breastfed	58(33.7%)	8(30.8%)	
Mixed breast milk and formula	46(26.7%)	4(15.4%)	0.34‡
Exclusively breastfed	68(39.5%)	14(53.9%)	
Prenatal morbidity			
None	82(47.7%)	10(38.5%)	
&Any chronic disease	90(52.3%)	16(61.5%)	0.38

*p-value for association between characteristic and child's weight-for-age percentile. Calculated using t-test for continuous variables and using chi square for categorical variables.

†Calculated using Fisher's exact test;

‡Calculated using Cochran-Armitage trend test

&Any chronic disease includes gestational diabetes, asthma, hypertension, depression, diabetes mellitus, heart diseases, allergies, migraine headaches, epilepsy, anxiety, treated for cancer

Table 2

Methylation fraction at *H19* and *IGF2* DMRs at birth by percentile of child's weight at age ~1-year

Differentially Methylated Region	≤85th percentile of weight-for-age (n=172)		>85th percentile weight-for-age (n=26)		
	Mean methylation fraction	95% CL	Mean methylation fraction	95% CL	p-value*
***H19* DMR**					
Mean	59.3	58.2, 60.3	62.7	59.9, 65.5	0.03
CG1	61.6	60.3, 62.9	65.6	62.2, 69.0	0.03
CG2	57.8	56.7, 58.9	59.8	56.8, 62.8	0.22
CG3	58.9	57.7, 60.0	62.3	59.2, 65.4	0.04
CG4	59.0	57.8, 60.2	63.1	60.0, 66.3	0.02
***IGF2* DMR**					
Mean	47.5	46.6, 48.4	47.2	44.8, 49.7	0.84
CG1	41.7	40.3, 43.1	39.1	35.5, 42.8	0.19
CG2	50.9	49.8, 51.9	50.4	47.7, 53.2	0.78
CG3	51.2	49.9, 52.6	49.7	46.1, 53.3	0.43

*p-value comparing unadjusted lsmeans, DMR= differentially methylated regions

Table 3

DNA Methylation Differences between Obese or Overweight status in Breast and non-Breastfed African Americans and Whites

Differentially Methylated Region	≤85th percentile of weight-for-age (n=172)		>85th percentile of weight-for-age (n=26)		
	Mean methylation fraction	95% CL	Mean methylation fraction	95% CL	p-val
***H19* DMR**					
All participants (n=204)	59.3	58.3, 60.4	62.4	59.5, 65.4	0.05
Never breastfed (n=70)	59.8	57.6, 61.9	68.3	62.3, 74.2	0.01
Ever breastfed (n=133)	59.2	57.9, 60.5	59.6	56.2, 63.1	0.82
P-value for cross-product term for breastfeeding and *H19* DMR methylation					0.20
African Americans (n=85)	61.5	59.4, 63.6	63.4	58.9, 68.0	0.46
Whites (n=108)	58.1	56.8, 59.4	61.9	57.7, 66.2	0.09
P-value for cross-product term for race and *H19* DMR methylation					0.58
***IGF2* DMR**					
All participants	47.5	46.5, 48.4	47.2	44.6, 49.7	0.83
Never breast fed	46.7	45.1, 48.2	45.4	40.9, 49.9	0.59
Ever breastfed	47.8	46.6, 49.0	48.2	44.9, 51.4	0.82
P-value for cross-product term for breastfeeding and *IGF2* DMR methylation					0.71
African Americans	47.5	45.9, 49.0	46.0	42.5, 49.4	0.43
Whites	47.4	46.2, 48.7	48.2	44.4, 51.9	0.71
P-value for cross-product term for race and *IGF2* DMR methylation					0.87

-Models for All participants adjusted for maternal cigarette smoking, breastfeeding, race and postnatal caloric intake

-Breastfeeding-restricted models adjusted for maternal cigarette smoking, race and postnatal caloric intake

-Race/ethnicity-restricted models adjusted for maternal cigarette smoking, breastfeeding and postnatal caloric intake

Table 4

* ORs and 95% CIs for the association between DNA Methylation at birth and Obese or Overweight status in Breast and non-Breastfed African Americans and Whites

Differentially Methylated Region	OR	95% CL	p-val
[#] ***H19*** **DMR**			
All participants (n=204)	3.12	1.13,8.60	0.03
Never breastfed (n=70)	22.27	2.07,239.84	0.01
Ever breastfed (n=133)	1.25	0.34,4.67	0.74
P-value for cross-product term for breastfeeding and *H19* DMR methylation			0.03
African Americans (n=85)	2.38	0.62,9.10	0.21
Whites (n=108)	4.40	0.95,20.37	0.06
P-value for cross-product term for race and *H19* DMR methylation			0.46
[μ] ***IGF2*** **DMR**			
All participants	1.19	0.36,3.93	0.78
Never breast fed	1.91	0.22,16.69	0.56
Ever breastfed	0.69	0.14,3.43	0.65
P-value for cross-product term for breastfeeding and *IGF2* DMR methylation			0.25
African Americans	2.33	0.48,11.34	0.30
Whites	0.57	0.06,5.16	0.62
P-value for cross-product term for race and *IGF2* DMR methylation			0.23

-Models for All participants adjusted for maternal cigarette smoking, breastfeeding, race and postnatal caloric intake

-Breastfeeding-restricted models adjusted for maternal cigarette smoking, race and postnatal caloric intake

-Race/ethnicity-restricted models adjusted for maternal cigarette smoking, breastfeeding and postnatal caloric intake

[#] *H19* DMR mean methylation fraction ≥ 75th percentile

[μ] *IGF2* DMR mean methylation fraction ≤ 25th percentile