Dietary fat intake during early pregnancy is associated with cord blood DNA methylation at IGF2 and H19 genes in newborns

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Abstract
Maternal fat intake during pregnancy affects fetal growth, but mechanisms underlying this relationship are unclear. We performed an exploratory study of the associations of fat consumption during pregnancy with cord blood DNA methylation of the insulin-like growth factor 2 (IGF2) and H19 genes. We used data from 96 uncomplicated full-term pregnancies of mothers of whom majority had normal body mass index (BMI) (66%) in Project Viva, a prospective pre-birth cohort. We assessed maternal diet with validated food frequency questionnaires during the first and second trimesters and measured DNA methylation in segments of the IGF2- and H19-differentially methylated regions (DMRs) by pyrosequencing DNA extracted from umbilical cord blood samples. Mean (SD) age was 32.8 (4.1) years and prepregnancy BMI was 24.0 (4.4) kg/m². Mean DNA methylation was 56.3% (3.9%) for IGF2-DMR and 44.6% (1.9%) for H19-DMR. Greater first trimester intake of omega-6 polyunsaturated fat (effect per 1% of calories at the expense of carbohydrates) was associated

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1 | INTRODUCTION

Epidemiologic studies and animal models suggest that maternal prenatal nutrition during critical periods of fetal development may have long-term health consequences for the offspring (Anjos et al., 2013; Emmett et al., 2015; Hibbeln et al., 2007; Robinson, 2001; Venter et al., 2017). While there are several mechanisms by which intrauterine exposures may program offspring development, many studies suggest that epigenetic alterations might be involved (Sullivan & Grove, 2010; Sutton et al., 2016; Vickers, 2014; Zheng et al., 2014).

Specifically, DNA methylation programming events during fetal development might adapt in response to environmental cues, such as maternal nutritional status and environmental exposures (Gabory et al., 2011; Perera & Herbstman, 2011).

One potential biomarker of developmental programming events is imprinted genes. Unlike most genes, which are equally expressed from the paternally inherited and maternally inherited alleles, imprinted genes are preferentially expressed from either the paternal or maternal allele. One remarkable and characteristic feature of imprinted genes is that around 80% are proximally co-located in clusters with the maternally expressed growth factor 2 (IGF2) gene, which shares regulatory elements with the maternally expressed H19 gene located 100 kilobases downstream and reciprocally imprinted (Bell & Felsenfeld, 2000). Expression of IGF2 and H19, regulated by epigenetic marks at IGF2 and H19 DMRs, critically regulates fetal growth signals and embryonic development (Ratajczak, 2012). Loss of imprinting or deviation of DNA methylation at the IGF2 and H19 locus results in growth disorders, including Beckwith–Weidemann and Silver–Russell syndromes, and higher risk for certain cancers (Azzi et al., 2014; Chen et al., 2010). The IGF2 gene is expressed throughout fetal development to promote growth, and the maternally expressed H19 produces a noncoding RNA that can regulate IGF2 expression during development, which limits fetal growth (Gabory et al., 2009).

Variability in exposure to folic acid (Haggarty et al., 2013; Hoyo et al., 2011; Hoyo et al., 2014; Steegers-Theunissen et al., 2009) and famine (Heijmans et al., 2008) during the embryonic period have been associated with impacts on IGF2 and H19 DMR methylation in children, suggesting potential plasticity of IGF2 methylation by nutritional modification. Omega-3 polyunsaturated fatty acids (PUFA) have been found to play important roles in one-carbon metabolism (Ahmed et al., 2014; Assies et al., 2014; Kulkami et al., 2011), thus possibly influencing DNA methylation. Fats are a major component of nutrition, and different kinds impact health outcomes based on if they have beneficial (omega-3 and monounsaturated fatty acids) or adverse (trans fat) physiological effects. In a randomized controlled trial of docosahexaenoic acid (DHA, an omega-3 fatty acid) supplementation from mid-pregnancy to parturition, DHA supplementation resulted in higher cord blood IGF2-DMR methylation in infants of overweight mothers and lower H19-DMR methylation in infants of normal weight mothers (Lee et al., 2014). It is less clear, however, how intake of other fats during fetal development influences IGF2 and H19 DNA methylation, and whether there is a time-sensitive window to epigenetic modification by fat intake. In this exploratory study, we examined the association between prenatal intake of different types of fat at two points during pregnancy and IGF2 and H19 DNA methylation in cord blood among a subset of mother–child pairs in a prospective birth cohort in Massachusetts.

2 | MATERIALS AND METHODS

2.1 | Study population

This study included participants in Project Viva, a prospective prebirth cohort that recruited women during their initial obstetric care visit (median: 9.9 weeks of gestation) between 1999 and 2002 at Atrius Harvard Vanguard Medical Associates, a multi-site/multi-specialty practice in Eastern Massachusetts. Details of the cohort have been described previously (Oken et al., 2015). Research assistants conducted in-person interviews with women at recruitment and at 26–28 weeks of gestation, and clinicians collected venous umbilical cord blood at delivery. We collected sociodemographic data, such as age, prepregnancy weight and height, smoking history, and household...
income, through in-person questionnaires and interviews at enrollment. We calculated total gestational weight gain by subtracting self-reported pre-pregnancy weight from the last prenatal weight (within 4 weeks of delivery) from medical records and obtained mode of delivery, infant birth weight in grams, and date of delivery from the hospital medical records. We also calculated length of gestation in days by subtracting the date of the last menstrual period from the date of delivery. If gestational age according to the second trimester ultrasound differed from that according to the last menstrual period by >10 days, we used the ultrasound result to determine gestational duration. Sex-specific birth-weight-for-gestational age Z scores (BW/GA, an index as fetal growth) were calculated using 1999–2000 U.S. National Reference Data (Oken et al., 2003). Of 2128 singleton live births in the cohort, 1018 venous umbilical cord blood samples were collected at the time of delivery, and consent for genetic and epigenetic analyses was obtained from 511 women. Of these, we excluded the offspring of women who did not complete first and second trimester dietary questionnaires (n = 69), had a history of gestational diabetes, pre-eclampsia, or pregnancy-induced hypertension (n = 42), had an unplanned pregnancy (n = 160), and delivered before gestation week 40 or after gestation week 42 (n = 86), leaving 154 infants eligible for this analysis. We excluded unplanned pregnancies to achieve greater homogeneity among the sample and reduce extraneous variation in DNA methylation. From the eligible pool, we randomly selected 50 male and 50 female infants to have their samples sent for pyrosequencing, of which four samples were excluded to achieve greater homogeneity among the sample and reduce extra-

### 2.2 | Dietary assessment

We assessed diet using a 140-item, self-administered food frequency questionnaire (FFQ) based on a well-validated FFQ used in other cohorts (Yuan, Spiegelman, Rimm, Rosner, Stampfer, Barnett, Chavarro, Rood, et al., 2017a; Yuan, Spiegelman, Rimm, Rosner, Stampfer, Barnett, Chavarro, Subar, et al., 2017b) and adapted and calibrated for use among pregnant women (Fawzi et al., 2004). The first trimester FFQ assessed diet intake since the last menstrual period and was completed by participants at enrollment. We queried vitamin and supplement intake in the first trimester with dose, duration, and brand/type of multivitamin, prescribed prenatal vitamin and supplements. The second trimester FFQ was self-completed at 26–28 weeks of gestation and assessed diet “during the past 3 months.” The second trimester FFQ was the same as the first trimester except that we assessed use of vitamins/supplements as part of the self-completed FFQ (Rifas-Shiman et al., 2006). Instruments used in the second and first trimester can be found the Project Viva website under investigators, data and collection forms: https://www.hms.harvard.edu/viva/.

Nutrient intakes were estimated by summing the nutrient contribution of all food and supplement items. We obtained nutrient estimates from the Harvard nutrient-composition databases, which was based on the nutrient database of the U.S. Department of Agriculture (U.S. Department of Agriculture, 2020). We have previously validated the estimation of omega-3 PUFA intake through the FFQ against erythrocyte and plasma levels of fatty acids (Fawzi et al., 2004; Oken et al., 2014).

### 2.3 | Cord blood sample collection and hormone level measurement

Venous cord blood samples were promptly stored in a dedicated refrigerator (4°C) and transported for processing within 24 h. Trained laboratory staff processed the samples on the same day. Whole blood samples were centrifuged to separate the buffy coat from plasma and red blood cells (RBCs), and the buffy coat was transferred into an RBC lysis solution to facilitate further lysis of RBCs. The solution was then centrifuged to obtain white blood cell (WBC) pellet and remove the lysis solution containing RBCs. DNA was extracted from only WBCs using the Qiagen Puregene Kit (Valencia, CA). Aliquots were then stored at −80°C until analysis. DNA was sodium bisulfite converted using the EZ DNA Methylation-Gold Kit (ZymoResearch, Irvine, CA).

The details of cord blood hormone measurement in this cohort were described previously (Oken et al., 2016). In brief, cord blood IGF2 and insulin-like growth factor binding protein 3 (IGF-BP3) levels were measured using the following commercial assays: ELISA, Alpco Diagnostics (Salem, NH) for IGF2 and ELISA, R&D Systems (Minneapolis, MN) for IGF-BP3. Day-to-day variability for each of these assays were below 10%.

### 2.4 | Quantification of IGF2 and H19 DMRs by pyrosequencing

The human IGF2-H19 locus contains three common DMRs: IGF2-DMR0 (located in first intron), IGF2-DMR2 (located within exon 8), and H19-DMR (St-Pierre et al., 2012). In this study, DNA methylation analysis covered IGF2-DMR0, which we refer to as the IGF2-DMR, and H19-DMR. Regions of the IGF2-DMR and H19-DMR were selected based on the literature and amplified using Promega GoTaq Hot Start Green Master Mix (Byun et al., 2007). Primers for the assays were IGF2-F—GGTTAATTTTTTTGGGTGAATTA, IGF2-R—CAAAAAACTTTACAAAAAACCC, H19-F—TAGGGTTTTTTGGT AGGTATAGGTT, and H19-R—AAATATAAAAAATTTTATAAACACT, where the reverse primers were biotinylated. Polymerase chain reaction (PCR) products were bound to streptavidin-Sepharose beads (Qiagen). Biotinylated strands were separated from nonbiotinylated strands using a vacuum prep tool and annealed to the sequencing primer. Samples were pyrosequenced on a Qiagen Q96MD machine according to the manufacturer’s protocol. Positive, negative and methylation controls were run to ensure successful sequencing. We measured the percent of cytosines that were methylated in segments of the IGF2- and H19-DMRs (IGF2: two CpG sites, H19: six CpG sites) as well as for each specific CpG site.
2.5 Statistical analysis

We modeled fatty acid intake as a continuous variable using multivariable nutrient density method (Willett, 2012). Specifically, we simultaneously included terms for the energy derived from protein, specific types of fatty acids (omega-6 PUFA, omega-3 PUFA, saturated, monounsaturated, and trans fatty acids) and total energy intake. Fatty acid density was calculated by multiplying specific fatty acid intake (g/day) by 9 kcal/g and dividing by the participants’ total energy intake. Similarly, protein density was calculated by multiplying protein intake (g/day) by 4 kcal/g and dividing by the participants’ total energy intake. Dietary intake of fatty acids was calculated by multiplying the frequency of consumption of each food item by its nutrient content and summing the nutrient contributions of all foods based on U.S. Department of Agriculture food composition data, taking into account types of margarine and fats used in cooking and baking. The correlation coefficients between intakes from FFQ and dietary records were 0.57–0.62 for total fat, 0.68–0.75 for SFAs, 0.48–0.51 for PUFAs and 0.58–0.60 for monounsaturated fats from previous studies (Sun et al., 2007; Yuan, Spiegelman, Rimm, Rosner, Stampfer, Barnett, Chavarro, Rood, et al., 2017a; Yuan, Spiegelman, Rimm, Rosner, Stampfer, Barnett, Chavarro, Subar, et al., 2017b). Correlation between trans fat intake assessed by the FFQ and the composition of trans fat in adipose tissue was 0.51 in a previous study (London et al., 1991). The coefficients of the model can be interpreted as an isocaloric substitution of specific fatty acids for carbohydrates.

We selected covariates based on prior knowledge through the use of directed acyclic graphs. All models were adjusted for maternal age at enrollment (years), prepregnancy body mass index (BMI, kg/m²), annual household income (≤$70,000 or >$70,000), trimester-specific gestational weight gain (kg), trimester-specific total energy intake (kcal), gestational age at delivery (weeks), mode of delivery (cesarean section or vaginal delivery) and infant sex. To examine the relationship between maternal fat intake and offspring’s DNA methylation patterns, we applied multivariable linear mixed models for the DMRs (e.g., methylation at multiple CpG sites) and multivariable linear models for individual CpG sites. Since a previous study found the association between omega-3 intake and IGF2 and H19 DNA methylation was modified by maternal baseline BMI (Lee et al., 2014), we tested for effect modification by prepregnancy BMI (<25 kg/m²) using cross product-terms in the multivariable linear models for the DMRs (e.g., methylation at multiple CpG sites) and multivariable linear models for individual CpG sites. We report estimates of associations as % change in fatty acids at the expense of carbohydrates. We used 1–5% changes in fatty acids depending on the mean (standard deviation [SD]) of calories relative to the total. Two-sided P values <0.05 were considered statistically significant. We performed statistical analyses with SAS v9.4 (SAS Institute, Cary, NC).

3 RESULTS

The analysis included 96 mothers (Table 1) who were mostly white (88%) and never smokers (65%), with a mean (SD) age of 32.8 (4.1) years and a prepregnancy BMI of 24.0 (4.4) kg/m², with 66% of mothers having a normal BMI. Mean (SD) intake of first trimester

| Table 1 Demographic and pregnancy characteristics of the subsample of women in project viva (n = 96) |
|-----------------------------------------------|
| Demographic and pregnancy characteristics | N (%) or mean (SD) |
| Maternal age at enrollment, years | 32.8 (4.1) |
| Maternal race, % White | 84 (88%) |
| Maternal never smoker, % | 62 (65%) |
| Maternal smoked during pregnancy, % | 5 (5%) |
| Prepregnancy BMI, kg/m² | 24.0 (4.4) |
| Gestational weight gain up to 91 days, kg | 2.8 (2.9) |
| Gestational weight gain up to 181 days, kg | 9.4 (3.9) |
| Annual income, %>$ 70,000 | 66 (69%) |
| Gestational age at birth, weeks | 40.7 (0.5) |
| Mode of delivery, % cesarean section | 21 (22%) |
| Dietary characteristics | Mean (SD) |
| First trimester total energy intake (kcal/d) | 2071 (564) |
| Second trimester total energy intake (kcal/d) | 2099 (528) |
| First trimester SFA intake, % calories | 10.7 (2.6) |
| Second trimester SFA intake, % calories | 11.2 (2.2) |
| First trimester MUFA intake, % calories | 10.8 (2.5) |
| Second trimester MUFA intake, % calories | 11.3 (2.4) |
| First trimester PUFA intake, % calories | 6.0 (1.8) |
| Second trimester PUFA intake, % calories | 6.3 (1.4) |
| First trimester omega-3 intake, % calories | 0.5 (0.2) |
| Second trimester omega-3 intake, % calories | 0.5 (0.2) |
| First trimester omega-6 intake, % calories | 5.3 (1.6) |
| Second trimester omega-6 intake, % calories | 5.5 (1.3) |
| First trimester trans fat intake, % calories | 0.9 (0.3) |
| Second trimester trans fat intake, % calories | 1.0 (0.3) |
| Newborn DMR methylation and birth characteristics | Mean (SD) |
| IGF2-DMR DNA methylation, % | 56.3 (3.9) |
| IGF2 CpG site 1 DNA methylation, % | 50.4 (5.0) |
| IGF2 CpG site 2 DNA methylation, % | 62.2 (5.5) |
| H19-DMR DNA methylation, % | 44.6 (1.9) |
| H19 CpG site 1 DNA methylation, % | 44.8 (2.2) |
| H19 CpG site 2 DNA methylation, % | 43.9 (2.4) |
| H19 CpG site 3 DNA methylation, % | 43.1 (2.0) |
| H19 CpG site 4 DNA methylation, % | 43.1 (2.0) |
| H19 CpG site 5 DNA methylation, % | 48.5 (2.6) |
| H19 CpG site 6 DNA methylation, % | 44.1 (2.2) |
| Birth weight for gestational age, Z score | 0.53 (0.95) |
| Cord blood IGF2, ng/ml | 417 (87.2) |

Note: BMI, body mass index; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.
Mean (SD) DNA methylation was 56.3% (3.9%) at IGF2-DMR and 44.6% (1.9%) at H19-DMR for the cord blood samples. Within the H19-DMR region, DNA methylation levels at the six individual CpG sites were correlated with one another, with correlation coefficients ranging from 0.65 to 0.88. The two CpGs measured within the IGF2-DMR region were less strongly correlated ($r = 0.26$). Of the covariates

| TABLE 2 | Demographic and pregnancy characteristics and IGF2 and H19 DMR DNA methylation |
|--------------------------------------|---------------------------------|---------------------------------|
| Characteristics                      | IGF2-DMR methylation: % per unit change in characteristics (95% confidence interval) | H19-DMR methylation: % per unit change in characteristics (95% confidence interval) |
| Prepregnancy BMI, kg/m²               | −0.08 (−0.3, 0.1)               | 0.0 (−0.1, 0.1)                |
| Total gestational weight gain, kg     | −0.05 (−0.2, 0.1)               | 0.0 (−0.1, 0.1)                |
| Female infant (vs. male)              | 0.8 (−0.8, 2.4)                 | −0.04 (−0.9, 0.8)              |
| Gestational age at delivery, weeks    | 1.4 (0.02, 2.8)                 | 0.2 (−0.5, 1.0)                |
| Cesarean section (vs. vaginal delivery)| 2.8 (1.0, 4.6)                 | −0.08 (−0.9, 1.0)              |
| Mother’s age at enrollment, years     | 0.1 (−0.05, 0.3)                | −0.0 (−0.1, 0.1)               |
| Annual household income > $70,000 (vs.| 1.2 (−0.6, 2.9)                 | 0.7 (−0.3, 1.6)                |
| ≤$70,000)                            |                                 |                                |

Note: Estimates adjusted for all characteristics listed in table. Bolded values are significant at $P$ value <0.05.

| TABLE 3 | First and second trimester fat intake and IGF2-DMR methylation changes in 96 mother–child pairs |
|--------------------------------------|---------------------------------|---------------------------------|
| IGF2 methylation levels: % per unit change in fatty acids at expenses of carbohydrate (95% confidence interval) |
| Individual CpG sites                 | 1                              | 2                              | DMR (average) |
| Total fat*, 5% calories              |                                 |                                 |                |
| First trimester                      | −0.7 (−1.5, 0.1)               | −0.7 (−1.7, 0.3)               | −0.7 (−1.4, 0.0) |
| Second trimester                     | 0.1 (−0.9, 1.1)                | −0.8 (−1.9, 0.4)               | −0.3 (−1.2, 0.5) |
| SFA†, 2% calories                    |                                 |                                 |                |
| First trimester                      | 0.05 (−0.9, 1.0)               | −0.04 (−1.2, 1.1)              | 0 (−0.8, 0.8)  |
| Second trimester                     | 1.4 (0.2, 2.6)                 | 1.5 (0.1, 2.9)                 | 1.4 (0.5, 2.4)  |
| Trans fat†, 1% calories              |                                 |                                 |                |
| First trimester                      | 6.9 (2.2, 11.7)                | −4.5 (−10.3, 1.4)              | 1.2 (−2.9, 5.4) |
| Second trimester                     | 1.4 (−2.6, 5.5)                | −3.8 (−8.6, 1.0)               | −1.2 (−4.5, 2.2) |
| MUFA†, 2% calories                   |                                 |                                 |                |
| First trimester                      | −1.3 (−2.5, 0.0)               | 1.2 (−0.3, 2.6)                | 0.0 (−1.1, 1.0) |
| Second trimester                     | −0.8 (−1.9, 0.4)               | −1.0 (−2.3, 0.4)               | −0.9 (−1.8, 0.1) |
| PUFA†, 2% calories                   |                                 |                                 |                |
| First trimester                      | −1.3 (−2.4, −0.2)              | −1.9 (−3.2, −0.5)              | −1.6 (−2.5, −0.6) |
| Second trimester                     | −1.2 (−2.6, 0.2)               | −0.7 (−2.4, 0.9)               | −0.9 (−2.1, 0.2) |
| Omega 3 fat†, 1% calories            |                                 |                                 |                |
| First trimester                      | −0.02 (−7.8, 7.7)              | 5.1 (−4.3, 14.5)               | 2.5 (−4.2, 9.3) |
| Second trimester                     | −4.2 (−12.9, 4.5)              | 4.5 (−5.8, 14.8)               | 0.2 (−7.2, 7.5) |
| Omega 6 fat†, 1% calories            |                                 |                                 |                |
| First trimester                      | −0.7 (−1.9, 0.4)               | −1.7 (−3.1, −0.3)              | −1.2 (−2.2, −0.2) |
| Second trimester                     | −0.2 (−1.4, 0.9)               | −0.8 (−2.2, 0.5)               | −0.5 (−1.5, 0.4) |

Note: Bolded values are significant at $P$ value <0.05.

Abbreviations: MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

*Main multivariate model is adjusted for % of calories from protein, trimester-specific total energy intake, maternal age, maternal prepregnancy BMI, trimester-specific gestational weight gain (gestational weight gain to 91 days for first trimester models and gestational weight gain to 182 days for second trimester models), gestational age at delivery, infant sex, mode of delivery, and household income.

†Further adjusted for fat subtypes (e.g., saturated, monounsaturated, polyunsaturated, and trans fat intake).

‡Further adjusted for fat subtypes (e.g., saturated, monounsaturated, omega3, omega 6, and trans fat intake).
### Table 4: First and second trimester fat intake and H19-DMR methylation changes in 96 mother-child pairs

|                   | Individual CpG sites |               |               |               |               |               |               |                   |
|-------------------|----------------------|---------------|---------------|---------------|---------------|---------------|---------------|-------------------|
|                   |                      | 1             | 2             | 3             | 4             | 5             | 6             | DMR (average)     |
|                   | H19 methylation levels: % per unit change in fatty acids at expenses of carbohydrate (95% confidence interval) |               |               |               |               |               |               |                   |
|                   |                      | First trimester |               |               |               |               |               |                   |
|                   |                      | Second trimester |               |               |               |               |               |                   |
|                   |                      |               |               |               |               |               |               |                   |
| **Total fat**, 10% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | −0.3 (−0.7, 0.1)     | 0.02 (−0.4, 0.5) | 0.0 (−0.4, 0.4) | 0.04 (−0.3, 0.4) | −0.1 (−0.6, 0.4) | 0.2 (−0.2, 0.6) | −0.03 (−0.4, 0.3) |
| Second trimester  | 0.0 (−0.5, 0.5)      | 0.2 (−0.3, 0.7) | 0.3 (−0.1, 0.7) | 0.3 (−0.1, 0.8) | 0.2 (−0.4, 0.7) | 0.5 (0.01, 0.9) | 0.2 (−0.2, 0.7)  |
| **SFA**, 2% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | 0.0 (−0.5, 0.5)      | 0.3 (−0.2, 0.8) | 0.0 (−0.4, 0.5) | 0.1 (−0.3, 0.6) | 0.1 (−0.5, 0.8) | −0.1 (−0.6, 0.4) | 0.1 (−0.4, 0.6)  |
| Second trimester  | −0.2 (−0.8, 0.4)     | 0.2 (−0.4, 0.9) | −0.2 (−0.7, 0.3) | 0.1 (−0.4, 0.6) | 0.1 (−0.6, 0.8) | 0.2 (−0.4, 0.8) | 0.0 (−0.5, 0.6)  |
| **Trans fats**, 1% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | −0.6 (−2.9, 1.8)     | −0.7 (−3.2, 1.8) | −0.3 (−2.5, 1.8) | −1.1 (−3.3, 1.1) | −1.2 (−4.1, 1.7) | 0.3 (−2.1, 2.7) | −0.6 (−2.9, 1.7) |
| Second trimester  | 1.2 (−0.9, 3.2)      | 1.2 (−0.9, 3.3) | 0.9 (−0.9, 2.7) | 0.0 (−1.8, 1.8) | −0.4 (−2.9, 2.0) | 0.4 (−1.6, 2.4) | 0.5 (−1.3, 2.4)  |
| **MUFA**, 2% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | −0.3 (−0.9, 0.3)     | −0.5 (−1.1, 0.2) | −0.2 (−0.7, 0.4) | −0.2 (−0.8, 0.4) | −0.1 (−0.8, 0.7) | −0.1 (−0.7, 0.5) | −0.2 (−0.8, 0.4) |
| Second trimester  | −0.1 (−0.7, 0.5)     | −0.5 (−1.1, 0.2) | 0.0 (−0.5, 0.5) | −0.1 (−0.6, 0.4) | 0.0 (−0.7, 0.7) | −0.2 (−0.7, 0.4) | −0.2 (−0.7, 0.4) |
| **PUFA**, 2% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | 0.3 (−0.3, 0.8)      | 0.6 (0.0, 1.2) | 0.4 (−0.1, 0.9) | 0.6 (0.0, 1.1) | 0.0 (−0.7, 0.7) | 0.5 (−0.1, 1.0) | 0.4 (−0.1, 0.9)  |
| Second trimester  | 0.3 (−0.4, 1.0)      | 0.6 (−0.2, 1.3) | 0.7 (0.0, 1.3) | 0.9 (0.3, 1.5) | 0.3 (−0.5, 1.2) | 0.9 (0.2, 1.6) | 0.6 (−0.1, 1.3)  |
| **Omega 3 fats**, 1% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | −4.0 (−7.7, −0.3)    | −2.9 (−6.9, 1.1) | −5.1 (−8.5, −1.7) | −4.8 (−8.2, −1.4) | −4.2 (−8.8, 0.4) | −4.9 (−8.7, −1.2) | −4.3 (−7.9, −0.8) |
| Second trimester  | −0.4 (−4.7, 3.9)     | −2.7 (−7.3, 1.8) | −0.3 (−4.1, 3.5) | 0.1 (−3.7, 4.0) | 0.5 (−4.7, 5.7) | 0.5 (−3.7, 4.7) | −0.3 (−4.4, 3.7) |
| **Omega 6 fats**, 1% calories |                      |               |               |               |               |               |               |                   |
| First trimester   | 0.7 (0.1, 1.2)       | 0.7 (0.1, 1.3) | 0.9 (0.4, 1.4) | 0.9 (0.4, 1.4) | 0.6 (−0.1, 1.2) | 0.9 (0.3, 1.5) | 0.8 (0.3, 1.3) |
| Second trimester  | 0.2 (−0.4, 0.7)      | 0.6 (0.0, 1.1) | 0.4 (−0.1, 0.9) | 0.5 (0.0, 1.0) | 0.1 (−0.5, 0.8) | 0.5 (−0.1, 1.0) | 0.4 (−0.1, 0.9) |

Note: Bolded values are significant at P value <0.05.
Abbreviations: MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

*Main multivariate model is adjusted for % of calories from protein, trimester-specific total energy intake, maternal age, maternal prepregnancy BMI, trimester-specific gestational weight gain (gestational weight gain to 91 days for first trimester models and gestational weight gain to 182 days for second trimester models), gestational age at delivery, infant sex, mode of delivery, and household income.

Further adjusted for fat subtypes (e.g. saturated, monounsaturated, polyunsaturated, and trans fat intake).
Further adjusted for fat subtypes (e.g. saturated, monounsaturated, omega3, omega 6, and trans fat intake).
considered as potential confounders or outcome predictors, only mode of delivery (2.8%; 95% confidence interval [CI]: 1.0%, 4.6% for cesarean section versus vaginal delivery) and gestational age at delivery (1.4%; 95% CI: 0.02%, 2.8% per a week increase in gestation) were significantly associated with DNA methylation at the IGF2-DMR; none were related to H19-DMR DNA methylation (Table 2).

Maternal PUFA and total fat intakes during the first trimester were inversely associated with IGF2-DMR DNA methylation (Table 3). Higher first trimester PUFA intake (effect per 2% of calories) was associated with lower IGF2-DMR DNA methylation in cord blood (−1.6%; 95% CI: −2.5%, −0.6%). This association was driven by intake of omega-6 PUFA. Specifically, higher intake of omega-6 fats by 1% of calories was associated with lower IGF2-DMR DNA methylation (−1.2%; 95% CI: −2.2%, −0.2%). When each of the two individual CpG sites was analyzed, we also observed similar associations with first trimester omega-6 fat intake: \( \beta = −1.3\%; 95\% \text{ CI: −2.4\%, −0.2\%} \) and \( \beta = −1.9\%; 95\% \text{ CI: −3.2\%, −0.5\%} \) for the two individual CpG sites of IGF2. Intake of other fats during the first trimester, including omega-3 PUFA, was not found to have statistically significant differential methylation of the IGF2-DMR. When second trimester intake was considered, only intake of saturated fat was associated with IGF2-DMR DNA methylation (\( \beta = 1.4\%; 95\% \text{ CI: 0.5\%, 2.4\%} \) for a 2% increment in calories from saturated fat).

Next, we examined the associations of maternal fat intake with DNA methylation at the H19-DMR (Table 4). Omega-3 PUFA intake during first trimester was positively associated with H19-DMR DNA methylation. Specifically, a 1% increase in calories from omega-3 fat at expense of carbohydrate was associated with significantly lower cord blood DNA methylation levels at H19-DMR (−4.3%; 95% CI: −7.9%, −0.8%). Similar magnitude and direction of associations were found for most individual CpG sites (Table 4). In addition, the association of omega-3 PUFA with H19-DMR was stronger for intake of long-chain omega-3 fatty acids (\( \beta = −6.5\%; 95\% \text{ CI: −13.5\%, −0.47\%} \)) than of alpha-linolenic acid (\( \beta = −3.2\%; 95\% \text{ CI: −7.4\%, 1.0\%} \)). On the other hand, omega-6 PUFA intake during the first trimester was positively associated with the newborn's H19-DMR DNA methylation; however, omega-3 and omega-6 fat intake during the second trimester were not associated with H19-DMR DNA methylation. Intakes of saturated, trans and mono-unsaturated fats, regardless of the trimester, were not associated with H19 DMR methylation. Furthermore, the associations between fat intake and IGF2 and H19-DMR DNA methylation were not modified by pre-pregnancy BMI (data not shown).

Lastly, we examined whether IGF2-DMR or H19-DMR methylation levels were associated with cord blood IGF2 levels and BW/GA Z score. Neither the IGF2-DMR nor the H19-DMR was associated with cord blood IGF2 levels or BW/GA Z score in multivariable adjusted analyses (Table 5).

### TABLE 5

| Cord blood IGF2, ng/ml per 1% increment in DMR methylation | BW/GA, Z score per 1% increment in DMR methylation |
|------------------------------------------------------------|--------------------------------------------------|
| IGF2-DMR methylation, % | \( \beta^a (95\% \text{ CI}) \) | \( \beta^a (95\% \text{ CI}) \) |
| H19-DMR methylation, % | \( \beta^a (95\% \text{ CI}) \) | \( \beta^a (95\% \text{ CI}) \) |

\[ a \text{ Unadjusted model.} \]

\[ b \text{ Model is adjusted for maternal age, maternal prepregnancy BMI, total gestational weight gain, gestational age at delivery, infant sex, mode of delivery, and household income.} \]

\[ c \text{ Model is adjusted for maternal age, maternal prepregnancy BMI, total gestational weight gain, gestational age at delivery, infant sex, mode of delivery, household income, and IGF2-BP3.} \]

\[ d \text{ Model is adjusted for maternal age, maternal prepregnancy BMI, total gestational weight gain, infant sex, mode of delivery, and household income.} \]
(Chao & D’Amore, 2008; Kadakia & Josefson, 2016). High or low levels of IGF2 may affect development differently depending on the specific period of pregnancy, which is why we separately analyzed dietary fat consumption during the first trimester versus the second trimester. For example, elevated IGF2 levels during late pregnancy may affect growth of digestive organs in particular, with greater susceptibility in male fetuses (Wallace et al., 2015; White et al., 2018).

Expression of IGF2 and H19 is regulated by methylation at several DMRs in the gene promoter region (Delaval & Feil, 2004). Earlier studies have shown that loss of imprinting at H19 and IGF2 DMRs underlie the pathogenesis of Beckwith–Wiedemann syndrome, characterized by overgrowth, and Russell–Silver syndrome, characterized by prenatal growth restriction (Reik et al., 1995; Smith et al., 2007; Sun et al., 1997; Weksberg et al., 1995). In a normal pediatric population, higher placental H19-DMR DNA methylation was associated with higher cord blood IGF2 levels (St-Pierre et al., 2012). On the other hand, deviation of IGF2-DMR methylation (either hyper- or hypomethylation) can unbalance IGF2 expression and has been associated with higher placenta weight and fetal growth (St-Pierre et al., 2012). There is considerable evidence that early-life exposures to environmental and nutritional factors may alter epigenetic processes and fetal programming (Geraghty et al., 2015; Heijmans et al., 2008; Lillycrop & Burdge, 2015; Marsit, 2015). For example, Heijmans et al. (2008) found that individuals exposed to famine early in gestation during the Dutch Hunger Winter had 5.2% less DNA methylation of the imprinted IGF2 DMR than their unexposed siblings, decades after the exposure. While maternal fat intake is critical in normal fetal development (Mennitti et al., 2015), its role in IGF2 and H19 methylation has received less attention.

Higher maternal omega-3 PUFA intake and its associated biomarkers have been linked to reduced fetal growth as well as lower body fat and abdominal fat in childhood (Donahue et al., 2011; Oken et al., 2004; Vidakovic, Gisti, et al., 2016a; Vidakovic, Santos, et al., 2016b). Even maternal intake of long-chain PUFA during lactation may reduce height in early adolescence of offspring, especially in boys (Lauritzen et al., 2016). Long-chain omega-3 fatty acids have also been found to induce alterations in the synthesis of proteins like S-adenosylmethionine and adenosine kinase that are involved in one-carbon metabolism (Ahmed et al., 2014; Kulkinin et al., 2011). It is possible that maternal omega-3 fat intake may influence DNA methylation at key gene loci, such as IGF2, which in turn, modulates embryonic growth and metabolic risks in later life. To our knowledge, only one human study to date has investigated the associations of omega-3 fat intake during pregnancy and DNA methylation levels at IGF2 and H19 imprinted regions in offspring (Lee et al., 2014). In that randomized controlled trial, 261 Mexican women were randomly assigned to receive long-chain omega-3 supplementation (400 mg of DHA) or placebo (a mixture of corn and soy oil) from gestational week 18–22 to parturition. The authors found that there was no significant difference in IGF2 DMR DNA methylation between DHA-supplemented group and the control group of the expectant mothers with normal BMI, as reported in our study. However, in the stratified analyses, IGF2 DMR DNA methylation was 1.66% higher, on average, in the DHA group than the control group in infants of overweight mothers (P value, interaction = 0.08), and H19-DMR DNA methylation was 1.45% lower, on average, in the DHA group than the control group in infants of normal weight mothers (P value, interaction = 0.07). In partial agreement with this study, we found that regardless of pre-pregnancy BMI status, maternal omega-3 fat intake during first trimester had a positive, yet nonsignificant, association with the IGF2-DMR and an inverse association with the H19-DMR; the latter association was stronger for long-chain omega-3 than for alpha linoleic acid. Lee et al. (2014) evaluated supplementation of omega-3 until delivery, while we only estimated intake in the first and second trimesters. Furthermore, while these associations were similar between the first and second trimester, the magnitude was stronger for the first trimester, suggesting that early gestation may be a critical period for establishing and maintaining epigenetic marks (Reik & Walter, 2001).

Interestingly, the time-dependent manner of our association is consistent with the Dutch Hunger Winter study, which found that exposure to famine in early but not late gestation was associated with less IGF2-DMR methylation (Heijmans et al., 2008). However, they found that the famine was associated with lower IGF2-DMR methylation, whereas we found that hypomethylation was associated with higher fat intake. Imprinting methylation variation might occur from early evasion of demethylation, maintenance or adaptive response events during fetal development, potentially explaining associations observed during the first trimester (Monk et al., 2019). We found that greater maternal omega-6 fat intake during the first trimester was associated with lower DNA methylation at IGF2-DMR but a higher DNA methylation of the H19-DMR, suggesting that omega-6 intake during early pregnancy may have the opposite effect of omega-3 fats on fetal growth and IGF2 epigenetic regulation. Supporting this hypothesis, a few studies have found associations between higher omega-6 PUFA or higher omega-6/omega-3 ratio during pregnancy and higher body and abdominal fat and blood pressure in childhood (Donahue et al., 2011; Oken et al., 2004; Vidakovic et al., 2015; Vidakovic, Gisti, et al., 2016a; Vidakovic, Santos, et al., 2016b). The mechanism through which omega-6 is involved in epigenetic regulation of IGF2 and H19 is still unclear, but omega-6 may be involved with epigenetic regulation of fatty acid desaturase (FADS)2 and or FADS1 (Mennitti et al., 2015). Lastly and somewhat unexpectedly, we found that saturated fat intake during the second trimester was positively associated with DNA methylation of the IGF2-DMR. In the present study, we did not find significant associations between IGF2 and H19-DMR DNA methylation and cord blood IGF2 levels. Possible explanations include the small sample size and the fact that we did not measure IGF2 and H19 methylation in tissues releasing IGF2 protein. An alternative explanation was that our population was selected from uncomplicated full-term pregnancies within the larger cohort, which aimed to reduce extraneous variation in DNA methylation. Any effect of methylation at IGF2 and H19 on cord blood hormone levels or fetal growth could have been masked if the associations were mediated through gestational age.

This exploratory study has some limitations. First, we did not examine allelic-specific DNA methylation patterns of the IGF2 and
H19 genes but surmise that a change in methylation at IGF-DMR is due to a paternal allele while a change in H19-DMR is from a maternal allele. Second, DNA was extracted from cord blood nucleated cells, which has a mixture of different cell types, and thus, our results might reflect shifts in leukocyte composition or other nucleated cells. However, Murphy et al. (2012) has showed that DMR methylation profiles of IGF2 and H19 imprinted genes are comparable across different cord blood cell fractions. Third, we had a small sample size. However, we had sufficient variations in fat intake and DNA methylation and were able to identify significant differences that have been reported in a previous study (Lee et al., 2014). Fourth, we tested many associations, and none of the significant associations would have withstood using Bonferroni or Benjamini–Hockberg correction. Furthermore, a majority of study participants were white and of high socioeconomic status and their overall diet quality found to be above average with regards to increased consumption of fruits, vegetables, and seafood (Lin et al., in press). Therefore, the generalizability of our population to other populations is unclear. However, the strict selection criteria of our study sample limited extraneous variability and aided in increasing the internal validity of our results. The magnitudes of DNA methylation differences in relation to fat intake were relatively small and the variation in IGF2 and H19 DMR methylation did not translate into significant differences in cord blood IGF2 levels or fetal growth. Lastly, dietary intake was measured with questionnaires completed by study participants, which could contribute to self-report, recall and social desirability bias. Measurement errors are inevitable in estimates of fatty acid intake. Nonetheless, the FFQ has been validated against multiple diet records and reasonable correlation coefficients between these assessments were observed. Residual confounding from different diet patterns and other micronutrients is possible. Given the limitations acknowledged above, our findings should be interpreted with caution, and future studies with larger sample sizes and stratification of offspring by sex are warranted.

In conclusion, we investigated the trimester-specific associations of different types of dietary fat intake with DNA methylation levels at IGF2 and H19 imprinted regions in cord blood. We observed that maternal omega-6 and omega-3 intake during early gestation was associated with differential IGF2 and H19 DNA methylation at birth. In addition, saturated fat intake during mid-pregnancy was positively associated with IGF2-DMR DNA methylation. Our results add to the growing body of literature implicating that prenatal maternal intake of different types of fat may be associated with epigenetic regulation of IGF2. Further investigations with larger sample sizes are needed to confirm these findings, evaluate whether differences persist into childhood, and to assess phenotypic consequences according to small changes in DNA methylation of imprinted IGF2 and H19 genes.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS
JEC, KHM, AB, and EO designed research; AB and HEL quantified IGF2 and H19 DNA methylation data; Y-HC, AJG, SLR-S, JEC and AC conducted research; Y-HC, AJG and SLR-S analyzed data; Y-HC, AC, and RPF wrote the article; AJG, SLR-S, HEL, M-FH, AC, EO, RPF, and JEC contributed to the interpretation of the data. All authors were involved in critical revision of the manuscript for important intellectual content; and Y-HC, JEC and AC had primary responsibility of the final content. All authors read and approved the final manuscript.

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