MATERNAL ADHERENCE TO A MEDITERRANEAN DIET DURING PREGNANCY, INFANT DNA METHYLATION AT BIRTH AND WEIGHT GAIN IN INFANCY

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ABSTRACT

Sarah Gonzalez-Nahm: Maternal Adherence to a Mediterranean Diet During Pregnancy, Infant DNA Methylation at Birth and Weight Gain in Infancy
(Under the direction of Diane Rowley)

The purpose of this dissertation is to examine the association between maternal adherence to a Mediterranean diet during pregnancy, infant DNA methylation at birth at 5 differentially methylation regions (DMRs) of imprinted genes, and weight gain in the first year of life. The first paper of this dissertation uses multinomial logistic regression models to determine the association between maternal adherence to a Mediterranean diet in early pregnancy and infant DNA methylation at birth. Results suggested an association between a lower level of methylation at the *MEG3 IG* DMR and lower adherence to a Mediterranean diet. This association was only evident in girls. The second paper focused on the association between infant DNA methylation at birth and weight gain between birth and age 1, as measured by change in weight-for-length Z scores. Results of linear regressions showed that a higher methylation level at the *MEG3* DMR was associated with a lower weight gain in the first year of life. This association was evident in boys and in infants with lower than the median birth weight for the study sample. In the third aim, the potential mediation by DNA methylation in the relationship between maternal Mediterranean diet during pregnancy and weight gain in the first year of life was explored. However, the presence of mediation was not assessed due to sample size and methodological limitations. Overall these findings suggest that maternal adherence to a Mediterranean diet during early pregnancy affects infant DNA methylation of select imprinted genes at birth, and infant DNA methylation at select imprinted genes is associated with weight gain in infancy. However, no differentially methylated regions included in this study were associated with both maternal adherence to a Mediterranean diet and weight gain in infancy.

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LIST OF ABBREVIATIONS

BMI   body mass index

CDC   Centers for Disease Control and Prevention

CI   confidence interval

CpG   cytosine-phosphate-guanine

DAG   directed acyclic graph

DLK1   Delta-like homolog 1

DMR   differentially methylated region

DNA   Deoxyribonucleic acid

EER   estimated energy requirement

EMM   effect measure modification

FFQ   food frequency questionnaire

IGF2   Insulin growth factor 2

KCAL   kilocalorie

KG   kilogram

LAZ   length-for-age

MDS   Mediterranean diet score

MEG3   Maternally expressed gene 3

MEG3 IG Maternally expressed gene 3 intergenic

MUFA   monounsaturated fatty acid

NEST   Newborn Epigenetic Study

OR   odds ratio

PLAGL1   Pleiomorphic adenoma gene-like 1

SD   standard deviation

SFA   saturated fatty acid

SRS	Silver Russell Syndrome
WLZ	weight-for-length
WAZ	weight-for-age

CHAPTER 1- INTRODUCTION

Childhood Obesity

Childhood obesity is a major public health concern in the United States and around the world. Obesity in childhood is a risk factor for obesity later in life[1–3], and is associated with a number of chronic diseases in both childhood[4,5] and adulthood[6]. In the United States, approximately 17% of children and adolescents ages 2-19 years are obese[7]. While overall childhood obesity rates have not changed much in recent years, the disparity in obesity rates between African Americans and Latinos, and non-Hispanic whites has continued. An alarming 24.3% of Black children and 21.2% of Hispanic children are obese, while only 14% of non-Hispanic White children are obese[7].

Obesity is a multifactorial condition, with environmental and biological risk factors and mechanisms. Environmental factors, such as diet and feeding practices[8,9], physical activity[8], the built environment[10], and social factors, such as race[7,11,12] and socioeconomic status[11,12] have been associated with the development and incidence of obesity in young children. Identification of early obesity predictors may help prevent the onset of obesity later in life. Weight gain in infancy has been shown to be a predictor of obesity later in life[13–15]. Research has shown that rapid weight gain in infancy increases obesity risk as early as 2-4 years of age[13,16,17], but also in adolescence[2,18] and adulthood[14,15].

Evidence has also pointed to the potential in-utero origins of obesity[19–22]. According to the in-utero origins theory, there is a mismatch between the in-utero and postnatal environments that predispose an individual to accumulate fat mass more readily than others[19–22]. Gaining a better understanding of the in-utero origins of obesity could help with the identification of those

"high risk" individuals. This could allow for better targeting of public health interventions for obesity prevention and health over the life course.

Dutch famine studies have demonstrated that there are critical or sensitive periods, during which environmental factors or metabolic insults may have a greater influence on health[23,24]. The in-utero period is a critical time window, during which a growing embryo or fetus may be negatively affected by maternal exposures. These effects may present themselves early in life and last throughout the life course[4], or may manifest themselves later in life[20,21]. Mechanisms for the *in utero* origins of obesity have yet to be established, however there is a growing literature suggesting potential epigenetic processes[25–27].

Epigenetics

Epigenetics is the study of changes to gene expression caused by mechanisms other than the underlying DNA sequence[28,29]. DNA methylation is the most commonly studied epigenetic mechanism in epidemiological models due to its stability and the ease with which it can be measured[30]. DNA methylation occurs at cytosines that are followed by guanines i.e. CpG dinucleotides, which exist within differentially methylated regions (DMRs)[28,31]. Changes in DNA methylation as a result of early exposures can be measured at specific sites[32,33], genome-wide[34,35], or globally[36,37]. DNA methylation can be measured in many cell types, including leukocytes from umbilical cord blood, as well as a variety of tissues. In human studies, tissue-specific DNA methylation is not readily obtainable due to ethical issues, therefore cord blood or peripheral blood are typically used. There is evidence suggesting that there are no significant differences between DNA methylation at DMRs regulating imprinted genes from conceptal tissues and cord blood[38].

As in-utero exposures are of interest, it is important to consider imprinted genes. Imprinted genes are monoallelically expressed, and this monoallelic expression is controlled by DNA methylation, which depends on which parent the allele came from[29,39]. Methylation of

regulatory sequences of imprinted genes is established during gametogenesis and is stably maintained throughout somatic division[40,41]. Parental origin-specific methylation profiles are spatially and temporally stable, such that methylation changes at imprinted gene DMRs resulting from *in utero* exposures can been seen later in life. For example, exposure to famine conditions in-utero has been detected decades post-exposure[23,24].

Epigenetic modifications can occur as a result of a variety of exposures. Thus far, animal models and epidemiological studies have shown that nutritional manipulations, such as the addition of methyl donors or the restriction of protein, can alter DNA methylation[42–44]. Environmental toxicants, such as cigarette smoke[45], cadmium[46], and lead[47,48] have also been found to affect the human epigenome. However, the phenotypes associated with these epigenetic changes are not well-known.

There is still insufficient and inconsistent evidence linking epigenetic mechanisms to obesity and weight gain in humans. Studies vary by DNA locus chosen, epigenetic mechanism examined, and timing of the outcome or exposure. Many studies looking at the epigenetic changes associated with obesity have not focused on the in-utero period[49], or have focused on non-imprinted genes[50]. To date, the insulin growth factor 2 (*IGF2*)/*H19* domain has been associated with fetal growth[51,52], child/adolescent weight and adiposity[53,54], and adult obesity and chronic disease[23,33,55]. Other imprinted genes that have been found in association with growth and/or obesity include maternally expressed gene 3 (*MEG3*)[55] and pleiomorphic adenoma gene-like 1 (*PLAGL1*)[55,56]. Methylation in non-imprinted genes, such as peroxisome proliferator-activated receptor (*PPAR*)[57], matrix metallopeptidase 9 (*MMP9*)[58], and retinoid x receptor alpha (*RXRA*)[50] has also been found in association with obesity. However, the heritability, malleability and significance of the epigenetic changes in non-imprinted genes is unknown. More research is needed to understand the role of and key players in DNA methylation of imprinted genes as a potential intermediate marker in the relationship between maternal and infant exposures and obesity in childhood.

Obesity and Assessment of Infant Weight Gain

Obesity in children ages 2-19 years is defined as a body mass index (BMI) greater than or equal to the 95th percentile for age and sex. There is currently no formal definition for obesity in children younger than two years of age[7]. However, a variety of measurements are regularly used to assess body weight[59], depending on the availability of information. Weight-for-length Z scores, weight-for-age Z scores, and BMI are often used to measure body mass and fatness in infants, as they are easily and inexpensively obtained. BMI, calculated as an individual's weight divided by their height squared, can be useful if comparing infant weight to weight later in childhood, when BMI is regularly used and accepted as a measure of obesity. However, it is not generally recommended to use BMI as an obesity measure, as there is insufficient research on the meaning of BMI calculated from recumbent length vs height and how that relates to obesity. Weight-for-length Z (WLZ) scores and Weight-for-age Z (WAZ) scores have also been used as ways to measure obesity. However, WLZ scores are generally regarded as a better measure of body fatness in infancy than WAZ scores, as they measure relative weight. There is also evidence showing an association between WLZ scores and obesity. According to World Health Organization (WHO) standards, obesity is classified as a WLZ score greater than 3 and overweight is classified as a WLZ score greater than 2[60]. Weight gain in infancy, as measured by change in WLZ scores has also been associated with weight and obesity later in life[13]. Weight gain can be measured continuously, or as a binary variable, in which rapid weight gain has been previously defined as a change in anthropometric Z scores of +0.67 SD[61] from the mean.

Maternal Diet

Maternal diet and nutritional status during pregnancy can influence the fetal environment, and may lead to metabolic programming of the fetus that results in increased risk of obesity and chronic disease[62–65] There is evidence to suggest that both maternal over[66] and

under[67] nutrition can affect offspring health in similar ways. Studies conducted on survivors of the Dutch famine of 1944-45 found that individuals affected by famine in-utero had a higher incidence of a number of chronic diseases, including type 2 diabetes[23]. In addition, a study of mothers in India found that consuming micronutrient-rich foods during pregnancy was associated with fetal size at birth[68]. Animal models have shown that protein restriction during pregnancy in rats can result in hypertension[69], increased fat deposition[67], altered feeding behavior and preferences[70], and other cardio-metabolic outcomes in offspring[71]. Conversely, a diet that is excessive in certain macronutrients, such as fat, can also have an effect on fetal biomarkers, and can have lifelong consequences. Murabayashi, et al[66]. found that rat fetuses of mothers fed a high fat diet during gestation, had higher levels of plasma glucose and insulin, and inflammatory change in adipose tissue compared to those whose mothers were fed a normal fat diet. Another study found that children whose mothers consumed a diet high in sugar during pregnancy had an increased odds of obesity at age 5 compared to children whose mothers did not have a high sugar intake during pregnancy[72].

Maternal Diet and Epigenetics

Maternal diet can also affect the epigenome. It has been shown that individuals who experienced famine conditions in-utero exhibit a lower level of methylation at the *IGF2* region and have a greater incidence of obesity and chronic diseases than their siblings who did not experience famine conditions *in utero*[23]. Although severe caloric restriction related to famine conditions may not be generalizable, a recent study also found that even subtle seasonal changes in the diet, which are common in developing countries, may affect the epigenome[73]. There is also evidence to suggest that methyl donor nutrients (folate, vitamin B12, choline, genistein, methionine, betaine) may affect DNA methylation. Studies have found that folic acid may be associated with increased methylation at the *IGF2* DMR[43,74,75]. In addition, agouti mouse models have shown that an increase in maternal dietary genistein intake can influence offspring

DNA methylation and may even be protect offspring from becoming obese later in life, despite a genetic predisposition to become obese[32]. Waterland and colleagues[76] also found that the addition of methyl donors to the diet of pregnant obese agouti mice can prevent the amplification of obesity in future generations, suggesting that epigenetic mechanisms are affected by this dietary change. There is also evidence to suggest that a maternal high fat diet can result in global hypomethylation, which may be reversed or improved with the addition of methyl donor nutrients to the diet[77].

Mediterranean Diet

The consumption patterns of individuals living in the Mediterranean have been studied extensively. The literature suggests that a high adherence to a Mediterranean diet is generally characterized by a high intake of vegetables, legumes, fruits and nuts, fish, grains, a high monounsaturated fat (MUFA) to saturated fat (SFA) ratio, moderate alcohol consumption, and low consumption of other meats and dairy products[78,79]. Many variations exist in terms of actual foods consumed as part of a Mediterranean diet based on geographical region[80,81]. Despite this, several indexes and scores have been created to measure adherence to a Mediterranean diet pattern, including the Mediterranean diet score (MDS), a median-based score developed by Trichopoulou, et al[78,79], the Mediterranean Adequacy Index[82] (MAI), and the Mediterranean Diet Quality Index[83] (DQI). The MDS has been showed to reliably capture what is commonly understood as a Mediterranean diet pattern[84], and is widely used in research.

Research has shown that a high adherence to a "Mediterranean diet" may lead to improved health, lower inflammation[85], healthier weight[86], and greater survival in adults[78,79]. In addition, maternal consumption of a Mediterranean diet pattern during pregnancy has been associated with a lower risk of fetal growth restriction[80] and smaller waist circumference at age 4[87]. There is evidence showing that bioactive food components present in the Mediterranean diet pattern, such as phenolic compounds can affect DNA methylation[88,89]. However the

epigenetic effects of overall maternal adherence to a Mediterranean diet pattern have not been studied.

Study Overview

To address the gaps in the literature, this study aims to

Aim 1: Estimate the relationship between maternal Mediterranean diet adherence and infant DNA methylation at birth

Aim 2: Determine the association between infant DNA methylation at birth and infant weight gain between birth and age one

Aim 3: Assess the relationship between maternal Mediterranean diet adherence, infant DNA methylation at birth, and infant weight gain between birth and age one

Differentially Methylation Regions

In this study, we focus on the following five DMRs of imprinted genes due to their role in growth[52,56], weight[53,56], chronic disease[90] and parental obesity[33,55]:

1) *MEG3* and 2) *MEG3* intergenic (IG): Location: chromosome 14q32.2[38].

Both the *MEG3 IG* and *MEG3* DMRs are thought to regulate the *MEG3*/Delta-like 1 homolog (DLK1) region, however, it is thought that the *MEG3 IG* DMR may function hierarchically as an upstream regulator in the methylation patterns of the *MEG3* DMR[91]. *MEG3* is found on the *MEG3* promoter, and *MEG3 IG* is located between the *MEG3* and DLK1 promoters. *MEG3* is a maternally expressed gene, meaning that only the maternal allele is expressed, and is believed to contain a tumor suppressor gene[92]. Its suppression has been associated with a variety of tumors, such as Wilm's tumors and pituitary tumors[39]. In addition, a decrease in methylation of the *MEG3* DMR in infants at birth has been associated with maternal pre-pregnancy obesity[55]. Changes in methylation at the *MEG3* DMR have also been associated with penicillin use[93], maternal depression during pregnancy[94], maternal folate consumption[74], and renal cancer[95]. In addition, hypermethylation at the *MEG3* DMR and expression of *MEG3*

long non-coding RNAs has been associated with insulin resistance and type 2 diabetes[90]. More drastic changes to *MEG3* and *MEG3 IG*, such as loss of imprinting and microdeletions have resulted in paternal/maternal uniparental disomy 14[91].

3) *PLAGL1* (also known as *ZAC1*): Location: chromosome 6q24.2[55].

 PLAGL1 is a tumor suppressor gene, and is thought to be part of a network of co-regulated genes believed to play an important role embryonic and postnatal growth[56]. In addition, *PLAGL1* has been shown to be hypermethylated in infants at birth in association with maternal obesity[55]. Also, loss of imprinting at *PLAGL1* is associated with transient neonatal diabetes mellitus syndrome (TNDM) in infants[96].

4) *IGF2*: Location: chromosome 11p15.5[38].

 IGF2 is located upstream of the imprinted promoters of *IGF2*[38]. Changes in *IGF2* methylation have been associated with fetal growth[51,52], maternal sociodemographic factors[97], lead exposure[47,48], in-utero cigarette smoke exposure[45], paternal obesity[33], renal cancer, and Wilm's tumors[39].

5) *H19*: Location: chromosome 11p15.5[38].

 H19 is a maternally expressed gene, and is located at the imprinting control region for the *IGF2*/*H19* imprinted domain, near the *H19* promoter[38]. *H19* is altered with Leukemia and Wilms' tumors[39]. Changes in *H19* methylation have been associated with increased weight for age in infants[53], Silver-Russel-Syndrome (SRS)[98] and Beckwidth Wiedemann syndrome[99].

CHAPTER 2- AIM1: MATERNAL ADHERENCE TO A MEDITERRANEAN DIET IS ASSOCIATED WITH INFANT SEX-SPECIFIC DNA METHYLATION AT INTERGENIC DIFFERENTIALLY METHYLATED REGION REGULATING THE DLK1/MEG3 IMPRINTED DOMAIN

Introduction

The developmental origins of health and disease hypothesis (DOHaD) posits that *in utero* exposures play a critical role in the risk of adult disease[100]. Maternal diet during pregnancy is an important exposure that is part of the *in utero* environment. Although mechanisms are still poorly understood, a growing consensus suggests that these exposures may act, at least in part, through epigenetic mechanisms[25,31,21], i.e., changes in gene expression caused by mechanisms other than the underlying DNA sequence[28,29]. DNA methylation is the most studied mechanism in epidemiologic studies, in part due to the stability of the DNA molecule and covalent bonding.

DNA methylation plays an integral role in fetal development, including establishing the monoallelic expression of genomically imprinted genes in a parent-of-origin manner during gametogenesis and the early embryo. These DNA methylation marks are faithfully maintained throughout somatic division[101,30,41,40], which makes the methylation marks in these regulatory regions a reliable indicator of exposures that occur during these early developmental stages (i.e. embryogenesis and gametogenesis)[23,57,101]. Therefore, the parental origin-specific methylation profiles are spatially and temporally stable, such that methylation changes at differentially methylated regions (DMRs) of imprinted genes resulting from early *in utero* exposures are observed later in life. Exposure to famine conditions *in utero* were detected in a regulatory region of imprinted *IGF2* 60 years after exposure[57]. Moreover, stability of DNA methylation at the same region was seen three years later in adult controls of a colorectal cancer study[102].

Maternal intake of methyl donor nutrients during the prenatal period has been shown to affect DNA methylation in offspring[73,103,32,104], suggesting that maternal factors, such as diet, can stably alter the *in utero* environment and health outcomes over the lifecourse[42,57,105,106]. Both maternal over- and under-nutrition can influence the offspring *in utero* environment and lead to fetal programming[21-25]. However, the majority of such studies have examined single nutrients, or have been conducted using animal models in well-controlled environments. Studies looking at overall diet patterns allow for the exploration of food interactions and synergy that cannot be studied when evaluating nutrients in isolation, or studying animal diets with a prescribed distribution of macro and micronutrients. Foods have a variety of components and effects on the body, and some may act in synergy with or may interfere with the absorption of other diet components[107]. Epidemiological studies exploring overall diet patterns in relation to epigenetic outcomes are needed to gain a better understanding of the influence of diet on DNA methylation.

A Mediterranean diet pattern has been recommended for its overall health benefits and potential for disease prevention[108]. These benefits may extend to the *in utero* period. The Mediterranean diet has been well-studied for its ability to reduce inflammation, and improve longevity and overall health among adults[85,109,110]. Consumption of a Mediterranean diet pattern during pregnancy has been associated with a lower risk of preterm birth[111], lower risk of infant growth restriction in a Spanish Mediterranean population[80], and more recently, a lower child waist circumference at age 4 years[87]. The Mediterranean diet pattern has also been associated with higher intake of certain nutrients, such as folate[112] and phenols[113] that have been shown to modify epigenetic mechanisms[75,114]. There are few published studies on the influence of the Mediterranean diet on epigenetic outcomes, and they have been limited to adult populations. There is some evidence suggesting an association between adherence to a Mediterranean diet in adulthood and increased *LINE-1* methylation[115]. Another study observed an association between a low consumption of the fruit and nuts component of the Mediterranean diet and lower

methylation at *LINE-1* among healthy non-pregnant women[116]. No studies have looked at the potential epigenetic mechanisms of the *in utero* effects of the Mediterranean diet pattern.

In this study we assessed the associations between maternal adherence to a Mediterranean diet pattern during early pregnancy and infant DNA methylation at birth in an ethnically diverse cohort. DNA methylation at birth was studied at five DMRs of imprinted genes. These regions were selected for their involvement in growth[117], obesity[33,55], and common chronic disease[90]. The selected regions include the *MEG3 IG* DMR and the *MEG3* DMR, which are involved in regulating the delta-like 1 homolog/maternally expressed gene 3 (*DLK1/MEG3*) imprinted domain on chromosome 14q32.2; the *IGF2* DMR and the *H19* DMR , which are involved in imprinting of the insulin growth factor 2/*H19* (*IGF2/H19*) domain on chromosome 11p15.5, which are located upstream of the imprinted promoters of *IGF2* and at the imprinting control region for the *IGF2/H19* imprinted domain near the *H19* promoter, respectively; and the *PLAGL1* DMR, which resides at the pleiomorphic adenoma gene-like 1 (*PLAGL1*) locus on chromosome 6q24.2. We expect that a low adherence to a Mediterranean diet pattern will result in aberrant DNA methylation at these five DMRs.

Methods

Study Sample and Data Collection

This study includes mother-infant pairs, who had completed a preconception or first trimester Food Frequency Questionnaire (FFQ) and who had infant DNA methylation data available from cord blood analysis as part of the Newborn Epigenetic Study (NEST). Recruitment and enrollment strategies have been described in detail elsewhere[94]. Briefly, between 2009 and 2011, women were recruited from 5 prenatal clinics and obstetric facilities in Durham, North Carolina. Eligibility criteria include being at least 18 years of age, and intention to use one of the qualifying obstetric facilities for delivery. Women were excluded if they were HIV positive, planned to relinquish custody of the child, or planned to move away from the area in

the following three years. Of 2548 women who met the eligibility criteria, 1700 (67%) were consented and enrolled. Upon enrollment, mothers completed questionnaires providing information on socio-demographic factors and lifestyle characteristics. At delivery, birth outcomes were abstracted from medical records and infant cord blood specimens were obtained to assess offspring methylation. Medical records were abstracted to verify gestational diabetes diagnosis and other medical conditions, birth weight and the newborn's sex.

Of 1700 who enrolled, 396 women were excluded for a variety of reasons, including infant death during or soon after birth, being illiterate, underage, refusing further participation, or who could no longer be found. Given the malleability of DNA methylation patterns in early gestation, we are only including women who completed FFQs relating to preconception or the first trimester (N= 870). Mothers who responded to the FFQ were significantly different than mothers who did not complete and FFQ with respect to race, education, age, BMI, and smoking status (data not shown). Women with extreme implausible energy intakes, defined in our study as an intake of less than 500 kcal/day or greater than 7000 kcal/day, were excluded from our study (N=36). The ratio of estimated energy requirement (EER) to reported energy intake was calculated to assess possible over and under-reporting (defined as EER:kcal greater or less than ± 2 SD[118]). Rather than exclude the possible over/under reporters, a sensitivity analysis was conducted to assess the influence of possible over and under reporting of energy intake on our results. DNA methylation was analyzed from cord blood for the first 550 study infants after exclusions for infant death, illiteracy, being underage, refusal of further participation, and attrition. The mother-infant pairs with analyzed DNA methylation were significantly different from those whose DNA methylation had not been analyzed with respect to race and maternal age. However, not all infants whose mothers completed a 1st trimester or preconception FFQ were part of the subsample with analyzed DNA methylation. Our study includes the 390 women from the NEST study who completed a first trimester or preconception food frequency questionnaire

(FFQ), who did not have extreme high or low reported caloric intakes, and whose infants had DNA methylation data available from cord blood analysis.

Dietary data were collected through a Block FFQ[119] that had been modified to represent diet patterns in North Carolina. Diet data collection was attempted at enrollment, and at least once during each trimester. The FFQ collected data on intake frequencies of over 150 food items and supplements, and was administered to reflect intake during three periods: 1) the peri-conception period, 2) the first trimester, and 3) the second and third trimesters. For this study, only peri-conceptional and first trimester FFQs will be used, as methylation markers of imprinted genes are malleable in the first days of pregnancy. FFQ responses were analyzed by NutritionQuest (www.nutritionquest.com). Reported intake portions and frequencies were assessed and converted to grams for statistical analysis.

DNA Methylation

Infant cord blood specimens were collected at birth. Samples were collected in EDTA-containing vacutainer tubes and centrifuged using standard protocols to allow for collection of plasma and buffy coat, with buffy coat used for DNA extraction (Qiagen; Valencia, CA). Specimens were stored at -80°C until the time of analysis. DNA was extracted using Puregene reagents according to the manufacturer's protocol (Qiagen), and quantity and quality were assessed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific; Wilmington, DE). Infant genomic DNA (800 ng) was modified by treatment with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research; Irvine, CA). Bisulfite treatment of denatured DNA converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged, allowing for quantitative measurement of cytosine methylation status. Pyrosequencing was performed using a PyroMark Q96 MD pyrosequencer (Qiagen). Pyrosequencing assay design, genomic coordinates, assay conditions, and assay validation are described in detail elsewhere[38]. Briefly, assays were designed to query established imprinted gene DMRs using the PyroMark Assay Design Software (Qiagen). PCR conditions were optimized to produce a single, robust

amplification product. Defined mixtures of fully methylated and unmethylated control DNAs were used to show a linear increase in detection of methylation values as the level of input DNA methylation increased (Pearson r is 0.99 for all DMRs). Once optimal conditions were defined, each DMR was analyzed using the same amount of input DNA from each specimen (40 ng, assuming complete recovery following bisulfite modification of 800 ng DNA). Percentage of methylation for each CpG cytosine was determined using Pyro Q-CpG software (Qiagen). Pyrosequencing assays were performed in duplicate for all specimens whose values fell more than two standard deviations above or below the means in which case the average of the two runs was used. The values obtained represent the mean methylation for the CpG sites contained within the sequence being analyzed.

Diet Assessment

Given the malleability of methylation markers of imprinted genes in the early stages of gestation, mothers' Mediterranean diet adherence was assessed using data from preconception and first trimester FFQs. Intakes were converted to grams/1000 kcals to account for differences in caloric intake, and scored with a modified version of Trichopolou's Mediterranean Diet Score (MDS)[78]. Briefly, the MDS assigns values of zero or one to each of nine indicated components, using the population medians of each component among the participants as cut-points. People whose consumption of presumed beneficial components (vegetables, legumes, fruits/nuts, cereals, fish) was at or above the median consumption were assigned a value of one, and a value of zero otherwise. People whose consumption of presumed detrimental components (meat and dairy products) was below the median consumption were assigned a value of one, and zero otherwise. A value of one was given to those consuming a moderate level of alcohol, and a value of one was assigned to those whose ratio of monounsaturated fatty acid to saturated fatty acid intake (MUFA:SFA) was at or above the median (and 0 otherwise).

For this study, the MDS was modified as follows:

1) Fruits and nuts were separated into 2 groups

2) The grains category was refined refinement to reflect only whole grain intake

3) The alcohol group was removed, as reported alcohol intake for mothers during pregnancy in this study was extremely low and alcohol consumption during pregnancy is not advised

4) Dairy was assessed as a "beneficial" food group rather than a "detrimental" food group, as intake of calcium is important during pregnancy.

The diet components for this study were as follows:

1) Fruit (including fresh, dried, and frozen fruit, but excluding fruit juice)

2) Vegetables (excluding vegetable juice and white potatoes)

3) Nuts and seeds (including nut butters)

4) Beans and legumes (including soy beans)

5) Whole grains and whole grain products

6) Dairy (including full fat dairy, but excluding dairy desserts)

7) Fish

8) The ratio of mono-unsaturated fat to saturated fat intake

9) Meats (including red meat, pork, poultry, game, excluding processed meats)

Intake at or above the study population median of "beneficial" food groups was assigned a score of 1 and a score of 0 otherwise. Below the median intake of "detrimental" foods received a score of 1, and 0 otherwise. The possible range of modified diet score values was 0-9. A higher modified diet score was representative of a greater adherence to a Mediterranean diet pattern.

Statistical Analysis

Chi-square and Kruskal-Wallis tests were conducted to assess associations between potential covariates and Mediterranean diet adherence. Multinomial logistic regression models were used to estimate the associations between maternal adherence to a Mediterranean diet and infant DNA methylation at birth. Continuous modified Mediterranean diet scores (MMDS) were categorized to reflect 3 groups of roughly the same size, with scores between 0-3 being considered "low adherence", scores of 4-5 being considered "medium adherence", and scores of 6-9 being considered "high adherence". "High adherence" was used as the reference category. Mean DNA methylation values were used in this analysis, as previously reported Cronbach's alpha for correlations among methylation values from all CpGs measured at each DMR was >0.89[94]. Normality of the percent methylation of each DMR was assessed using the Kolmogorov-Smirnov test. As 4 of the 5 DMRs tested in this sample were not normally distributed (data not shown), DNA methylation was then assessed in tertiles ("hypo-methylation", "moderate methylation", and "hyper-methylation"). Given the theoretical 50% expected methylation of imprinted genes, the "mid-level" category of DNA methylation was used as the referent. Likelihood ratio tests (LRT) were performed to test for the interaction of association between an infant's sex and maternal diet on infant DNA methylation at birth (α=0.20). The addition of an interaction term for infant's sex In 3 of the 5 DMRs assessed (*MEG3*, *MEG3 IG*, *H19*) was significant, therefore sex-specific adjusted models are presented in addition to overall models.

Covariates considered in the analysis were maternal race/ethnicity (Black, White, and Other), maternal education (greater or less than college education), maternal age at delivery, the sex of the infant, maternal smoking at any point during pregnancy (yes/no), gestational diabetes diagnosis (yes/no), self-reported maternal BMI prior to pregnancy, infant gestational age, maternal gestational weight gain (in kg), parity (primiparous/multiparious), supplement use during pregnancy (yes/no), maternal physical activity during pregnancy (light, moderate, intense

activity), maternal methyl donor intake (sum of total folate from diet and supplements, total choline from diet), processed meats, total energy intake, % total fat in the diet, plate. Covariates of interest were added one by one into the original, unadjusted model. Those that changed the estimates by more than 10% or that were deemed important from the literature were included into the final model. Final adjusted models included the following covariates: maternal pre-pregnancy BMI, maternal age, maternal smoking during pregnancy, maternal education. As the role of dairy in the Mediterranean diet is controversial, we also conducted a sensitivity analysis, in which we removed dairy from the diet score. All statistical analyses were conducted using SAS 9.4 (SAS Institute, Inc).

Results

The characteristics of the study population by Mediterranean diet adherence level are displayed in table 1. Women with the highest adherence to a Mediterranean diet pattern were more likely to be White, have completed a college degree or more, were on average older (mean: 31.1 years), had lower gestational weight gain (mean: 14.3 kg), and were less likely to be smokers (4.4%). Women with the lowest adherence to a Mediterranean diet pattern were more likely to be Black, have earned less than a college degree, were on average younger (mean: 25.9 years), had greater gestational weight gain (mean: 15.0 kg), and were more likely to be smokers (23.4%). No statistically significant differences were found between diet adherence groups with respect to parity, pre-pregnancy BMI, gestational age, gestational diabetes diagnosis, or sex of the infant. In our study, 12.9% of the population were considered possible energy under-reporters and 9.7% were considered possible energy over-reporters based on EER:kcal ratio (Appendix 2). Both over and under-reporters were less likely to be White. Women who may have under-reported their energy intake, were more likely to have a college education or greater, have a higher BMI (mean BMI=30.3), and had significantly lower reported intake of dairy compared to possible "moderate" energy reporters. Possible over-

reporters of energy intake were on average younger (mean age=25.2) and had a significantly greater reported intake of vegetables, non-processed meats, and dessert foods compared to possible "moderate" energy reporters.

Maternal diet characteristics by Mediterranean diet adherence category are shown in Appendix 1. Women's total caloric intake, % calories from protein, % calories from saturated fat, % calories from omega-3 fatty acids, fruit, vegetables, legumes, nuts, whole grains, dairy, fish, non-processed meats, and MUFA:SFA were significantly different by Mediterranean diet adherence category, with women in the highest diet adherence group ingesting the fewest calories, having greater protein, omega-3, fruit, vegetable, nut, legume, fish, dairy, whole grain intake, greater MUFA:SFA, and lower saturated fat and non-processed meat intake than those in lower adherence categories.

Results from the total unadjusted models (table 2) show that a low adherence to a Mediterranean diet pattern during pregnancy is associated with a higher odds of infant lower methylation at the *MEG3 IG* region (OR=2.80; 95% CI=1.35-5.82). This association is strongest and statistically significant only in girls (OR=5.35, 95% CI=1.56-18.36 – unadjusted data not shown). At the *MEG3* region, low Mediterranean diet adherence was associated with a lower odds of lower methylation (OR=0.51; 95% CI=0.26-1.03). This relationship was also statistically significant in girls only (OR=0.24, 95% CI=0.07-0.77). In general, sex-specific models showed a difference in magnitude, and in some cases, direction of association in one sex compared to the other (see table 3 for sex-specific adjusted estimates).

After adjustment, the associations at the *MEG3 IG* DMR persisted (OR=3.17; 95% CI= 1.38-7.27), even after the stringent Bonferroni correction (0.05/5=0.01). In girls this association was further strengthened (OR=7.40, 95% CI= 1.88-29.09), however confidence intervals became wider. Notably, after adjustment the strength of association between medium Mediterranean diet adherence and methylation of the *MEG3 IG* DMR in girls, and the odds of higher methylation in boys at the *PLAGL1* and *H19* DMRs in association with low adherence to

a Mediterranean diet pattern increased and became statistically significant. However the confidence intervals became wider, and the results were no longer statistically significant after Bonferroni correction (*MEG3 IG*: OR= 3.34, 95% CI= 1.10-10.21; *H19*: OR= 4.46, 5% CI= 1.32-15.08; *PLAGL1*: OR= 3.24, 95% CI= 1.02-10.26).

The results of the sensitivity analysis, in which we removed dairy from the diet score are available in the appendix (Appendix 3). In this alternate model we observed an attenuation and loss of statistical significance of our results (Adjusted OR= 1.31; 95% CI= 0.56-3.06). Adding dairy as a covariate did not substantially change our results, but adding calcium did (Adjusted OR= 1.8; 95% CI= 0.90-3.62). Women who had at or above the median consumption of dairy had a significantly greater calcium intake, but not significantly different saturated fat or total fat than women who reported below the median consumption of diary (Appendix 4). And women who had at or above the median dairy intake were also more likely to be White, have a higher level of education, and were on average older than women who reported below the median intake of dairy. Women who had a higher intake of dairy were more likely to be high adherers to a Mediterranean diet when the score incorporated dairy than when it did not (results not shown).

Discussion

We observed a decreased level of methylation at the *MEG3 IG* DMR among girls in response to low and medium maternal adherence to a Mediterranean diet pattern after adjusting for maternal age, maternal education, maternal pre-pregnancy BMI, and maternal smoking. Deregulation of the *MEG3 IG* DMR has been found in cancer tissues[120], and it is believed that the *MEG3 IG* region may be an upstream regulator of the *MEG3* DMR[91], which has been associated with type 2 diabetes[90]. As the Mediterranean diet has been associated with improvements in type 2 diabetes[121], it is possible that the lower levels of *MEG3 IG* methylation seen in our study may be indicative of a protective effect against type 2 diabetes. However, the public health significance of our findings is unclear at this time, as outcomes associated with

lower levels of methylation at *MEG3 IG* are not yet known. Our study's small sample size did not allow for mediation analysis to study child outcomes, therefore future studies will need to investigate the phenotypes associated with maternal diet and methylation at the *MEG3 IG* DMR to better understand the implications of these results.

This is one of a few studies that has looked at and reported sex-specific differences in DNA methylation of imprinted genes[45,122]. Tobi, et al observed sex-specific associations between prenatal famine exposure and methylation at the *LEP*, *INSIGF*, and *GNAS* DMRs. Murphy, et al found an increase in *IGF2* methylation in association with prenatal smoking that was most prominent in boys. Thus far no other published studies have reported sex-specific associations at the *MEG3 IG* DMR. Our findings support the idea that DNA methylation may occur in a sex-specific manner, and contribute to the growing literature on sex-specific DNA methylation.

Surprisingly, no statistically significant associations were observed at the *IGF2* DMR in this study. Previous studies looking at maternal nutrition during pregnancy have found associations between supplementation with B vitamins and increases in *IGF2* methylation[43,75]. Others have found decreased *IGF2* methylation in response to famine or undernutrition[23]. Perhaps this is because the Mediterranean diet represents a more subtle or even different diet exposure than the dietary factors included in these studies. For example, while a Mediterranean diet pattern has been associated with a higher folate consumption and blood folate concentration[112,123], it may not represent the same level as a 400 mcg supplementation of folic acid. In addition, a low adherence to a Mediterranean diet pattern is not generally characterized by the overall calorie or protein restriction that is experienced during famine. It is also possible that the dietary driver of DNA methylation changes are not methyl donors, but other foods or compounds in the diet, such as polyphenols or unsaturated fatty acids, which may not have an effect on the *IGF2* DMR.

A strength of this study is its use of a measure of overall diet rather than nutrients in isolation. Thus far, the study of nutrition in relation to DNA methylation has largely focused on the intake of individual nutrients, with many studies conducted using animal models. In general, nutrients are not consumed in isolation in the human diet, and in some cases, nutrients and food components can interact with one another, enhance or weaken the effect of others, or affect biological processes in the body[107]. This has been seen in the case of plant phenols[124] and measurement of starches with regard to the glycemic index[125]. Investigating the potential effects of foods and overall diet is important and will have more applicable public health implications. To our knowledge this is the first study to assess the effects of overall maternal diet pattern on infant DNA methylation. Therefore this study presents an important contribution to this emerging literature.

We found that 12.9% of our sample possibly under-reported their energy intake and 9.7% possibly over-reported energy intake, which is within the range of values seen in previous literature assessing energy reporting bias among pregnant and non-pregnant women[126–128]. We did see differences in reported intake of specific food groups by energy reporting category, however our sensitivity analysis showed that the possibility of over and under-reporting had little effect on our findings, as adjusting for the possible over- and under- reporters did not substantially change the results of our study.

Changes related to the scoring of dairy resulted in an attenuated and non-significant association between maternal low adherence to a Mediterranean diet during pregnancy and infant methylation at the *MEG3 IG* DMR. The women in our sample who had a higher dairy intake also had a higher calcium, but not fat intake, and the addition of calcium, and not dairy, to the model resulted in a strengthening of the association. This suggests that calcium may be a driver in the association between maternal Mediterranean diet adherence and *MEG3 IG* methylation. Calcium has been previously associated with DNA methylation in animal models[129,130], however its association with the regions included in this study has not been

studied. Another possible reason for this is that sociodemographic factors may be underlying the association between dairy intake and *MEG3 IG* methylation, as women with above the median diary intake were more likely to be White, have a higher education level, and were on average older than those with below the median dairy consumption. Currently there is no consensus on how to asses dairy intake when assessing Mediterranean diet adherence during pregnancy. Dairy has been assessed as both "beneficial"[80,131], "detrimental"[87,132]. We decided to present our main findings including dairy as a beneficial component, as we believe dairy to be an important component of a balanced diet, and dairy intake was not associated with a greater saturated fat intake compared to those who consumed a lower level of dairy. In addition, there is literature supporting the importance of dairy as a daily component of a Mediterranean diet pattern[133,134]. However, it is important to note that the removal of dairy from the Mediterranean diet assessment may alter findings.

The Mediterranean diet score, from which the score in this study was based, has been shown to be a reliable indicator of adherence to a Mediterranean diet pattern[84]. However, assessing diet with a Mediterranean diet score in non-Mediterranean populations may present a problem. The specific foods consumed in non-Mediterranean regions may be different from those consumed in Mediterranean regions, as food preferences, access, and availability are dictated by the environment, and may differ[135]. It is important to acknowledge this, as the components of the diet pattern may provide different health benefits. For example, olive oil makes up a large part of the monounsaturated fatty acids consumed in Mediterranean populations. However, in other populations, for example the United States, a large proportion of monounsaturated fat consumption comes from animal fats[136], which do not confer the same health benefits linked to olive oil.

A limitation of this study was its small sample size. This likely resulted in imprecise or unstable adjusted estimates, as evidenced by wide confidence intervals. Repeated testing is also a limitation in our study, however the statistical significance of our main finding persisted

even after the stringent Bonferroni correction. In addition, the generalizability of our results to prior NEST findings is limited, as women-infant pairs in our reduced sample were significantly different from those in the larger NEST cohort. As the first study to report on overall maternal diet pattern and infant DNA methylation of imprinted genes, this study provides important preliminary data, however larger studies, using a more representative sample will be needed to gain a better idea of the magnitude of the associations reported here. Another limitation was the possibility of residual confounding for our adjusted estimates. Because lifestyle and resources are highly associated with sociodemographic factors such as maternal education, and age, it is possible that other unmeasured factors associated with methylation also influenced our results.

In conclusion, our study suggests that low and medium adherence to a Mediterranean diet pattern in early pregnancy alters DNA methylation at the *MEG3 IG* DMR in a sex-specific manner. Associations between maternal diet in early pregnancy and methylation changes at the *MEG3*, *H19*, and *PLAGL1* regions may also exist, however a larger study may be needed to uncover these associations. While our study was small, as the first study of its kind, it provides important preliminary data, and suggests that sex-specific analyses may be important in studies relating diet to DNA methylation of imprinted genes. Studies have shown an association between DNA methylation and weight[56], adiposity[54], and chronic disease[23,90] at the sites included in this study. However, the significance of our results is still unclear, as child outcomes resulting from the altered DNA methylation profiles observed in this study are still unknown. Future studies should focus on child health outcomes associated with DNA methylation changes seen as a result of maternal diet during pregnancy. In addition, these results should be interpreted with caution. In order to make more concrete inferences, more and larger studies will be necessary to find consistent trends in DNA methylation and contribute to the larger body of literature. The results of this study highlight the potential importance of overall maternal diet during pregnancy in the study of infant DNA methylation. DNA methylation and other epigenetic

markers can be viewed as a way, in which adverse environmental exposures are recorded in the body over the life course. Researchers should look to maternal diet as a modifiable risk factor that may help remediate the damage done by environmental exposures.

Table 1. Characteristics of mothers and infants in the NEST cohort by Mediterranean diet adherence category

	Low Mediterranean adherence		Medium Mediterranean adherence		High Mediterranean adherence	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Race[1]						
Black	58 (50)		47 (30.5)		19 (15.8)	
White	30 (25.9)		49 (31.8)		52 (43.3)	
Other	28 (24.1)		58 (37.7)		49 (40.8)	
Missing						
Maternal education[1]						
Less than college	88 (77.9)		96 (64.0)		51 (43.2)	
College degree or greater	25 (22.1)		54 (36.0)		67 (56.8)	
Missing	3		4		2	
Maternal age[2]		25.9 (5.4)		28.0 (5.7)		31.1 (5.1)
Parity						
Primiparous	42 (36.2)		61 (39.6)		44 (36.7)	
Multiparous	74 (63.8)		93 (60.4)		76 (63.3)	
Maternal pre-pregnancy BMI		26.8 (7.0)		27.3 (6.9)		26.8 (7.0)
Missing	2		0		1	
Gestational weight gain[2] (kg)		15.1 (7.8)		12.6 (6.4)		14.3 (6.9)
Missing	2		1		2	
Gestational age		38.5 (2.2)		38.4 (1.9)		38.2 (2.5)
Maternal smoking[1]						
Yes	26 (23.6)		19 (12.8)		5 (4.4)	
No	84 (76.4)		130 (87.2)		110 (95.7)	
Missing	6		5		5	
Gestational diabetes						
Yes	3 (2.6)		10 (6.6)		10 (8.5)	
No	112 (97.4)		141 (93.4)		108 (91.5)	
Missing	1		3		2	
Infant sex						
Male	63 (54.3)		82 (51.9)		68 (56.7)	
Female	53 (45.7)		75 (48.1)		52 (43.3)	

[1] Results of chi-square test for differences between groups of diet adherence were statistically significant (P <0.01).

[2] Results of Kruskal-Wallis test for differences between groups of diet adherence were statistically significant (P < 0.01).

Table 2. Unadjusted and Adjusted[1] total estimates for the association between adherence to a Mediterranean diet pattern during pregnancy (low and medium adherence, compared to high adherence) and infant DNA methylation (hypo- and hyper-methylation compared to moderate methylation).

	Unadjusted				Adjusted			
	Hypo-methylation		Hyper-methylation		Hypo-methylation		Hyper-methylation	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
MEG3 IG								
Low adherence	2.80 (1.35, 5.82)	0.01	1.70 (0.82, 3.52)	0.15	3.17 (1.38, 7.27)	0.01	1.63 (0.72, 3.67)	0.24
Medium adherence	1.80 (0.92, 3.54)	0.09	1.57 (0.83, 2.97)	0.17	1.95 (0.95, 3.98)	0.07	1.61 (0.83, 3.13)	0.16
MEG3								
Low adherence	0.53 (0.27, 1.06)	0.07	1.20 (0.61, 2.34)	0.60	0.73 (0.33, 1.61)	0.44	1.24 (0.58, 2.62)	0.58
Medium adherence	1.32 (0.69, 2.52)	0.41	1.64 (0.83, 3.24)	0.15	1.63 (0.81, 3.30)	0.17	1.64 (0.80, 3.38)	0.18
IGF2								
Low adherence	1.26 (0.65, 2.43)	0.50	0.62 (0.32, 1.21)	0.16	1.40 (0.66, 2.98)	0.38	0.99 (0.47, 2.11)	0.98
Medium adherence	1.14 (0.61, 2.13)	0.69	0.91 (0.50, 1.66)	0.76	1.41 (0.71, 2.78)	0.33	1.32 (0.69, 2.52)	0.40
H19								
Low adherence	1.33 (0.66, 2.68)	0.42	0.99 (0.50, 1.97)	0.98	1.79 (0.82, 3.92)	0.15	1.65 (0.75, 3.63)	0.22
Medium adherence	1.03 (0.54, 1.95)	0.94	0.82 (0.44, 1.52)	0.53	1.14 (0.58, 2.23)	0.71	0.98 (0.50, 1.90)	0.95
PLAGL1								
Low adherence	0.92 (0.47, 1.79)	0.80	1.11 (0.58, 2.12)	0.75	0.99 (0.46, 2.14)	0.98	2.11 (0.98, 4.56)	0.06
Medium adherence	0.97 (0.53, 1.77)	0.91	0.88 (0.48, 1.62)	0.69	1.03 (0.53, 1.97)	0.94	1.27 (0.65, 2.48)	0.48

[1] Adjusted for maternal pre-pregnancy BMI, maternal education, maternal smoking during pregnancy, maternal age at delivery

Table 3. Sex-specific adjusted[1] estimates of the association between maternal adherence to a Mediterranean diet pattern during pregnancy (low and medium adherence compared to high adherence) and infant DNA methylation at birth (hypo- and hyper-methylation compared to moderate methylation).

	Males				Females			
	Hypomethylation		Hypermethylation		Hypomethylation		Hypermethylation	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
MEG3 IG								
Low MDS	1.77 (0.60, 5.23)	0.30	0.81 (0.25, 2.62)	0.72	7.40 (1.88, 29.09)	0.0004	3.41 (0.95, 12.21)	0.06
Med MDS	1.27 (0.49, 3.32)	0.62	1.13 (0.43, 2.96)	0.81	3.34 (1.10, 10.21)	0.03	1.99 (0.75, 5.33)	0.17
MEG3								
Low MDS	1.08 (0.36, 3.22)	0.89	1.11 (0.36, 3.38)	0.86	0.30 (0.08, 1.15)	0.08	1.39 (0.49, 3.96)	0.53
Med MDS	1.45 (0.53, 3.97)	0.47	1.49 (0.53, 4.17)	0.45	2.03 (0.73, 5.65)	0.18	1.88 (0.66, 5.34)	0.23
IGF2								
Low MDS	1.63 (0.53, 4.96)	0.39	1.16 (0.37, 3.60)	0.80	1.23 (0.42, 3.55)	0.71	0.82 (0.28, 2.35)	0.70
Med MDS	1.82 (0.69, 4.75)	0.22	1.49 (0.58, 3.81)	0.41	1.14 (0.42, 3.06)	0.80	1.24 (0.50, 3.13)	0.64
H19								
Low MDS	2.60 (0.77, 8.77)	0.12	4.46 (1.32, 15.08)	0.02	1.34 (0.47, 3.88)	0.59	0.70 (0.22, 2.19)	0.54
Med MDS	1.16 (0.44, 3.02)	0.77	1.10 (0.42, 2.86)	0.85	1.15 (0.44, 3.04)	0.77	1.04 (0.40, 2.70)	0.94
PLAGL1								
Low MDS	0.95 (0.31, 2.93)	0.93	3.24 (1.02, 10.26)	0.05	0.94 (0.31, 2.85)	0.92	1.48 (0.50, 4.36)	0.48
Med MDS	0.84 (0.33, 2.09)	0.70	1.32 (0.51, 3.40)	0.56	1.06 (0.40, 2.82)	0.91	1.16 (0.44, 3.07)	0.77

[1] Adjusted for maternal pre-pregnancy BMI, maternal education, maternal smoking during pregnancy, maternal age at delivery

CHAPTER 3- AIM 2: INFANT DNA METHYLATION AT BIRTH AND WEIGHT GAIN IN THE FIRST YEAR OF LIFE

Introduction

Understanding what factors influence a child's risk of obesity is crucial to the development of new strategies for obesity prevention. Obesity in early childhood is a risk factor for obesity later in life[2,3,5] and for a number of chronic diseases in both childhood[4] and adulthood[137]. Early identification of obesity or its risk factors may help prevent the progression of obesity and its consequences later in life[6]. Rapid weight gain in infancy has been associated with obesity and overweight later in childhood[13,15], and adulthood[15]. The causes of this rapid weight gain are not well understood.

In accordance with the developmental origins of disease hypothesis, the intrauterine environment is hypothesized to influence an individual's later susceptibility for chronic diseases[19], including obesity[21,22]. Epigenetic modifications have been proposed as a mechanism for the *in utero* origin of later obesity, and a growing literature has found supporting evidence[32,50,138]. Epigenetic modifications are changes to gene expression caused by mechanisms other than the underlying DNA sequence. DNA methylation is the most studied epigenetic mechanism in humans, due in part, to its stability. DNA methylation that controls the monoallelic expression of imprinted genes is established during gametogenesis and is stably maintained throughout somatic division[29,40,41,101,139], and may therefore provide a register of *in utero* exposures. A study of famine survivors found that adults who experienced famine *in utero* had hypo-methylation of the imprinted *IGF2* gene compared to their siblings who had not experienced famine *in utero*[23]. In addition, a colorectal cancer study found that methylation status of the *IGF2*/*H19* imprinted locus of adult controls was maintained 3 years later[140].

While interest in epigenetic research has increased, there is still insufficient and inconsistent evidence linking epigenetic mechanisms to obesity and weight gain in humans. Studies vary by DNA locus chosen, epigenetic mechanism examined, and timing of the outcome or exposure. The most consistent association between imprinted genes and obesity has been seen with the *IGF2* locus. Studies have found a relationship between the *IGF2* domain and fetal growth[51,52,141,142], and body composition or weight in children[52–54]. More research is needed to uncover consistent associations between additional differentially methylated regions (DMRs) and weight gain, and better understand the role of DNA methylation as a potential intermediate marker in the relationship between maternal and infant exposures and weight gain in childhood.

This study aims to assess the association between DNA methylation at 5 imprinted genes and weight gain between birth and age 1. In this analysis we include two differentially methylated regions (DMRs) involved in regulating the delta-like 1 homolog/maternally expressed gene 3 (*DLK1/MEG3*) imprinted domain on chromosome 14q32.2 (the *MEG3 IG* DMR and the *MEG3* DMR), two that are involved in imprinting of the insulin growth factor 2/*H19* (*IGF2/H19*) domain on chromosome 11p15.5 (the *IGF2* DMR and the *H19* DMR) which are located upstream of the imprinted promoters of *IGF2* and at the imprinting control region for the *IGF2/H19* imprinted domain near the *H19* promoter, respectively, and one at the pleiomorphic adenoma gene-like 1 (*PLAGL1*) locus at 6q24.2. These regions were selected for their association with growth[51,52,56], chronic disease[23,90], and parental obesity[55].

Methods

Study Sample and Data Collection

Study participants were recruited as part of the Newborn Epigenetic Study (NEST). Recruitment and enrollment strategies have been described in detail elsewhere[94]. Briefly, between 2009 and 2011, women were recruited from 5 prenatal clinics and obstetric facilities in Durham, North Carolina. Eligibility criteria include being at least 18 years of age, and intention to

use one of the qualifying obstetric facilities for delivery. Women were excluded if they were HIV positive, planned to relinquish custody of the child, or planned to move away from the area in the following three years. Upon enrollment, mothers completed questionnaires providing information on socio-demographic factors, lifestyle characteristics, and anthropometrics (the mother's and the father's height, highest and lowest weight ever, and current and usual weight). At delivery, birth outcomes were abstracted from medical records and infant cord blood specimens were obtained to assess offspring methylation, and at one year, data was collected on child anthropometrics, feeding, and lifestyle.

1700 pregnant women consented and were enrolled in the NEST cohort. Of those, 396 women were excluded for miscarriage (N=109) or infant death during or soon after birth (N=4), being illiterate (N=1), underage (N=1), refusing further participation (N=146), moving away from the area (N=21), or delivering at a hospital not included in the study (N=114). DNA methylation data was analyzed for the first 550 infants in the study. Infants with analyzed DNA methylation were not significantly different than infants whose DNA methylation had been analyzed with respect to race, maternal education, maternal smoking status, maternal pre-pregnancy BMI, maternal age, or weight at age 1 (data not shown). Weight and length measurements at birth and one year were available for 322 infants who had analyzed DNA methylation data. Infant birth weight and length were abstracted from medical records by study personnel. Infant weight and length at age 1 were reported by parents at 1-year follow-up through secure mail-in postcards (30% of sample) or abstracted from medical records (70% of sample). Weight-for-length and weight-for-age Z scores were calculated using Centers for Disease Control and Prevention (CDC) standards[143]. Infants with a WLZ greater than 5 or less than -4, or a LAZ greater than 3 or less than -5, were identified and further examined (N=9). Of those, 4 were implausible and were excluded from analysis. Preterm infants (born before 37 weeks gestation) were also excluded from the study (N=18), as their growth and weight gain trajectories differ from those of term infants[144]. The current study refers to the 300 full term infants with available

DNA methylation data on at least one the 5 DMRs of interest, and who had plausible length and weight measurements at birth and age 1. Infants included in this study had on average greater birth weight and gestational age compared to infants not included in the study (data not shown).

Infant Anthropometric Data

Lengths and weights were used to calculate weight-for-length and length-for-age Z scores at birth and age 1 using reference data from the CDC[143]. Change in Z scores was calculated by subtracting the Z score at birth from the Z score at age 1 (WLZ age 1-WLZ birth). Two outcomes were assessed as a way to capture child relative weight gain and gain in length: change in weight-for-length Z scores (WLZ) between birth and age one, and change in length-for-age Z scores (LAZ) between birth and age 1. Child relative weight gain and gain in length were modeled as a continuous variable, using linear regression. In addition, WLZ was dichotomized, and rapid weight gain at age 1 was defined as a change in weight-for-length Z score between birth and age 1 of 0.67 SD or greater[61].

DNA Methylation

Specimen collection and DNA methylation methods have been described in detail elsewhere[38]. Briefly, infant cord blood specimens were collected at birth. Samples were collected in EDTA-containing vacutainer tubes and centrifuged using standard protocols to allow for collection of plasma and buffy coat, with buffy coat used for DNA extraction (Qiagen; Valencia, CA). Specimens were stored at -80°C until the time of analysis. DNA was extracted using Puregene reagents according to the manufacturer's protocol (Qiagen), and quantity and quality were assessed using a Nanodrop 1000 Spectrophotometer (Thermo Scientific; Wilmington, DE).

Infant genomic DNA (800 ng) was modified by treatment with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research; Irvine, CA). Bisulfite treatment of denatured DNA converts all unmethylated cytosines to uracils, leaving methylated cytosines unchanged, allowing for quantitative measurement of cytosine methylation status. Pyrosequencing was

performed using a PyroMark Q96 MD pyrosequencer (Qiagen). Pyrosequencing assay design, genomic coordinates, assay conditions, and assay validation are described in detail elsewhere[33]. Briefly, assays were designed to query established imprinted gene DMRs using the PyroMark Assay Design Software (Qiagen). PCR conditions were optimized to produce a single, robust amplification product. Defined mixtures of fully methylated and unmethylated control DNAs were used to show a linear increase in detection of methylation values as the level of input DNA methylation increased (Pearson r is 0.99 for all DMRs). Once optimal conditions were defined, each DMR was analyzed using the same amount of input DNA from each specimen (40 ng, assuming complete recovery following bisulfite modification of 800 ng DNA). Percentage of methylation for each CpG cytosine was determined using Pyro Q-CpG software (Qiagen). Pyrosequencing assays were performed in duplicate for all specimens whose values fell more than two standard deviations above or below the means in which case the average of the two runs was used. The values obtained represent the mean methylation for the CpG sites contained within the sequence being analyzed.

Statistical Analysis

Frequencies and means of sociodemographic variables were calculated, and chi square and T tests were conducted to determine if there were significant difference in sociodemographic variables by level of change in WLZ score. All potential covariates were regressed on the exposure (DNA methylation) and the main outcome (change in weight-for-length Z scores) to test preliminary associations. Linear and logistic regressions were conducted to test the association between DNA methylation and early weight gain and growth outcomes.

Potential covariates were determined based on directed acyclic graphs (DAG). Sex and birth weight were chosen as potential effect measure modifiers (EMM), as DNA methylation[45,122] and obesity[145] are believed to vary by sex, and weight gain may vary by birth weight[14,61], with the heaviest term infants gaining more rapid weight gain than term infants with average birth weights. Birth weight was assessed dichotomously, with a cut point above or below the study

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sample median. Interaction terms were tested via likelihood ratio test (α=0.15). As birth weight

has been associated with methylation of *PLAGL1*[56,93,94] and *IGF2* at birth, and with infant weight

gain, it is thought that birth weight may be on the causal path between infant DNA methylation

at birth and infant weight gain. Given the possibility for birth weight to be either a mediator or

modifier in the relationship between infant DNA methylation and weight gain in infancy, it was

not included as a potential confounder in our main models. However, we did conduct a

sensitivity analysis to determine effects of including it as a confounder in our models. The

following covariates were tested as potential confounders: maternal education (less than a

college degree/college degree or greater), maternal gestational diabetes (yes/no), maternal pre-

pregnancy BMI, maternal smoking at any time during pregnancy (yes/no), gestational weight

gain, parity (primiparous, multiparous), maternal age at delivery, self-report status of

anthropometric measurements, and date of length and weight measurements relative to infant's

first birthday. Covariates were tested in the model one at a time, and were kept in the model if

they changed the estimate by more than 10%. Final models included maternal education,

maternal pre-pregnancy BMI, maternal smoking, maternal age, birth weight, self-report status,

and date of weight and length measurements. The interaction terms for sex and birth weight

were significant, therefore sex-specific and birth-weight category specific WLZ score models will

be presented in addition to total models.

Despite breastfeeding being a potentially important covariate, it was excluded from all

models due to excessive missingness (~40%). As breastfeeding was likely missing not at

random, with non-breastfeeding women being more likely to have missing data on

breastfeeding, we conducted a sensitivity analysis using simulations to add breastfeeding to our

models to determine the potential impact of its exclusion. Breastfeeding defined as non-

exclusive breastfeeding duration, and was categorized as 0=0-1 months, 1=1-6 months, 2=

greater than 6 months. Breastfeeding was assigned to different possible combinations of

variable values in 10% increments, and regressions were run with each possible scenario of

values. Breastfeeding was not included in models, as the simulation results showed that it did

not change our estimate by more than 10% (condensed table available in appendix 6).

Mean DNA methylation values for each DMR were used in regression models, as

previously reported Cronbach's alpha for correlations among methylation values from all CpGs

measured at each DMR was >0.89[35]. DNA methylation was assessed in tertiles ("hypo-

methylation", "moderate methylation", and "hyper-methylation"), as percent methylation values

were generally not normally distributed at the DMRs of interest, and both hypo- and hyper-

methylation have been associated with health outcomes, depending on the DMR[55,94]. Given the

theoretical 50% expected methylation of imprinted genes, the "moderate" category of DNA

methylation was used as the referent. All statistical analysis was completed using SAS 9.4.

Results

Study population characteristics are presented in table 4. The racial/ethnic breakdown of

the women in the study is as follows: 38.3% African American, 29.7% White/Caucasian, and

32.0% other races/ethnicities including Hispanic, Asian/Pacific Islander. The majority of women

in the study completed less than a college degree (68%) and reported not smoking at any point

during pregnancy (87%). The mean maternal age for women in this study was 28.0 years (±5.7).

The mean maternal pre-pregnancy BMI for women in the study was 27.4 (±7.2), and the mean

birthweight of infants born to mothers in this study was 3385 grams (±496). There were no

significant differences in sociodemographic variables between infants above and below 0.67 SD

of change in WLZ between birth and age 1.

Change in WLZ Scores Between Birth and Age 1

Unadjusted linear regression estimates (see table 5) show that infants with a higher level

of methylation at the *MEG3* DMR at birth have a lower change in WLZ score between birth and

age 1 (β=-0.6, 95% CI= -1.1, -0.2) compared to infants with moderate methylation. This

association is evident in boys (β=-1.4; 95% CI= -2.1, -0.6) and not girls (β=-0.1, 95% CI= -0.8,

0.6). Sex-specific estimates are available in table 6. Infants with lower methylation at the *H19*

DMR at birth show a greater change in WLZ score between birth and age 1 compared to those

with moderate methylation (β=0.5; 95% CI=0.0, 1.0), however there were no sex-specific

differences in estimates at this DMR. Only girls showed statistically significant differences in

change in WLZ score estimates between birth and age 1 at the *PLAGL1* DMR. Girls with either

higher or lower methylation showed a smaller change in WLZ scores between birth and age 1 at

the *PLAGL1* DMR compared to girls with moderate methylation (hypo: β=-0.8; 95% CI=-1.5, -

0.2; hyper: β= -0.9; 95% CI=-1.6, -0.2). However, no overall association was seen. All

statistically significant associations remained after adjustment. Birth weight-specific results

showed that infants with either a lower than the median birth weight had smaller weight gain

between birth and age 1 in association with either a higher or lower level of *MEG3* methylation

(hypo: β=-0.9; 95% CI=-1.7, -0.2; hyper: β=-0.9; 95% CI=-1.6, -0.2). At the *H19* DMR, infants

with a higher than median birth weight had greater weight gain between birth and age 1 in

association with lower methylation (β=0.8; 95% CI=0.0, 1.5). When WLZ between birth and age

1 was dichotomized at +0.67 SD, results similar to those from linear models were seen in

association with *MEG3* hyper-methylation (Adjusted OR= 0.5; 95% CI= 0.3, 0.9), however

stratified estimates were not calculated due to small numbers. Results of our sensitivity analysis

showed that inclusion of breastfeeding did not substantially alter results (range of β estimates in

relation to hyper-methylation at *MEG3*: -0.62, -0.63. See Appendix 5). In addition, results of our

sensitivity analysis including birth weight as a confounder did not substantially alter our findings

(Appendix 6).

Change in LAZ Scores Between Birth and Age 1

Results of LAZ models are available in table 7. Infants with hyper-methylation at the *H19*

DMR had a decrease in LAZ score between birth and age 1 (β=-0.4; 95% CI= -0.7, 0.0). This

change was no longer statistically significant after adjustment.

Discussion

        We observed slower relative weight gain, as measured by changes in WLZ between birth and age 1 among infants who had a higher level of methylation at the *MEG3* DMR, and greater weight gain among infants with lower methylation at the *H19* DMR. We also observed sex- and birth weight-specific differences in the association between methylation at the *MEG3* and *PLAGL1* DMRs and weight gain between birth and age 1. We observed a slower weight gain in association with a higher level of MEG 3 methylation only in boys, and slower weight gain in association with both higher and lower levels of PLAGL1 methylation in girls only. In addition, infants with lower than the sample median birth weight, who also had either a higher or lower level of *MEG3* methylation had smaller weight gain between birth and age 1 compared to those with moderate methylation. No associations were observed between methylation at the 5 DMRs included in this study and gain in length between birth and age 1. This study adds to a growing body of epidemiologic literature on growth outcomes associated with DNA methylation at birth, and shows that DNA methylation at the *MEG3*, *PLAGL1*, and *H19* DMRs may be associated with weight gain in infancy.

        Higher methylation at the *PLAGL1* DMR has been previously associated with maternal obesity[55], and greater birth weight[56] and weight at age 1[56], however no association had been previously seen between methylation at the *PLAGL1* DMR and change in Z scores or BMI between birth and age 1[56]. In addition, prior literature suggests an association between a higher percent methylation at *H19* in association with overweight status at age one, defined as a weight-for-age BMI at or above the 85th percentile[53]. These results are not consistent with our findings, however the outcome of our study is weight change over time and not weight status at one point in time. In addition, we measured weight gain relative to infant length, which may result in infants being classified differently with respect to obesity, as infants who are heavy, but tall may not be classified as obese. Hypo-methylation of the *H19* paternal allele has been previously associated with Silver Russel Syndrome (SRS), which is characterized by severe

growth restriction, insulin resistance, among other clinical characteristics. However in our study, lower *H19* methylation was associated with greater relative weight gain between birth and age 1. More research is needed to gain a better understanding of the relationship between infant relative weight gain and methylation at these DMRs.

Only one other study has examined the association between methylation at the *MEG3* DMR and early relative weight gain in a mediation model of the indirect effect of *MEG3* in the association between maternal lead exposure and rapid early weight gain[146]. No association was found between *MEG3* methylation and rapid weight gain, however the study was underpowered, and DNA methylation was assessed linearly. A lower percent methylation at the *MEG3* region has previously been associated with maternal obesity prior to pregnancy, with infants of mothers who were obese prior to pregnancy having lower methylation at *MEG3* compared to infants of mothers were not obese prior to pregnancy[55]. Our study results showed that greater methylation at *MEG3* was associated with slower weight gain, which is associated with a lower risk of obesity. Therefore our results mirror those findings, suggesting that perhaps a lower percent methylation may be associated with weight and weight gain in both infants and adults. In addition, prior studies have shown that the *MEG3* DMR may play multiple roles in the body. Studies have linked the *MEG3* DMR to insulin resistance[147] and type 2 diabetes[90]. In addition, *MEG3* may also play a role in the development of cancer, as *MEG3* is believed to transcribe a powerful tumor suppressor gene[92]. The results of this study add to the growing knowledge on the *MEG3* DMR. More long-term studies are needed to fully understand the role of these regions in health and weight gain.

Although previous literature supports the role of the *IGF2* DMR in weight gain and adiposity, we did not observe an association between the *IGF2* DMR and weight gain in the first year of life. However, these results are consistent with a study that found no significant difference in *IGF2* methylation between one-year old infants above or below the 85th percentile for weight-for-age[53], but did find an association between increased methylation at *H19* and

weight for age above 85th percentile at age 1. In addition, much of the evidence shows an association between the *IGF2* DMR and fetal growth[51,148]. In our study we restricted to term births only, therefore many of the infants with aberrant *IGF2* methylation may have been excluded from this study.

In our study, boys with hyper-methylation at the *MEG3* DMR and girls with hypo- and hyper-methylation at the *PLAGL1* DMR exhibited slower weight gain between birth and age 1 compared to those with moderate methylation. There is more than one plausible explanation for the sex-specific results seen in this study. The first possible explanation is that the weight-for-length Z scores in this study do not follow the CDC WLZ score distribution. Therefore, although Z scores take into account the different growth patterns of boys and girls, there may be unaccounted for differences in the distribution by sex, which may lead to differences in WLZ scores by sex. However, a comparison of WLZ scores at age 1 and change in WLZ scores between birth and age 1 did not reveal any significant differences by sex, suggesting that our results do follow the sex-specific Z score distributions. A third possibility is that the study WLZ score distribution differs from the CDC distribution in only one sex, which results in sex-specific differences in WLZ scores. However we did not see any consistent differences in anthropometric variables in one sex over the other, suggesting that this is not the case. A final explanation is that methylation at the highlighted DMRs is different in boys and girls. There is a growing body of literature supporting the idea of sex-specific methylation[23,45,149]. Sex-specific results have not been consistently seen across the same DMRs in different studies, however more studies are needed to gain a better understanding of this phenomenon.

In addition, our study results suggest that infants with lower than the median birth weight and either a lower or higher methylation at the *MEG3* DMR had a smaller relative gain in weight between birth and age 1 compared to those with moderate methylation. The literature suggests that infants at the higher end of the birth weight distribution may be at a higher risk for obesity[150]. As our study was restricted to term births only, infants with lower than the median

birth weight generally fell within the normal range of birth weight. These results suggest that infants of average birth weight may be more likely to have either higher or lower *MEG3* methylation, and may be at a decreased risk of obesity.

This study benefits from an ethnically diverse cohort, and prospectively collected data. This allows us to gain a better understanding of the timing of methylation with regards to our outcome of interest, weight gain. In addition, the use of weight-for-length rather than weight-for-age Z scores as our outcome of interest provides a better estimate of adiposity. However it is not without limitations. Our study's small sample size may have limited our ability to see significant differences among our population. Multiple testing is also a limitation in this study, as it may increase the possibility that our results are seen by chance. Moreover, we were unable to include breastfeeding as a covariate in our main analysis, due to excessive missing data. Our sensitivity analysis showed that inclusion of breastfeeding through multiple imputation did not alter our findings. However, previous literature has suggested that breastfeeding is associated with obesity and early weight gain[151], therefore it is possible that the lack of association in our study is related to measurement error or bias in our breastfeeding variable.

There is evidence to show that rapid weight gain in infancy and early childhood may be a strong predictor of obesity and adiposity later in life[14,15,17]. However, weight trajectories in mid-childhood are more predictive of adiposity in adult life[152,153]. It is possible that many who are classified as having rapid weight gain at age 1 will not become obese. Assessing weight status at various time points throughout early and mid-childhood, in addition to adolescence and adulthood, may provide more robust results.

Our study findings suggest that DNA methylation of the *MEG3*, *H19*, and *PLAGL1* DMRs at birth is associated with weight gain in the first year of life. Associations between methylation and infant relative weight gain at *MEG3* vary by sex and birth weight and associations at PLAGL1 vary by sex. Longitudinal studies are needed to determine whether or not DNA methylation at these DMRs is associated with obesity later in life. Determining the associations

between DNA methylation and early obesity risk is important, as DNA methylation may serve as a biological marker for the assessment of early obesity risk. However, gaining a better understanding of the exposures that affect methylation at these regions is also important, as exposures that modify methylation of regions that are associated with obesity risk may be a good target for early obesity prevention efforts.

Table 4. Characteristics of mother-infant pairs by change in WLZ score category between birth and age 1

	Overall		WLZ change ≥ 1 SD		WLZ change < 1 SD	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
All infants	300 (100)		124 (41.3)		176 (58.7)	
Weight age 1[1] (kg)		10.1 (1.4)		10.9 (1.4)		9.5 (1.1)
Length age 1 (cm)		75.0 (3.9)		75.5 (4.4)		74.7 (3.5)
Race						
Black	115 (38.3)		50 (40.3)		65 (36.9)	
White	89 (29.7)		33 (26.6)		56 (31.8)	
Other	96 (32.0)		41 (33.1)		55 (31.3)	
Education						
Less than college degree	195 (67.9)		87 (72.5)		108 (64.7)	
College degree or more	92 (32.1)		33 (27.5)		59 (35.3)	
Missing			4		9	
Maternal smoking						
No	245 (86.6)		105 (89.0)		140 (84.9)	
Yes	38 (13.4)		13 (11.0)		25 (15.2)	
Missing	17		6		11	
Maternal age		28.0 (5.7)		27.6 (5.8)		28.2 (5.7)
Missing		11		3		8
Sex (of the infant)						
Male	159 (53)		71 (57.3)		88 (50)	
Female	141 (47)		53 (42.7)		88 (50)	
Birth weight (grams)		3385.0 (496.0)		3344.4 (446.3)		3413.6 (527.5)
Parity[2]						
Primiparous	121 (40.3)		57 (46.0)		64 (36.4)	
Multiparous	179 (59.7)		67 (54.0)		112 (63.6)	
Breastfeeding						
0-1 months	52 (27.5)		21 (25.9)		31 (28.7)	
1-7 months	44 (23.3)		21 (25.9)		23 (21.3)	
7 months+	93 (49.2)		39 (48.2)		54 (50)	
Missing	111		43		68	
Maternal BMI		27.4 (7.2)		27.8 (7.0)		27.2 (7.4)
Missing		3				
Gestational weight gain (kg)		13.7 (7.5)		13.6 (7.5)		13.8 (7.6)
Missing		3				
Gestational diabetes						
Yes	22 (7.5)		9 (7.3)		13 (7.6)	
No	273 (92.5)		114 (92.7)		159 (92.4)	
Missing	5		1		4	

[1] Results of T-test show statistically significant differences α <0.05.

[2] Results of chi-square test show statistically significant differences α <0.05.

Table 5. Unadjusted and adjusted[1] results of linear regression of the association between infant DNA methylation at birth and change in infant Z scores between birth and age 1, and logistic regression of the association between infant DNA methylation at birth and rapid weight gain between birth and age 1.

| | Change in WLZ 0-1 | | | | | | Rapid weight gain (≥0.67 SD change in WLZ) | | | | | |
| | Unadjusted | | | Adjusted | | | Unadjusted | | | Adjusted | | |
	β	95 % CI	P	β	95 % CI	P	β	95 % CI	P	β	95 % CI	P
MEG3												
Hypo-methylation	-0.42	(-0.90, 0.07)	0.11	-0.43	(-0.95, 0.09)	0.10	0.71	(0.39, 1.31)	0.27	0.68	(0.36, 1.31)	0.25
Hyper-methylation	-0.63	(-1.11, -0.15)	0.01	-0.67	(-1.18, -0.17)	0.01	0.53	(0.29, 0.97)	0.04	0.49	(0.26, 0.94)	0.03
H19												
Hypo-methylation	0.48	(0.005, 0.96)	0.05	0.52	(0.02, 1.02)	0.04	1.57	(0.84, 2.94)	0.15	1.61	(0.83, 3.09)	0.16
Hyper-methylation	0.12	(-0.34, 0.59)	0.60	0.17	(-0.32, 0.65)	0.50	1.36	(0.74, 2.50)	0.32	1.43	(0.76, 2.70)	0.27
PLAGL1												
Hypo-methylation	-0.37	(-0.83, 0.09)	0.12	-0.34	(-0.82, 0.14)	0.16	0.90	(0.50, 1.60)	0.71	0.92	(0.51, 1.68)	0.79
Hyper-methylation	-0.31	(-0.77, 0.14)	0.18	-0.30	(-0.79, 0.18)	0.22	0.82	(0.46, 1.44)	0.48	0.82	(0.45, 1.50)	0.52
MEG3 IG												
Hypo-methylation	-0.13	(-0.64, 0.37)	0.61	-0.24	(-0.78, 0.30)	0.38	0.85	(0.45, 1.61)	0.63	0.67	(0.33, 1.34)	0.26
Hyper-methylation	-0.18	(-0.67, 0.31)	0.46	-0.30	(-0.80, 0.20)	0.24	0.77	(0.42, 1.43)	0.41	0.61	(0.31, 1.17)	0.14
IGF2												
Hypo-methylation	0.16	(-0.30, 0.62)	0.50	0.19	(-0.30, 0.69)	0.44	1.11	(0.62, 1.99)	0.72	1.26	(0.67, 2.38)	0.48
Hyper-methylation	-0.11	(-0.56, 0.35)	0.64	-0.13	(-0.61, 0.35)	0.59	0.74	(0.41, 1.33)	0.32	0.75	(0.41, 1.39)	0.36

[1] Adjusted for maternal education, maternal smoking, maternal pre-pregnancy BMI, maternal age, self-report of anthropometric measures, and date of anthropometric measurement at age 1.

Table 6. Stratified adjusted[1] estimates: sex-specific and birth-weight-specific adjusted estimates of linear regression of DNA methylation on change in weight-for-height Z score between birth and age 1.

| | Sex-specific | | | | | | Birth weight specific | | | | | |
| | Males | | | Females | | | Lower | | | Higher | | |
	β	95% CI	P	β	95% CI	P	β	95% CI	P	β	95% CI	P
MEG3												
Hypo-methylation	-0.70	(-1.44, 0.04)	0.06	-0.31	(-1.07, 0.44)	0.41	-0.91	(-1.66, -0.16)	0.02	-0.13	(-0.90, 0.65)	0.75
Hyper-methylation	-1.36	(-2.11, -0.61)	0.001	-0.12	(-0.82, 0.58)	0.74	-0.87	(-1.56, -0.18)	0.01	-0.63	(-1.47, 0.21)	0.14
H19												
Hypo-methylation	0.50	(-0.25, 1.25)	0.19	0.65	(-0.06, 1.35)	0.07	0.31	(-0.37, 0.99)	0.36	0.77	(-0.01, 1.54)	0.05
Hyper-methylation	0.00	(-0.72, 0.72)	1.00	0.30	(-0.38, 0.98)	0.38	-0.26	(-0.91, 0.38)	0.42	0.54	(-0.20, 1.27)	0.15
PLAGL1												
Hypo-methylation	0.19	(-0.52, 0.89)	0.60	-0.84	(-1.50, -0.18)	0.01	-0.42	(-1.06, 0.22)	0.20	-0.10	(-0.87, 0.67)	0.80
Hyper-methylation	0.22	(-0.50, 0.93)	0.55	-0.89	(-1.56, -0.22)	0.01	-0.06	(-0.76, 0.65)	0.87	-0.41	(-1.13, 0.32)	0.27
MEG3 IG												
Hypo-methylation	-0.01	(-0.74, 0.72)	0.98	-0.57	(-1.40, 0.26)	0.18	-0.25	(-0.99, 0.49)	0.50	-0.17	(-0.98, 0.63)	0.67
Hyper-methylation	-0.22	(-0.96, 0.52)	0.56	-0.52	(-1.24, 0.21)	0.16	-0.45	(-1.14, 0.24)	0.20	-0.16	(-0.91, 0.59)	0.68
IGF2												
Hypo-methylation	0.03	(-0.68, 0.74)	0.94	0.36	(-0.38, 1.10)	0.34	0.32	(-0.31, 0.95)	0.32	0.14	(-0.66, 0.94)	0.73
Hyper-methylation	-0.49	(-1.22, 0.24)	0.18	0.05	(-0.61, 0.72)	0.88	-0.30	(-0.95, 0.35)	0.37	-0.04	(-0.75, 0.68)	0.92

[1] Adjusted for maternal education, maternal smoking, maternal pre-pregnancy BMI, maternal age, self-report of anthropometric measures, date of anthropometric measurement at age 1.

Table 7. Unadjusted and adjusted[1] results of linear regression in the association between infant DNA methylation at birth and change in LAZ scores between birth and age 1.

	Change in LAZ 0-1					
	Unadjusted			Adjusted		
	β	95 % CI	P	β	95 % CI	P
MEG3						
Hypo-methylation	-0.20	(-0.54, 0.14)	0.26	-0.03	(-0.39, 0.33)	0.86
Hyper-methylation	0.05	(-0.29, 0.39)	0.76	0.10	(-0.25, 0.45)	0.58
H19						
Hypo-methylation	-0.17	(-0.52, 0.18)	0.34	-0.15	(-0.52, 0.22)	0.43
Hyper-methylation	-0.35	(-0.70, 0.00)	0.05	-0.31	(-0.67, 0.05)	0.09
PLAGL1						
Hypo-methylation	0.11	(-0.23, 0.45)	0.52	0.16	(-0.19, 0.51)	0.36
Hyper-methylation	-0.11	(-0.44, 0.22)	0.52	-0.09	(-0.45, 0.26)	0.60
MEG3 IG						
Hypo-methylation	0.05	(-0.32, 0.42)	0.79	0.10	(-0.29, 0.50)	0.60
Hyper-methylation	0.05	(-0.30, 0.41)	0.76	0.07	(-0.30, 0.44)	0.73
IGF2						
Hypo-methylation	0.09	(-0.26, 0.44)	0.60	0.11	(-0.27, 0.48)	0.57
Hyper-methylation	-0.12	(-0.47, 0.22)	0.49	-0.05	(-0.41, 0.31)	0.77

[1] Adjusted for maternal pre-pregnancy BMI, maternal education, maternal smoking, maternal age, self-report of anthropometric measures, date of anthropometric measurement at age 1.

CHAPTER 4- AIM 3: MATERNAL ADHERENCE TO A MEDITERRANEAN DIET PATTERN, INFANT DNA METHYLATION AT BIRTH, AND WEIGHT GAIN IN THE FIRST YEAR OF LIFE: A SIMPLE MEDIATION ANALYSIS

Introduction

Obesity is a major global public health concern. Obesity leads to an increased risk of chronic diseases, such as diabetes and cardiovascular disease. Rapid weight gain in early childhood has consistently been found to be a predictor of adiposity, and obesity later in life[13,15]. Maternal diet during pregnancy may also be associated with child overweight and adiposity, however this association has not been seen consistently across studies. A study of mice showed that a maternal diet high in fat resulted in greater fat mass accumulation in offspring at birth[154]. Epidemiological evidence suggests that a diet high in sugar and saturated fat during pregnancy is associated with a greater odds of overweight/obesity at age 5[72]. However, there is also literature showing no association between maternal dietary pattern during pregnancy and body composition at age 6[155]. More research is needed to gain a better understanding of the association between maternal dietary patterns during pregnancy and infant weight gain and adiposity.

Mechanisms underlying obesity risk from *in utero* exposures or early childhood weight gain are not well understood. Epigenetic mechanisms have been proposed as possibly playing a role in the onset of early childhood obesity[21,27,156]. Epigenetic modifications are changes to the genome that do not alter the underlying DNA sequence[21,29]. DNA methylation is the most commonly studied epigenetic mechanism due to its stability and the ability to measure it from easily obtained specimens, such as cord blood and peripheral blood[28–30].

The Mediterranean diet pattern has been shown to have a positive impact on health, inflammation, and chronic disease[78,85]. However its potential *in utero* effects on offspring have

not been well-studied. There is evidence suggesting that adherence to a Mediterranean diet pattern may lower the risk of fetal growth restriction[80]. A more recent study showed that adherence to a Mediterranean diet pattern during pregnancy was associated with child waist circumference at age 4[87]. There is also some evidence pointing to an association between adherence to a Mediterranean diet and epigenetic mechanisms in adults. A greater adherence to a Mediterranean diet in adulthood has been associated with *LINE1* methylation[115]. However no studies to date have looked at the potential for epigenetic mechanisms to underlie the association between maternal adherence to a Mediterranean diet and infant outcomes.

In this study we aimed to conduct a mediation analysis to assess the relationship between maternal adherence to a Mediterranean diet in early pregnancy, infant DNA methylation at birth, and weight gain in the first year of life. DNA methylation at birth at five differentially methylated regions (DMRs) of imprinted genes was included in the study of this aim. These regions have been selected for their involvement in growth[52,56], obesity[53,55], and common chronic disease[90]. The selected regions include the *MEG3 IG* DMR and the *MEG3* DMR, which are involved in regulating the delta-like 1 homolog/maternally expressed gene 3 (DLK1/*MEG3*) imprinted domain on chromosome 14q32.2[38]; the *IGF2* DMR and the *H19* DMR , which are involved in imprinting of the insulin growth factor 2/*H19* (*IGF2*/*H19*) domain on chromosome 11p15.5, which are located upstream of the imprinted promoters of *IGF2* and at the imprinting control region for the *IGF2*/*H19* imprinted domain near the *H19* promoter, respectively[38]; and the *PLAGL1* DMR, which resides at the pleiomorphic adenoma gene-like 1 (*PLAGL1*) locus on chromosome 6q24.2[55].

Methods And Discussion

I proposed to analyze the potential mediation of infant DNA methylation at birth in the association between maternal adherence to a Mediterranean diet using methodology outlined by Baron and Kenny[157]. However a small sample size and non-linear associations resulted in

this type of mediation analysis not being feasible. In this section I will describe the study sample, review mediation methodology as outlined by Baron and Kenny, and discuss the limitations of this methodology and why mediation analysis was not possible.

Study Sample

This study was restricted to mother-infant pairs who completed an FFQ during preconception or 1st trimester, who had plausible weight and length measurements, and who had analyzed DNA methylation. 1700 pregnant women consented and were enrolled in the NEST cohort. Of those, 396 women were excluded for a variety of reasons, including infant death during or soon after birth, being illiterate, underage, refused further participation, or who could no longer be found. DNA methylation data was analyzed for only the first 550 infants in the study .Weight and length measurements at one year were available for 740 term infants, and preconception/1st trimester FFQ data were available for 870 women. However not all infants whose mothers completed an FFQ also had weight and length measurements at age 1 or analyzed DNA methylation. Given the malleability of DNA methylation patterns in early gestation, only women who completed FFQs relating to preconception or the first trimester were included. Extreme implausible energy intakes, defined in our study as an intake of less than 500 kcal/day or greater than 7000 kcal/day (N=36), were excluded from our study. Preterm infants (born before 37 weeks gestation) were also excluded from this study (N=184), as their growth and weight gain trajectories differ from those of term infants[144]. In addition, infants with implausible weight and length measurements were excluded (N=7). The analytic sample for this aim was therefore reduced to 189 full term mother-infant pairs with available DNA methylation data on at least one of the 5 DMRs of interest, who completed a first trimester or preconception FFQ, who did not report extreme high or low energy intakes, and who had plausible length and weight measurements at birth and age 1.

Mediation

According to Baron and Kenny, in order for mediation to exist, variations in the exposure variable must significantly account for variations in the outcome; variations in the exposure must significantly account for variations in the mediator; and variations in the mediator must significantly account for variations in the outcome in the presence of the exposure. After controlling for the potential mediator, the previously significant relationship between the exposure and outcome should decrease or become non-significant[157,158]. Perfect mediation will exist when the exposure has no effect on the outcome variable when controlling for the mediator. Partial mediation exists when the beta estimate from the exposure on the outcome is decreased in the presence of the mediator[157]. This methodology is not adequate for assessing mediation of non-linear associations, exposure-mediator interactions, or multiple mediators[158,159].

In aims 1 and 2 of this research we found non-linear associations. In aim 1 of this dissertation we observed a non-linear association between maternal adherence to a Mediterranean diet and infant methylation at the *MEG3 IG* region. In aim 2 we also observed a non-linear association between infant methylation at the *MEG3* region and weight gain in infancy. Because of this, results of mediation analysis, as described by Baron and Kenny would likely not be meaningful, as the test of non-linear associations using methodology meant to assess linear associations would likely provide null results. In addition, our small sample of 189 mother-infant pairs was a barrier to assessing mediation. Fritz, et al determined through simulations that the sample size needed to achieve 0.8 power using the methodology described by Baron and Kenny would be at a minimum 562, up to 20,886[160].

The methodology for mediation analysis outlined by Baron and Kenny has been widely used in research, however it is not without limitations. This simple mediation methodology has been criticized for its inability to look at exposure-mediator interactions, overly relying on significance testing of results, its inability to assess non-linearities, and the inability to deal with

multiple mediators or variables that are both mediators and confounders[158,161,162]. In this study, theoretically there is a possibility for exposure-mediator interactions, as mediation by infant DNA methylation may differ based on maternal Mediterranean diet adherence level. Our results in aim 1 showed that both low and medium levels of Mediterranean diet adherence are associated with DNA methylation, and have overlapping confidence intervals, suggesting that exposure-mediator interaction is likely not present. However, it is also possible that EMM exists, as the association between exposure and mediator may only be visible in certain strata of a third variable, for example sex or birth weight. In aims 1 and 2 of this dissertation, sex and birth weight were found to be effect measure modifiers, therefore it is possible that mediation may only exist in girls or among those with lower than the median birth weight. Though not the purpose of this research, it is likely that more than 1 mediator exists in the association between maternal adherence to a Mediterranean diet during pregnancy and infant weight gain. For example, infant birth weight is also a possible mediator in this relationship. More sophisticated methodologies, such as counterfactual frameworks described by Vanderweele and others[161] would be needed to study these relationships.

CHAPTER 5- CONCLUSIONS

Overview of Findings

This dissertation examined the associations between maternal adherence to a Mediterranean diet and infant DNA methylation at birth (aim 1), as well as the association between infant DNA methylation at birth and weight gain in infancy (aim 2). In addition it assessed the potential for mediation of infant DNA methylation in the relationship between maternal Mediterranean diet and infant weight gain (aim 3). Our results from aim 1 suggest an association between a low adherence to a Mediterranean diet during pregnancy and greater infant DNA methylation at the *MEG3 IG* DMR at birth. This association was evident only in girls. In aim 2, we found that greater methylation at the *MEG3* DMR was associated with a smaller weight gain between birth and age 1 compared to infants with moderate levels of methylation at this site. This relationship was evident only in boys and in infants with birth weights that were less than the median birth weight for the study population. However there was no DMR that was associated with both maternal adherence to a Mediterranean diet and infant relative weight gain, suggesting that methylation at one the 5 study DMRs was not a mediator in the association between maternal diet and infant weight gain.

There is limited literature on the effects of maternal Mediterranean diet during pregnancy and childhood outcomes, and none have focused on the potential for mediation of epigenetic mechanisms. Most studies have been limited to fetal growth[80], birth defects[163] child respiratory outcomes[131]. Only one published study looked at the possible association between maternal Mediterranean diet intake and risk of child overweight[87]. They found no association with BMI at age 4, but they did find an association between maternal Mediterranean diet intake and waist circumference. This suggests that perhaps a more direct measure of adiposity, such as waist

circumference or tricep skinfolds will provide a better assessment of obesity risk. Also different timing of outcome assessment may have provided different results. Although there is a body of literature suggesting weight gain between birth and age 1 is a risk factor for obesity, there is also literature suggesting that adiposity in mid-childhood and adolescence is a predictor for obesity later in life[152,153]. Longitudinal studies that can measure obesity risk over time will be needed to gain a better understanding of the relationship between maternal diet and child obesity risk.

Sex and birth weight specific models were presented in the first two aims of this project. Therefore it is possible that such interaction effects exist in the relationship between maternal diet and infant weight gain that may be mediated by infant DNA methylation at birth. The use of mediation assessment methodologies that allow for the assessment of these interactions, such as counterfactual frameworks, are needed to study the mediation of infant DNA methylation at birth in the relationship between maternal diet and infant weight gain.

Limitations

This study was limited by a small sample size for studying DNA methylation, and the availability of only 9 DMRs for analysis through NEST, of which this study focused on 5. In humans, 1-5% of genes are thought to be imprinted[164], many of which have been identified in recent years. Studies looking at different sets of imprinted genes may provide different results. Newer technologies and methodologies allow for genome-wide DNA methylation analysis[165], which may allow for the expansion of gene sites to target for statistical analysis. It is also important to not completely discount the possibility that the DMRs assessed in this study may be mediators in the association between maternal diet and obesity later in life. A larger study may be able to show statistically significant differences where this study was not. However, it is also possible that no association exists. There is little prior evidence showing an association between DNA methylation at birth and weight gain in infancy. Although a statistically significant

association was seen in aim 2 between methylation at *MEG3* and relative weight gain between birth and age 1, the study was limited by multiple testing and a small sample size. Therefore it is also possible that these results are spurious, that no true association exists, and the mechanisms underlying weight gain in infancy are not related to DNA methylation. Replication of studies looking at the associations between maternal diet, infant DNA methylation, and infant weight gain is needed.

Selection Bias and Generalizability

The NEST cohort is a clinic-based population from Durham North Carolina. It benefits from a racially and ethnically diverse selection of mother-infant pairs, and prospectively collected data. While the study population may approximate Durham's racial/ethnic make-up, it differs with respect to the racial/ethnic make-up of births across the state of North Carolina. It should also be noted that mothers under age 18 were not included in this study, therefore the NEST population differs from Durham and the state of North Carolina with regards to maternal age. The results of this study are therefore not generalizable to mothers under the age of 18 or births across the state of North Carolina.

In aim 1, our small sample size was largely due to two constraining factors: limited availability of analyzed DNA methylation data and maternal FFQ non-response. Women-infant pairs not included in study aim 1 were more likely to be Black, have lower education, more likely to be smokers, and were on average heavier than from those included in the study (data not shown). In our study we observed that women of lower education and Black race were also more likely to have a lower adherence to a Mediterranean diet pattern, therefore it is possible that those who have low adherence to the Mediterranean diet are underrepresented in our study. If these likely "low adherers" also have low levels of methylation at the *MEG3 IG*, then the association we see in aim 1 is likely attenuated. However, if those likely "low adherers" have high or moderate methylation at *MEG3 IG*, then the association seen at the *MEG3 IG* in this

study is likely greater than it would have been had the entire study population been included in the analysis.

Recall and Information Bias

An additional limitation of this cohort is the use of self-reported measures, such as weight and length measurements at age 1. Approximately 30% of weights and lengths were reported by mothers rather than abstracted from medical records. We added a variable indicating self-reported measures in our models, however there is still possibility for residual confounding or measurement error. The diet data collected for this study also relied heavily on maternal self-report. Dietary measures from FFQs are prone to recall bias and social desirability bias. FFQ data have been found to be poor measures of energy intake[166–168], and have only moderate correlations with blood micronutrient measures[169,170]. Energy-adjusted estimates were used as a way to address this. We also conducted a sensitivity analysis to account for the potential for over and under-reporting of energy intake and found no significant difference in our results after accounting for possible over/under reporters. However, FFQ data can provide a reasonable estimate of general dietary patterns[171], which was the aim of this study. Therefore the use of FFQ data was justified despite its limitations.

Implications

The results of this research, though preliminary, support the idea that maternal diet during early pregnancy can modify infant DNA methylation of imprinted genes at birth, and that infant DNA methylation of select imprinted genes at birth can predict weight gain in infancy. The results of this research also support the idea that methylation can be sex-specific, and should encourage future researchers to assess sex-specific methylation and gain a better understanding of the biological mechanisms behind this. It will be important to determine how these findings can be incorporated into public health recommendations, as the public health implications are currently unclear.

The findings of this research suggest that *MEG3 IG*, *MEG3*, and *H19* may be important regions to continue to study in association with maternal diet and infant obesity risk. If methylation at a given DMR is consistently associated with both maternal diet and infant early obesity risk, this DMR could be used as a biological marker to measure obesity risk and target interventions. Also, maternal dietary interventions during pregnancy could be used to prevent aberrant methylation, and help prevent obesity over the life course. However, more and larger studies are needed to replicate the findings from this research. In addition, studies using more sophisticated methodologies for the assessment of mediation are needed to further explore the possibility of DNA methylation in the association between maternal diet and infant weight gain.

As the first study to explore associations between overall maternal diet patterns and infant DNA methylation, this dissertation presents an important contribution to the literature. Prior studies have focused on individual micronutrients in relation to DNA methylation rather than diet as a whole, thereby limiting their translatability into easily understood dietary recommendations. This research encourages the continuation of the study of maternal diet patterns, in particular the Mediterranean diet pattern in relation to infant DNA methylation and later child outcomes.

This research was conducted in collaboration with experts in maternal and child health, nutrition, epidemiology, and epigenetics. This interdisciplinary approach gave us a unique view and understanding of diet, DNA methylation, obesity, and health over the life course, while also focusing on the importance of public health significance in our research. More interdisciplinary research is needed to bring alternate solutions to public health problems, and move the field forward.

As the field of epigenetic epidemiology advances, it is important to remember that DNA methylation can be a useful tool to evaluate the embodiment of adverse environmental and possibly societal exposures over the life course. This can be especially useful in the study of health disparities, as DNA methylation markers may provide a way to assess the damaging

health effects of detrimental exposures across generations, and may provide insights into potential remediating strategies. Epigenetic epidemiology research should take an interdisciplinary approach, and continue to focus on modifiable exposures that affect DNA methylation, such as maternal diet, as they may be possible intervention points in the prevention of disease or the remediation of damage done by negative exposures throughout the life course or even across generations.

APPENDIX 1: TABLE OF DIET COMPONENTS BY MEDITERRANEAN ADHERENCE LEVEL AMONG MOTHERS IN THE NEWBORN EPIGENETIC STUDY[1]

	Diet score 0-3 (Low adherence)	Diet score 4-5 (Medium adherence)	Diet score 6-9 (High adherence)	P value
Calorie intake (kcal/day)	2954.3	2522.0	2144.2	<.0001
% kcals of protein	12.3	14.0	14.5	<.0001
% kcals of MUFA	11.9	11.8	12.0	0.851
% kcals of omega-3	0.6	0.7	0.7	0.011
% kcals of omega-6	5.2	5.7	5.8	0.118
%kcal of SFA	10.7	10.1	9.6	0.004
% Kcals of carbohydrates	58.7	57.8	58.0	0.925
Mean intake (g/1000 kcal/day) of diet score components				
Fruit group	134.5	186.0	235.2	<.0001
Vegetable group	51.5	104.7	153.4	<.0001
Legumes	12.0	25.8	38.4	<.0001
Nuts/seeds	1.0	3.2	4.7	<.0001
Whole grain	17.9	25.0	42.8	<.0001
Dairy	38.8	46.8	55.0	<.0001
Fish	2.5	4.2	6.1	<.0001
Meat	7.8	4.7	3.8	0.015
MUFA:SFA	0.5	0.6	0.7	<.0001

[1] Median intake (g/1000kcal/day) for each diet score component, used as cut point for diet score development of each diet score component: fruit: 143.5; vegetable: 87.0; legume: 15.4; nuts: 0.6; whole grain: 21.8; dairy: 30.4; fish: 1.9; meat: 2.9; MUFA:SFA: 0.5

APPENDIX 2. CHARACTERISTICS OF POSSIBLE OVER/UNDER-REPORTERS OF ENERGY INTAKE, NEWBORN EPIGENETIC STUDY

	Possible under-reporters		Possible plausible reporters		Possible over-reporters	
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)
Overall women	50 (12.8)		302 (77.4)		38 (9.7)	
Race[1]						
Black	21 (42)		90 (29.8)		13 (34.2)	
White	9 (18)		115 (38.1)		7 (18.4)	
Other	20 (40)		97 (32.1)		18 (47.4)	
Maternal Education[1]						
Less than college degree	32 (65.3)		168 (56.8)		35 (97.2)	
College degree or greater	17 (34.7)		128 (43.2)		1 (2.8)	
Maternal age[2]		30.0 (6.5)		28.5 (5.6)		25.2 (5.0)
Maternal smoking						
Yes	5 (10.2)		36 (12.4)		9 (25.7)	
No	44 (89.8)		254 (78.6)		26 (75.3)	
Maternal pre-pregnancy BMI[2]		30.3 (8.5)		26.7 (6.6)		25.0 (5.8)
Energy intake (kcal)[2]		1027.0 (249.7)		2473.2 (828.5)		5003.8 (1115.4)
Fruit intake (g/1000 kcal)		200.0 (159.1)		183.0 (166.2)		189.4 (143.1)
Vegetable intake (g/1000 kcal)[2]		127.7 (99.3)		100.8 (76.6)		96.5 (82.1)
Desserts (g/1000 kcal)[2]		19.0 (18.8)		25.6 (22.6)		27.4 (25.3)
Non-processed meats (g/1000 kcal)[2]		2.8 (3.9)		5.7 (8.7)		5.8 (9.5)
Monounsaturated fat intake (% of total kcals)		11.1 (4.6)		12.0 (3.1)		12.4 (3.7)
Polyunsaturated fat intake (% of total kcals)		6.4 (2.5)		6.5 (1.9)		6.7 (2.1)
Saturated fat intake (% of total kcals)		9.5 (3.0)		10.2 (2.7)		10.1 (2.6)

[1]Results of chi-square test α <0.05

[2] Results of ANOVA α < 0.05

APPENDIX 3. UNADJUSTED AND ADJUSTED[1] MULTINOMIAL LOGISTIC REGRESSION OF THE ASSOCIATION BETWEEN MATERNAL ADHERENCE TO A MEDITERRANEAN DIET DURING PREGNANCY AND INFANT DNA METHYLATION AT BIRTH – ANALYSIS EXCLUDING DAIRY FROM MDS SCORING, NEWBORN EPIGENETIC STUDY.

	Unadjusted				Adjusted			
	Hypo-methylation		Hyper-methylation		Hypo-methylation		Hyper-methylation	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
MEG3 IG								
Low adherence	1.68 (0.91, 3.12)	0.10	1.42 (0.76, 2.66)	0.27	1.80 (0.90, 3.62)	0.10	1.42 (0.71, 2.84)	0.33
Med adherence	1.34 (0.62, 2.91)	0.46	1.92 (0.93, 3.99)	0.08	1.45 (0.64, 3.31)	0.38	2.00 (0.93, 4.31)	0.08
MEG3								
Low adherence	0.81 (0.45, 1.48)	0.50	1.13 (0.63, 2.05)	0.68	1.08 (0.54, 2.13)	0.83	1.16 (0.60, 2.27)	0.66
Med adherence	1.21 (0.56, 2.60)	0.63	1.53 (0.71, 3.29)	0.27	1.57 (0.68, 3.60)	0.29	1.71 (0.76, 3.86)	0.20
IGF2								
Low adherence	1.41 (0.79, 2.49)	0.24	0.81 (0.46, 1.43)	0.46	1.56 (0.81, 2.99)	0.18	1.19 (0.63, 2.28)	0.59
Med adherence	1.17 (0.57, 2.40)	0.67	0.97 (0.48, 1.93)	0.92	1.42 (0.66, 3.04)	0.37	1.25 (0.60, 2.63)	0.55
H19								
Low adherence	1.10 (0.61, 2.00)	0.75	1.10 (0.61, 2.01)	0.75	1.39 (0.72, 2.70)	0.33	1.56 (0.79, 3.08)	0.20
Med adherence	0.65 (0.31, 1.37)	0.26	0.86 (0.42, 1.75)	0.68	0.74 (0.34, 1.60)	0.44	1.00 (0.47, 2.12)	0.99
PLAGL1								
Low adherence	0.84 (0.48, 1.49)	0.56	1.12 (0.64, 1.95)	0.69	0.86 (0.45, 1.66)	0.65	1.71 (0.89, 3.30)	0.11
Med adherence	1.10 (0.56, 2.19)	0.78	0.88 (0.43, 1.79)	0.72	1.19 (0.57, 2.47)	0.65	1.18 (0.54, 2.57)	0.68

[1] Adjusted for maternal education, maternal pre-pregnancy BMI, maternal smoking, maternal age, total dietary calcium intake

APPENDIX 4. CHARACTERISTICS OF STUDY SAMPLE BY DAIRY INTAKE, NEWBORN EPIGENETIC STUDY

	Below the median dairy intake		At or above the median dairy intake	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Race/ethnicity[1]				
Black	80 (39.6)		44 (23.4)	
White	44 (21.7)		87 (46.3)	
Other	78 (38.6)		57 (30.3)	
Maternal education[1]				
Less than college degree	143 (72.2)		92 (50.3)	
Greater than college degree	55 (27.8)		91 (49.7)	
Maternal age[2]		27.2 (5.8)		29.5 (5.5)
Maternal smoking				
Yes	27 (14.0)		23 (12.7)	
No	166 (86.0)		158 (87.3)	
Calcium (mg/1000 kcal)[2]		1236.8 (718.6)		1498.7 (771.9)
Saturated fat (g/1000 kcal)		28.0 (17.3)		29.4 (15.3)
Monounsaturated fat (g/1000 kcal)		34.7 (22.6)		33.3 (17.8)
Total fat (g/1000 kcal)		88.5 (53.1)		87.2 (44.9)
Total calories		2556.2 (1338.0)		2510.8 (2347.1)

[1] Results of chi-square test α< 0.001.

[2] Results of T-test α< 0.001

APPENDIX 5. CONDENSED RESULTS OF SIMULATIONS ADDING BREASTFEEDING TO REGRESSION OF THE ASSOCIATION BETWEEN INFANT *MEG3* METHYLATION AT BIRTH AND RELATIVE WEIGHT GAIN IN INFANCY, NEWBORN EPIGENETIC STUDY

Proportion BF 0-1 months	Proportion BF 1-6 months	Proportion BF 7+ months	Methylation	Mean β	Minimum β	Maximum β
0	0	1	Hypo-methylation	0.38	0.38	0.38
0	0	1	Hyper-methylation	0.62	0.62	0.62
0	0.5	0.5	Hypo-methylation	0.39	0.36	0.43
0	0.5	0.5	Hyper-methylation	0.62	0.59	0.64
0	0.8	0.2	Hypo-methylation	0.39	0.37	0.42
0	0.8	0.2	Hyper-methylation	0.62	0.61	0.63
0	1	0	Hypo-methylation	0.39	0.39	0.39
0	1	0	Hyper-methylation	0.62	0.62	0.62
0.1	0.5	0.4	Hypo-methylation	0.39	0.35	0.43
0.1	0.5	0.4	Hyper-methylation	0.62	0.58	0.64
0.1	0.8	0.1	Hypo-methylation	0.40	0.36	0.43
0.1	0.8	0.1	Hyper-methylation	0.62	0.60	0.64
0.2	0	0.8	Hypo-methylation	0.39	0.36	0.43
0.2	0	0.8	Hyper-methylation	0.62	0.60	0.64
0.2	0.5	0.3	Hypo-methylation	0.40	0.34	0.45
0.2	0.5	0.3	Hyper-methylation	0.62	0.57	0.67
0.9	0	0.1	Hypo-methylation	0.41	0.38	0.45
0.9	0	0.1	Hyper-methylation	0.63	0.62	0.65
1	0	0	Hypo-methylation	0.41	0.41	0.41
1	0	0	Hyper-methylation	0.63	0.63	0.63

APPENDIX 6. TABLE OF UNADJUSTED AND ADJUSTED RESULTS OF LINEAR REGRESSION OF THE ASSOCIATION BETWEEN INFANT DNA METHYLATION AT BIRTH AND CHANGE IN WLZ SCORES BETWEEN BIRTH AND AGE 1, AND LOGISTIC REGRESSION OF THE ASSOCIATION BETWEEN INFANT DNA METHYLATION AT BIRTH AND RAPID WEIGHT GAIN, INCLUDING BIRTH WEIGHT AS A COVARIATE, NEWBORN EPIGENETIC STUDY

	Change in WLZ 0-1						**Rapid weight gain (≥0.67 SD change in WLZ)**					
	Unadjusted			Adjusted[1]			Unadjusted			Adjusted[1]		
	β	95 % CI	P	β	95 % CI	P	β	95 % CI	P	β	95 % CI	P
MEG3												
Hypo-methylation	-0.42	(-0.90, 0.07)	0.11	-0.42	(-0.94, 0.10)	0.11	0.71	(0.39, 1.31)	0.27	-0.37	(0.36, 1.34)	0.27
Hyper-methylation	-0.63	(-1.11, -0.15)	0.01	-0.71	(-1.22, -0.20)	0.01	0.53	(0.29, 0.97)	0.04	-0.78	(0.24, 0.88)	0.02
H19												
Hypo-methylation	0.48	(0.005, 0.96)	0.05	0.52	(0.02, 1.02)	0.04	1.57	(0.84, 2.94)	0.15	1.61	(0.83, 3.10)	0.16
Hyper-methylation	0.12	(-0.34, 0.59)	0.60	0.20	(-0.29, 0.68)	0.42	1.36	(0.74, 2.50)	0.32	1.50	(0.79, 2.86)	0.21
PLAGL1												
Hypo-methylation	-0.37	(-0.83, 0.09)	0.12	-0.35	(-0.83, 0.13)	0.16	0.90	(0.50, 1.60)	0.71	0.91	(0.50, 1.66)	0.77
Hyper-methylation	-0.31	(-0.77, 0.14)	0.18	-0.28	(-0.77, 0.21)	0.26	0.82	(0.46, 1.44)	0.48	0.85	(0.46, 1.57)	0.61
MEG3 IG												
Hypo-methylation	-0.13	(-0.64, 0.37)	0.61	-0.27	(-0.81, 0.27)	0.32	0.85	(0.45, 1.61)	0.63	0.62	(0.31, 1.27)	0.19
Hyper-methylation	-0.18	(-0.67, 0.31)	0.46	-0.33	(-0.84, 0.18)	0.20	0.77	(0.42, 1.43)	0.41	0.57	(0.29, 1.11)	0.10
IGF2												
Hypo-methylation	0.16	(-0.30, 0.62)	0.50	0.18	(-0.32, 0.67)	0.49	1.11	(0.62, 1.99)	0.72	1.23	(0.65, 2.33)	0.53
Hyper-methylation	-0.11	(-0.56, 0.35)	0.64	-0.11	(-0.59, 0.36)	0.64	0.74	(0.41, 1.33)	0.32	0.77	(0.42, 1.42)	0.40

[1] Adjusted for maternal education, maternal pre-pregnancy BMI, maternal age, maternal smoking during pregnancy, self-report of anthropometric measures, date of anthropometric measurement, and birth weight

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