

Insulin-Like Growth Factor 2/H19 Methylation at Birth and Risk of Overweight and Obesity in Children

Ellen Perkins, MD¹, Susan K. Murphy, PhD^{2,3}, Amy P. Murtha, MD⁴, Joellen Schildkraut, PhD^{1,5}, Randy L. Jirtle, PhD⁶, Wendy Demark-Wahnefried, PhD⁸, Michele R. Forman, PhD⁹, Joanne Kurtzberg, MD⁷, Francine Overcash, MPH^{1,5}, Zhiqing Huang, MD, PhD², and Cathrine Hoyo, PhD¹

Objective To determine whether aberrant DNA methylation at differentially methylated regions (DMRs) regulating insulin-like growth factor 2 (*IGF2*) expression in umbilical cord blood is associated with overweight or obesity in a multiethnic cohort.

Study design Umbilical cord blood leukocytes of 204 infants born between 2005 and 2009 in Durham, North Carolina, were analyzed for DNA methylation at two *IGF2* DMRs by using pyrosequencing. Anthropometric and feeding data were collected at age 1 year. Methylation differences were compared between children >85th percentile of the Centers for Disease Control and Prevention growth charts weight-for-age (WFA) and children ≤85th percentile of WFA at 1 year by 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%) than in infants with WFA ≤85th percentile (59.3%; 95% CI, 58.2%-60.3%; *P* = .02). At the intragenic *IGF2* DMR, methylation levels were comparable between infants with WFA ≤85th percentile and infants 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, these findings suggest aberrant DNA methylation at sequences regulating imprinted genes may be useful identifiers of children at risk for the development of early obesity. (*J Pediatr* 2012;161:31-9).

The prevalence of obesity in children <5 years old has more than doubled since the 1990s, affecting 1 in 5 children in the United States, with minority populations disproportionately affected.^{1,2} Early childhood obesity and excessive infant weight gain have been associated with higher blood pressure³ and wheezing⁴ in childhood and obesity and metabolic and cardiovascular diseases in adulthood.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 children 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 effectors⁶ 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 genome⁷ and their regulation may be networked,⁶ 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.⁸

Numerous epidemiological studies have reported a small but consistently lower risk of rapid growth and obesity in breastfed children. Although epigenetic mechanisms have been proposed,⁹ the mechanism by which breastfeeding

BMI	Body mass index
CpG	Cytosine-phosphate-Guanine
DMR	Differentially methylated region
<i>IGF2</i>	Insulin-like growth factor 2
NEST	Newborn Epigenetics Study
WFA	Weight-for-age

From the ¹Department of Community and Family Medicine, ²Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, ³Department of Pathology, ⁴Department of Obstetrics and Gynecology, Division of Maternal-Fetal Medicine, ⁵Program of Cancer Detection, Prevention, and Control, ⁶Department of Radiation Oncology, and ⁷Department of Pediatrics, Duke University, Durham, NC; ⁸Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, AL; and ⁹Department of Epidemiology, MD Anderson Cancer Center, Houston, TX

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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 (Durham, North Carolina) 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 enrollment have been detailed elsewhere.¹⁰ In brief, 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 in the study. Inclusion criteria were age ≥ 18 years and English speaking. We excluded women who planned to give offspring up for adoption and women who were human immunodeficiency virus-positive, because the effect of anti-retroviral medications on the methylation profile is still unknown. Most women (>50%) were from Durham County, North Carolina, although the catchment area also included contiguous counties.

Between 2005 and 2009, 940 of the 1101 women (85%) approached consented to participate and were enrolled in NEST. Methylation analyses were performed on cord blood leukocyte DNA of the first 438 neonates (46%). The final sample includes the first 204 offspring who had methylation data and had reached 1 year of age by August 2010 and whose mothers completed a follow-up questionnaire. Follow-up is ongoing. The distribution of factors that may affect overweight status or obesity, including maternal age ($P = .54$), education ($P = .94$), race/ethnicity ($P = .43$), and sex of infant ($P = .80$) were comparable between the 940 infant-mother pairs enrolled and the 438 infant-mother pairs with DNA methylation data. These factors were also comparable between the first 204 infants in whom follow-up data have been collected to date and the 428 in whom methylation analyses were conducted. Most questionnaires (78%) were completed via 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 body mass index (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 1 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 by using the University of Minnesota's Nutrition Data System for Research (2008). Although a single 24-hour recall cannot adequately reflect total energy intake in 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 when the child was between 12 and 29 months of age. The median age of the child when the first follow-up questionnaire was mailed was 14 months, and the median time to returning the questionnaire was 1 month.

To verify the accuracy of the infant weights reported by mothers in the questionnaire, anthropometric measurements at child age 1 year were abstracted from the medical records of 72 infants who had the data available within 1 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 ($P < .0001$), suggesting that mothers accurately reported their offspring's weight.

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

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, because they contribute to long-term health in humans.¹¹⁻¹⁶ Genomic DNA was modified by treatment with sodium bisulfite by using high throughput methods as previously described.¹⁷ Bisulfite treatment of denatured DNA converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged. Pyrosequencing was performed by using a Biotage Pyromark MD pyrosequencing instrument (Qiagen). We evaluated two regions, including 3 cytosine-phosphate-Guanine (CpG) sites comprising the intragenic *IGF2* DMR, upstream of exon 3 (chr1p15.5, site 1: 2 109 519; site 2: 2 109 516; and site 3: 2 109 500; NCBI Human Genome Build 37.1; National Institutes of Health,

Bethesda, Maryland) and 4 CpG sites, hereafter referred to as the *H19* DMR, within a sequence motif that binds the CCCTC-binding factor 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). With the same CpGs evaluated here as estimates of DMR methylation, hypermethylation at the intergenic DMR upstream of *H19*^{18,19} (referred to as the *H19* DMR) and CpG hypomethylation at the intragenic DMR upstream of *IGF2* exon 3 (referred to as the *IGF2* DMR)¹⁹ have been associated with increased *IGF2* transcriptional activity and loss of imprinting and had been found dysregulated in multiple obesity-related cancer types.^{18,20-26} These sequences have been described extensively²⁷ and are available at National Center for Biotechnology Information. Pyrosequencing assays were designed with PSQ Assay Design Software (Biotage AB, Uppsala, Sweden). 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 institutional review boards of Duke University, University of Texas MD Anderson Cancer Center, and National Cancer Institute.

Obesity and overweight status in early childhood are often estimated with weight adjusted for length. In the absence of length measurements, we estimated rapid early growth by approximately 1 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, on the basis of the Centers for Disease Control and Prevention growth charts expected sex-specific smoothed for WFA.²⁸ Because WFA was not normally distributed, infants were dichotomized into overweight or obese status, when WFA was >85th percentile or non-obese or overweight when WFA was ≤85th percentile, although a cutoff point 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 cutoff points at >85th and >95th percentiles, and weight for length in the subset of infants in whom length data were available at age approximately 1 year. To test for potential bias associated with the length of time to return the questionnaire in infants who were breastfed and infants who were not, we used *t* tests to evaluate the association between time to return the questionnaire and both breastfeeding status ($P = .4$) and overweight or obesity status ($P = .5$).

For each of the 3 CpGs at the *IGF2* DMR and 4 CpGs at the *H19* DMR, the distribution of methylation percentages obtained from Pyrosequencing data were evaluated for normality by 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 in CpG dinucleotide percents for each DMR. The

correlation co-efficient for the 4 CpG dinucleotides at the intergenic *H19* DMR ranged from 93% to 96%, and the correlation co-efficients in the methylation fractions at the 3 CpG dinucleotides at the *IGF2* DMR ranged from 81% to 83%. On the basis of these high correlations, we used a single (average) mean for each DMR, to compare DMR methylation fractions between infants with a WFA >85th percentile and WFA ≤85th percentile in early childhood. Despite these high correlations in CpGs at each DMRs, we also repeated these analyses with 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 3 categories: "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

χ^2 tests were used to compare maternal and offspring characteristics in relation to overweight status or obesity (dichotomized at WFA >85th percentile of the Centers for Disease Control and Prevention growth charts). Least squares mean DNA methylation percentages at the *H19* and at the *IGF2* DMRs were then compared between children with WFA >85th percentile and children with WFA ≤85th percentile, by 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 with 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 <.10 was considered to be statistically significant. Factors found significantly associated with overweight status or obesity in **Table 1** were evaluated for potential confounding in these models, and those significantly associated were retained. Factors considered for potential confounding were maternal pre-pregnancy 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 in statistical models as continuous variables. Maternal morbidity (any versus none), mode of delivery (vaginal versus cesarean delivery), race/ethnicity (African-American, white, and other), sex (male versus female), cigarette smoking (never smoked, stopped during pregnancy, or quit before knowledge of pregnancy),

Table I. 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			.10
African-American	64 (37.7%)	15 (57.7%)	
White	97 (57.1%)	11 (42.3%)	
All others	9 (5.3%)	0	
Missing	2	0	
Maternal level of education			.32
Up to some college	88 (51.2%)	16 (61.5%)	
College graduate or graduate school	84 (48.8%)	10 (38.5%)	
Maternal BMI			.66
Mean, kg/m ²	26.4	27.2	
Range, kg/m ²	16.3-57.0	18.9-43.9	
BMI <25	94 (58.0%)	11 (44.0%)	.40
BMI 25-30	31 (19.1%)	7 (28.0%)	
BMI ≥30	37 (22.8%)	7 (28.0%)	
Missing	10	1	
Maternal self-reported health status			.74
Excellent	40 (23.3%)	6 (23.1%)	
Very good or good	118 (68.6%)	19 (73.1%)	
Fair or poor	14 (8.1%)	1 (3.9%)	
Maternal smoking			.24
Never smoked	107 (62.2%)	12 (46.2%)	
Smoked during pregnancy	51 (29.7%)	10 (38.5%)	
Quit before knowledge of pregnancy	14 (8.1%)	4 (15.4%)	
Mean maternal age at delivery, years			.32
Range, years	30	29	
Gestational age at birth			.24
Mean, weeks	38	39	
Range, weeks	26-42	34-41	
<35 weeks	13 (7.6%)	1 (3.9%)	.49
≥35 weeks	159 (92.4%)	25 (96.2%)	
Infant sex			.10
Male	96 (55.8%)	10 (38.5%)	
Female	76 (44.2%)	16 (61.5%)	
Birth weight			.13
Mean, g	3135.6	3347.6	
Range, g	760.0-5160.0	2070.0-4510.0	
Use of any childcare			.87
No	87 (51.8%)	13 (50.0%)	
Yes	81 (48.2%)	13 (50.0%)	
Missing	4	0	
Weight gain, pounds			<.01
Range, pounds	14.5	19.6	
Range, pounds	8.7-22.1	9.9-28.9	
Mean daily caloric intake, Kcal			.002
Range, Kcal	1061.2	1369.8	
Range, Kcal	187.9-2938.3	304.6-3998.9	
Missing			
Breastfeeding: first 12 months of life			.83 [†]
Never breastfed	58 (33.9%)	8 (30.8%)	
Ever breastfed	113 (66.1)	18 (69.2%)	
Breastfeeding: first 3 months of life			.34 [‡]
Never breastfed	58 (33.7%)	8 (30.8%)	
Mixed breast milk and formula	46 (26.7%)	4 (15.4%)	
Exclusively breastfed	68 (39.5%)	14 (53.9%)	
Prenatal morbidity			.38
None	82 (47.7%)	10 (38.5%)	
Any chronic disease [§]	90 (52.3%)	16 (61.5%)	

*P value for association between characteristic and child's WFA percentile. Calculated with *t* test for continuous variables and with χ^2 for categorical variables.

[†]Calculated with Fisher exact test.

[‡]Calculated with 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.

education (up to some college versus college or higher) were entered as categorical variables.

These analyses were repeated with 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 in aberrant and

normal categories by using a cutoff point ≥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, because hypomethylation at this DMR has been associated with *IGF2* deregulation.¹⁹ All statistical analyses were conducted with SAS software version 9.2 (SAS Institute, Cary, North Carolina).

Table II. Methylation fraction at *H19* and *IGF2* DMRs at birth by percentile of child's weight at age approximately 1 year

DMR	≤85th percentile of WFA (n = 172)		>85th percentile of WFA (n = 26)		P value*
	Mean methylation fraction	95% CI	Mean methylation fraction	95% CI	
<i>H19</i> DMR					
Mean	59.3	58.2-60.3	62.7	59.9-65.5	.03
CG1	61.6	60.3-62.9	65.6	62.2-69.0	.03
CG2	57.8	56.7-58.9	59.8	56.8-62.8	.22
CG3	58.9	57.7-60.0	62.3	59.2-65.4	.04
CG4	59.0	57.8-60.2	63.1	60.0-66.3	.02
<i>IGF2</i> DMR					
Mean	47.5	46.6-48.4	47.2	44.8-49.7	.84
CG1	41.7	40.3-43.1	39.1	35.5-42.8	.19
CG2	50.9	49.8-51.9	50.4	47.7-53.2	.78
CG3	51.2	49.9-52.6	49.7	46.1-53.3	.43

*P value comparing unadjusted least square means.

Results

Table I summarizes the distribution of sociodemographic characteristics, anthropometric measures, and lifestyle factors of the mother-infant pairs. Thirteen percent of infants were categorized as overweight or obese, on the basis of the WFA cutoff point >85th percentile. Compared with children with WFA ≤85th percentile, children with WFA >85th percentile were more likely to be female ($P = .10$) and African-American ($P = .10$) and have a larger birth weight ($P = .13$), a larger weight gain ($P < .01$), and a higher caloric intake during the first year of life ($P < .002$). Obese and overweight children, however, were comparable with non-obese children in maternal age at delivery, self-reported health status, chronic morbidity, gestational age at birth, educational level, maternal BMI before pregnancy, infant breastfeeding, and use of childcare outside the home.

Of the 204 mothers, 66% ($n = 133$) reported ever breastfeeding their offspring during the first year of life; these proportions were comparable in overweight or obese children (69%) and non-obese or overweight children (66%). During the follow-up period, 8.4% of infants ($n = 17$) had received a diagnosis of conditions that included eczema, seizure disorders, cerebral palsy, and tonsillitis. All the 17 children except two were breastfed, and only one child was obese or overweight. A combination of breast- and formula-feeding was reported in 27% of non-obese children and 15% of obese or overweight children, and exclusive breastfeeding for at least 3 months was reported by 54% of mothers of obese or overweight children, compared with 40% of non-obese children ($P = .34$). Never breastfeeding was comparable in obese or overweight children (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; $P = .95$).

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 1 year. In all infants, the average methylation fraction was 61% at the *H19* DMR (SD, 8%; IQR, 56%-63%) and 47% at the *IGF2* DMR (SD, 7%; IQR, 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 ($P = .03$). 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 and maternal pre-pregnancy BMI, 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%) compared with children who were neither overweight nor obese (59.2%; 95% CI, 57.9%-60.5%) who were ever breastfed ($P = .82$). However, in infants who were never breastfed, we noted an 8.4% ($P = .01$) methylation difference between overweight and obese children (68.3%; 95% CI, 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 ($P = .26$), although further adjusting for race/ethnicity reduced the cross-product term P value ($P = .20$). Repeating these analyses with linear mixed models to allow simultaneous entry of individual CpG dinucleotide methylation fractions in 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% ($P = .19$) higher methylation fraction at birth in children with higher weight gain at age 1 year (data not shown). In the subset of children with length data at age 1 year, methylation fraction differences of a similar magnitude were also observed between children with weight-for-length >85th percentile and children with weight-for-length ≤85th percentile ($P = .19$). After race/ethnicity stratification, we also found that methylation fractions of African-American children with WFA ≤85th percentile were comparable with African-American children with WFA >85th percentile ($P = .46$); however, white children with WFA >85th percentile had somewhat higher methylation fractions at birth than white children with WFA ≤85th percentile ($P = .09$). However, the cross-product term for methylation fraction

Table III. DNA methylation differences in obese or overweight status in breastfed and non-breastfed African-American and white children

DMR	≤85th percentile of WFA (n = 172)		>85th percentile of WFA (n = 26)		P value
	Mean methylation fraction	95% CI	Mean methylation fraction	95% CI	
<i>H19</i> DMR					
All participants (n = 204)	59.3	58.3-60.4	62.4	59.5-65.4	.05
Never breastfed (n = 70)	59.8	57.6-61.9	68.3	62.3-74.2	.01
Ever breastfed (n = 133)	59.2	57.9-60.5	59.6	56.2-63.1	.82
P value for cross-product term for breastfeeding and <i>H19</i> DMR methylation					.20
African-American (n = 85)	61.5	59.4-63.6	63.4	58.9-68.0	.46
White (n = 108)	58.1	56.8-59.4	61.9	57.7-66.2	.09
P value for cross-product term for race and <i>H19</i> DMR methylation					.58
<i>IGF2</i> DMR					
All participants	47.5	46.5-48.4	47.2	44.6-49.7	.83
Never breastfed	46.7	45.1-48.2	45.4	40.9-49.9	.59
Ever breastfed	47.8	46.6-49.0	48.2	44.9-51.4	.82
P value for cross-product term for breastfeeding and <i>IGF2</i> DMR methylation					.71
African-American	47.5	45.9-49.0	46.0	42.5-49.4	.43
White	47.4	46.2-48.7	48.2	44.4-51.9	.71
P value for cross-product term for race and <i>IGF2</i> DMR methylation					.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.

and race/ethnicity was associated with a *P* value of .58. We found no significant differences in *IGF2* DMR methylation fractions between overweight children and 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 breastfeeding and race, in the association between aberrant methylation and subsequent obesity or overweight status by repeating these analyses with logistic regression models. Hypermethylation was defined as DNA methylation fractions >75th percentile, and normal methylation was defined otherwise, at the *H19* locus (Table IV). We found that in all children, after adjusting for caloric intake and cigarette smoking, the 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 white children (OR, 4.4; 95% CI, 1.0-20.4) than in the 85 African-American children (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 were never breastfed (OR, 22.3; 95% CI, 2.1-239.8) compared with children who were ever breastfed (OR, 1.3; 95% CI, 0.3-4.7). The cross-product term for breastfeeding and the *H19* DMR

methylation fraction was statistically significant (*P* = .05). Further adjusting for race/ethnicity reduced the *P* value for the cross-product term to .03 (Table IV). At the *IGF2*

Table IV. ORs and 95% CIs for the association between DNA methylation at birth and obese or overweight status in breastfed and non-breastfed African-American and white children*

DMR	OR	95% CI	P value
<i>H19</i> DMR*			
All participants (n = 204)	3.12	1.13-8.60	.03
Never breastfed (n = 70)	22.27	2.07-239.84	.01
Ever breastfed (n = 133)	1.25	0.34-4.67	.74
P value for cross-product term for breastfeeding and <i>H19</i> DMR methylation			.03
African-American (n = 85)	2.38	0.62-9.10	.21
White (n = 108)	4.40	0.95-20.37	.06
P value for cross-product term for race and <i>H19</i> DMR methylation			.46
<i>IGF2</i> DMR†			
All participants	1.19	0.36-3.93	.78
Never breastfed	1.91	0.22-16.69	.56
Ever breastfed	0.69	0.14-3.43	.65
P value for cross-product term for breastfeeding and <i>IGF2</i> DMR methylation			.25
African-American	2.33	0.48-11.34	.30
White	0.57	0.06-5.16	.62
P value for cross-product term for race and <i>IGF2</i> DMR methylation			.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. †*IGF2* DMR mean methylation fraction ≤25th percentile.

DMR, we found no DNA methylation differences between overweight or obese children and children who are neither obese nor overweight.

Discussion

We found that children who were overweight or obese at age 1 year had higher methylation percentages at the *H19* DMR at birth compared with children who were neither overweight nor obese. Methylation differences of strikingly similar magnitude have been reported previously in relation to gene expression and several phenotypic differences.^{18,20,23,24,29} DNA methylation differences of a similar magnitude were found between Dutch famine survivors and their same-sex siblings³⁰ and in Gambians conceived in the nutritionally challenging rainy season compared with those conceived in the dry season.³¹ 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,⁹ 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. Higher circulating *IGF2* protein levels have been associated with obesity in adults.³² 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 children whose weight was within reference range was modified by breastfeeding status. Although 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 because 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, 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.³³ 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.³⁴ 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 2 years in the lower protein group.³⁵

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 with 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.³⁶ 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.³⁷ 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 breastfeeding that could influence methylation patterns and infant growth patterns. Only one of the 17 children with postnatal morbidity was overweight or obese. However, if confirmed in larger studies in which 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.³⁸ 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 compared with unexposed same-sex siblings at this DMR.³⁰ In general, adult survivors of the Dutch Famine have a higher risk of obesity and obesity-related chronic disease.³⁹

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 children⁴⁰⁻⁴² and adults,³² although inconsistently.^{43,44} A potential limitation is that we did not measure and control for the potential confounding effect of white blood cell counts, because their relative abundance in specimen may influence methylation percentages. However, adjusting for infection during parturition (a major cause of variation in white blood cell counts) 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 being evaluated, the methylation profiles were similar between polymorphonuclear and mononuclear cells,²⁷ 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 being studied, several other studies have shown the temporal stability of methylation at one of these DMRs,^{30,45} although at older ages.

Although findings from our exploratory race-stratified analyses were intriguing, disentangling potential epigenetic effects of race/ethnicity from those of breastfeeding 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 with weight gain as an outcome and weight-for-length in a subgroup of 72 infants in whom height data were recorded within 1 month of questionnaire administration revealed DNA methylation differences of a strikingly similar magnitude, although less stable. Further, mothers' inability to recall infant feeding practices in 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 OR for growth in infancy.⁴⁶ 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 breastfeeding; some of 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,⁴⁷ most mothers in our study were unable to report paternal height. Finally, we did not conduct subanalyses by weight-for-gestational-age at birth; however, on the basis of 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 ($P = .2$) or

methylation fraction ($P = .6-.7$), 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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Reprint requests: Cathrine Hoyo, PhD, Department of Community and Family Medicine, Duke University, PO Box 104006, Durham, NC 27710. E-mail: cathrine.hoyo@duke.edu

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