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RESEARCH ARTICLE

Low maternal adherence to a Mediterranean diet is associated with increase in methylation at the *MEG3-IG* differentially methylated region in female infants

Sarah Gonzalez-Nahm[1,*], Michelle A. Mendez[2], Whitney R. Robinson[3], Susan K. Murphy[4], Cathrine Hoyo[5], Vijaya K. Hogan[6] and Diane L. Rowley[7]

[1]Department of Health, Behavior and Society, Bloomberg School of Public Health, Johns Hopkins University, 624 N. Broadway, HH 904, Baltimore, MD 21205, USA, [2]Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, [3]Department of Epidemiology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, [4]Department of OB/GYN, Duke University Medical Center, Durham, NC USA, [5]Department of Biological Sciences, North Carolina State University, Raleigh, NC, USA NC, USA, [6]Department of Food, Health and Well-Being, W.K. Kellogg Foundation Battle Creek, MI, USA and [7]Department of Maternal and Child Health, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA

*Correspondence address. Department of Health, Behavior and Society, Bloomberg School of Public Health, Johns Hopkins University, 624 N. Broadway, HH 904, Baltimore, MD 21205, USA. Tel: 919-386-9423; Fax: 410-955-7241; E-mail: sarah.nahm@jhu.edu

Abstract

Diet is dictated by the surrounding environment, as food access and availability may change depending on where one lives. Maternal diet during pregnancy is an important part of the *in utero* environment, and may affect the epigenome. Studies looking at overall diet pattern in relation to DNA methylation have been lacking. The Mediterranean diet is known for its health benefits, including decreased inflammation, weight loss, and management of chronic diseases. This study assesses the association between maternal adherence to a Mediterranean diet pattern during pregnancy and infant DNA methylation at birth. Mediterranean diet adherence in early pregnancy was measured in 390 women enrolled in the Newborn Epigenetic Study, and DNA methylation was assessed in their infants at birth. Multinomial logistic regression was used to assess the association between adherence to a Mediterranean diet and infant methylation at the maternally expressed gene 3, maternally expressed gene 3 - intergenic region, *pleiomorphic adenoma gene-like 1, insulin-like growth factor 2 gene*, *H19, mesoderm-specific transcript, neuronatin, paternally expressed gene 3*, epsilon sarcoglycan *and paternally expressed gene* 10 promoter region, measured by pyrosequencing. Infants of mothers with a low adherence to a Mediterranean diet had a greater odds of hypo-methylation at the *MEG3-IG* differentially methylated region (DMR). Sex-stratified models showed that this association was present in girls only. This study provides early evidence on the association between overall diet pattern and methylation at the 9 DMRs included in this study, and suggests that maternal diet can have a sex-specific impact on infant DNA methylation at specific imprinted DMRs.

Key words: DNA methylation; sex-specific; epigenetics; Mediterranean diet; MEG3-IG

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Introduction

The developmental origins of health and disease hypothesis posits that *in utero* exposures play a critical role in the risk of adult disease [1]. 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 [2–4], i.e. changes in gene expression caused by mechanisms other than the underlying DNA sequence [5, 6] DNA methylation is the most studied mechanism in epidemiologic studies, in part due to the covalent nature of this modification coupled with the stability of the DNA molecule.

DNA methylation plays an integral role in fetal development, including establishing the monoallelic expression of genomically imprinted genes in a parent-of-origin-dependent manner. Imprinted gene DNA methylation is largely established in the developing gametes and these marks are faithfully maintained throughout somatic cell division [7–10], making the methylation status of these regulatory regions a reliable indicator of exposures that occur during early development [7, 11, 12]. Therefore, the parental origin-specific methylation profiles are normally spatially and temporally stable, such that methylation changes at differentially methylated regions (DMRs) of imprinted genes resulting from early exposures can be detected later in life. As an example, individuals who were exposed to famine *in utero* exhibited DNA methylation differences relative to unexposed siblings in a regulatory region of the imprinted insulin-like growth factor 2 gene (*IGF2*), 60 years after exposure [11]. Moreover, DNA methylation at the same region was found to be stable over a three year study period in adult controls of a colorectal cancer study [13].

Maternal intake of methyl donor nutrients during the prenatal period has been shown to affect DNA methylation in offspring [14–17] in a sex-specific manner [18], suggesting that maternal factors, such as diet, can alter the *in utero* environment and affect health outcomes over the lifecourse [11, 19–21]. Although both maternal over- and under-nutrition can influence the offspring *in utero* environment and lead to altered fetal programming [21–25], the majority of diet studies examines single nutrients, or has been conducted using animal models in well-controlled environments. Studies evaluating 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, and some may act in synergy with or interfere with the absorption of other dietary components [22]. 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 [23]. These benefits may be particularly important during the *in utero* period of development. The Mediterranean diet has been well-studied for its ability to reduce inflammation and improve longevity and overall health among adults [24–26]. Consumption of a Mediterranean diet pattern during pregnancy has been associated with a lower risk of preterm birth [27], lower risk of infant growth restriction in a Spanish Mediterranean population [28], and more recently, a lower child waist circumference at age 4 years [29]. The Mediterranean diet pattern has also been associated with higher intake of certain nutrients, such as folate [30] and phenols [31] that have been shown to modify epigenetic marks in the genome [32–35]. There are few published studies on the influence of the Mediterranean diet on epigenetic outcomes, and those available have been limited to adult populations. There is some evidence suggesting an association between adherence to a Mediterranean diet in adulthood and increased LINE1 methylation [36]. 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 [37]. No studies have evaluated the potential epigenetic outcomes of *in utero* exposure to 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 examined at nine DMRs of imprinted genes. These regions were selected for their involvement in growth [38], obesity [39, 40], and common systemic inflammatory chronic disease [41]. The selected DMRs include the intergenic (*MEG3-IG*) and the *MEG3* regions, 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 and expression of the *IGF2/H19* domain on chromosome 11p15.5 and 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; the pleiomorphic adenoma gene-like 1 (*PLAGL1*) DMR, which resides at the *PLAGL1* locus on chromosome 6q24.2; the mesoderm-specific transcript (*MEST*) promoter at 7q32.2; the neuronatin (*NNAT*) locus at 20q11.23; the paternally expressed gene 3 (*PEG3*) promoter region at 19q13.43; and the epsilon sarcoglycan and paternally expressed gene 10 (*SGCE/PEG10*) promoter region at 7q21.3. We expect that low adherence to a Mediterranean diet pattern will result in altered DNA methylation at these DMRs.

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 (results not shown). Women who may have under-reported their energy intake, were more likely to have a college education or greater (94.4%), have a higher BMI (mean BMI = 30.3), and had significantly lower

reported intake of dairy compared with 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, meats, nuts, and dessert foods compared with possible 'moderate' energy reporters. Controlling for over- and under-reporters of dietary intake did not change our estimates, therefore final analyses included potential over-and under-reporters.

Maternal diet characteristics by Mediterranean diet adherence category are shown in Table 2. 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. Overall infant methylation levels for each DMR, and those specific to the level of maternal Mediterranean diet adherence are presented in Table 3.

Results from the total unadjusted models (Table 4) showed that a low adherence to a Mediterranean diet pattern during pregnancy was associated with a higher odds of lower methylation at the *MEG3-IG* region (OR = 2.80; 95% CI = 1.35–5.82). This association was strongest and statistically significant only in girls (OR = 5.35, 95% CI = 1.56–18.36—unadjusted data not shown). A low adherence to a Mediterranean diet during pregnancy was also associated with a greater odds of both lower and higher infant methylation at the *NNAT* region (low: OR = 2.20; 95%CI = 1.00–4.87; high: OR = 2.43; 95%CI = 1.10–5.35), however no sex-specific differences were observed. At the *MEG3* region, unadjusted estimates showed that a lower adherence to a Mediterranean diet in pregnancy was associated with a lower odds of lower methylation only in girls (OR = 0.24, 95% CI = 0.07–0.77; data not shown). In general, sex-specific models showed a difference in magnitude, and in some cases, direction of association in one sex compared with the other (see Table 5 for sex-specific adjusted estimates).

After adjustment, the associations at the *MEG3* and *NNAT* DMRs were no longer significant. The association at the *MEG3-IG* DMR persisted in girls (OR = 7.40, 95% CI: 1.88–29.09), even after the stringent Bonferroni correction (0.05/9 = 0.006), 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 (*MEG3-IG*: OR: 3.34, 95% CI: 1.10–10.21; *H19*: OR: 4.46, 95% CI: 1.32–15.08; *PLAGL1*: OR: 3.24, 95% CI: 1.02–10.26).

Table 1: characteristics of mothers during pregnancy 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[a]						
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[a]						
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[b]		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[b] (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[a]						
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)	

[a]Results of chi-square test for differences between groups of diet adherence were statistically significant ($P < 0.01$).
[b]Results of Kruskal-Wallis test for differences between groups of diet adherence were statistically significant ($P < 0.01$).

Table 2: diet components by Mediterranean adherence level among mothers in the NEST study[a]

	Diet score 0–3 (Low adherence)	Diet score 4–5 (Medium adherence)	Diet score 6–9 (High adherence)	*P* value
Calorie intake (kcal/d)	2954.3	2522.0	2144.2	<0.0001
% kcals of protein	12.3	14.0	14.5	<0.0001
% 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/d) of diet score components				
Fruit group	134.5	186.0	235.2	<0.0001
Vegetable group	51.5	104.7	153.4	<0.0001
Legumes	12.0	25.8	38.4	<0.0001
Nuts/seeds	1.0	3.2	4.7	<0.0001
Whole grain	17.9	25.0	42.8	<0.0001
Dairy	38.8	46.8	55.0	<0.0001
Fish	2.5	4.2	6.1	<0.0001
Meat	7.8	4.7	3.8	0.015
MUFA:SFA	0.5	0.6	0.7	<0.0001

[a]Median intake (g/1000kcal/d) 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.

Table 3: infant DNA methylation levels (%) at birth; overall and by maternal Mediterranean diet adherence

	Overall Mean (SD)	Low adherence Mean (SD)	Medium adherence Mean (SD)	High adherence Mean (SD)
MEG3-IG	49.4 (3.4)	49.2 (3.5)	49.3 (3.3)	49.6 (3.4)
MEG3	72.1 (5.5)	72.9 (5.6)	71.9 (5.8)	71.6 (5.1)
NNAT	55.5 (5.7)	55.6 (5.0)	55.4 (5.7)	55.8 (6.1)
SGCE/PEG10	44.9 (6.6)	44.2 (6.0)	45.4 (6.1)	45.0 (7.8)
IGF2	51.5 (5.5)	50.7 (4.5)	51.4 (5.3)	52.5 (6.3)
H19	47.8 (4.1)	47.1 (4.8)	47.9 (3.7)	48.1 (3.8)
PLAGL1	57.1 (7.3)	57.6 (5.9)	56.8 (7.5)	57.1 (8.1)
MEST	43.4 (5.0)	43.7 (6.2)	43.2 (4.5)	43.2 (4.3)
PEG3	36.2 (3.6)	35.6 (2.6)	36.6 (4.4)	36.4 (3.0)

Also, the association between a medium adherence to a Mediterranean diet pattern and lower odds of higher methylation at the *NNAT* gene in girls and greater odds of higher methylation at the *SGCE/PEG10* gene in boys became significant after adjustment (*NNAT*: OR: 0.29, 95% CI: 0.10–0.88; *SGCE/PEG10*: OR: 2.59, 95% CI 0.99–6.75). However, confidence intervals became wider, and the results were no longer statistically significant after adjustment.

Discussion

We observed a decreased level of methylation at the *MEG3-IG* DMR among girls in response to lower maternal adherence to a Mediterranean diet pattern after adjusting for maternal age, maternal education, maternal pre-pregnancy BMI, and maternal smoking. The actual change in % methylation, however, was small (~1%). The functional significance of such a small difference on gene expression is not clear, however, a previous study showed that a 1% difference in methylation at the *IGF2* DMR was associated with a doubling (or halving) of the overall *IGF2* expression levels, depending on the direction of the methylation changes [42]. Deregulation of the *MEG3-IG* DMR has been found in cancer tissues [43], and it is believed that the *MEG3-IG* region may be an upstream regulator of the *MEG3* DMR [44], which has been associated with type 2 diabetes [41]. As the Mediterranean diet has been associated with improvements in type 2 diabetes [45], 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 size did not allow for mediation analysis to study child outcomes. Therefore, future studies should investigate the phenotypes associated with maternal diet and methylation at the *MEG3-IG* DMR to better understand the health implications of these results.

This is one of a few studies that has examined and reported sex-specific differences in DNA methylation of imprinted genes [42, 46]. We know of no other published studies have reported sex-specific associations at the *MEG3-IG* DMR although sex-specific effects at other genomic regions have been reported in relation to diet and other environmental exposures. Tobi *et al.* observed sex-specific associations between prenatal famine exposure and methylation at the *LEP*, *INSIGF*, and *GNAS* DMRs. Murphy *et al.* [42] found an increase in *IGF2* methylation in association with prenatal smoking that was most prominent in boys. Sex-specific methylation has also been observed in animal models in non-imprinted genes in relation to diet [47] and environmental chemical exposure [48], and epidemiological studies have also found the association between endocrine disrupting chemicals and obesity to be sex-specific [49–51]. It is still unclear why the effects of Mediterranean diet on *MEG3* regulatory sequences were pronounced in females in our study; however, this remains an active topic of investigation. 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 associated with a wide range of *in utero* exposures.

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, or circulating levels of several

Table 4: unadjusted and adjusted[a] total estimates for the association between adherence to a Mediterranean diet pattern during pregnancy and infant DNA 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
NNAT								
Low adherence	2.20 (1.00, 4.87)	0.05	2.43 (1.10, 5.35)	0.03	1.72 (0.71, 4.17)	0.23	2.16 (0.91, 5.15)	0.08
Medium adherence	0.75 (0.39, 1.45)	0.40	0.80 (0.42, 1.54)	0.51	0.66 (0.32, 1.35)	0.25	0.75 (0.37, 1.50)	0.41
SGCE/PEG10								
Low adherence	1.37 (0.70, 2.68)	0.35	1.34 (0.68, 2.63)	0.40	1.07 (0.49, 2.33)	0.87	1.38 (0.65, 2.93)	0.41
Medium adherence	1.45 (0.77, 2.75)	0.25	1.67 (0.88, 3.14)	0.11	1.31 (0.66, 2.62)	0.44	1.64 (0.85, 3.16)	0.14
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
MEST								
Low adherence	1.24 (0.62, 2.46)	0.54	1.20 (0.61, 2.37)	0.61	0.92 (0.42, 1.98)	0.83	1.06 (0.50, 2.27)	0.88
Medium adherence	1.22 (0.64, 2.35)	0.55	1.27 (0.67, 2.43)	0.46	1.00 (0.50, 1.98)	0.99	1.12 (0.57, 2.22)	0.74
PEG3								
Low adherence	1.37 (0.70, 2.68)	0.34	1.34 (0.68, 2.63)	0.07	1.261 (0.58, 2.72)	0.56	0.55 (0.25, 1.20)	0.14
Medium adherence	1.45 (0.77, 2.75)	0.72	1.67 (0.88, 3.14)	0.30	0.87 (0.43, 1.76)	0.69	0.73 (0.38, 1.40)	0.35

[a]Adjusted for maternal pre-pregnancy BMI, maternal education, maternal smoking during pregnancy, maternal age at delivery. Comparisons are for low and medium diet adherence, compared with high adherence, and low and high methylation compared with moderate methylation (assessed in tertiles).

B vitamins and increases in *IGF2* methylation [32, 35, 52]. Others have found decreased *IGF2* methylation in response to famine or undernutrition [12]. 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 [30, 53], 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 [22]. This has been seen in the case of plant phenols [54] and measurement of starches with regard to the glycemic index [55]. 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 patterns on infant DNA methylation. Therefore, this study makes 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 studies assessing energy reporting bias among pregnant and non-pregnant women [56–58]. We did see differences in reported intake of specific food groups by energy reporting category; however, our sensitivity analysis suggested 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.

The Mediterranean diet score (MDS), from which the score in this study was based, has been shown to be a reliable indicator of adherence to a Mediterranean diet pattern [59]. However, assessing diet with a MDS 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 [60]. 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

Table 5: sex-specific adjusted[a] estimates of the association between maternal adherence to a Mediterranean diet pattern during pregnancy and infant DNA methylation at birth

	Males				Females			
	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	1.77 (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
Medium adherence	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 adherence	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
Medium adherence	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
NNAT								
Low adherence	1.30 (0.40, 4.22)	0.67	2.59 (0.75, 8.89)	0.13	2.32 (0.56, 9.68)	0.25	2.06 (0.55, 7.69)	0.28
Medium adherence	0.66 (0.25, 1.72)	0.39	1.44 (0.54, 3.85)	0.46	0.62 (0.20, 1.92)	0.40	0.29 (0.10, 0.88)	0.03
SGCE/PEG10								
Low adherence	1.31 (0.42, 4.04)	0.64	3.06 (1.02, 9.16)	0.05	0.92 (0.30, 2.82)	0.88	0.70 (0.23, 2.12)	0.53
Medium adherence	1.59 (0.60, 4.19)	0.35	2.59 (0.99, 6.75)	0.05	1.17 (0.42, 3.26)	0.76	1.24 (0.48, 3.22)	0.66
IGF2								
Low adherence	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
Medium adherence	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 adherence	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
Medium adherence	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 adherence	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
Medium adherence	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
MEST								
Low adherence	0.52 (0.17, 1.65)	0.27	1.20 (0.42, 3.43)	0.73	1.25 (0.41, 3.81)	0.69	0.83 (0.26, 2.61)	0.74
Medium adherence	0.67 (0.25, 1.79)	0.42	0.77 (0.30, 2.03)	0.60	1.46 (0.53, 4.06)	0.46	1.60 (0.59, 4.31)	0.35
PEG3								
Low adherence	0.94 (0.32, 2.80)	0.91	0.39 (0.13, 1.22)	0.11	1.69 (0.55, 5.16)	0.36	0.76 (0.25, 2.30)	0.63
Medium adherence	0.55 (0.20, 1.48)	0.23	0.53 (0.21, 1.34)	0.18	1.44 (0.50, 4.09)	0.50	1.071 (0.41, 2.78)	0.89

[a]Adjusted for maternal pre-pregnancy BMI, maternal education, maternal smoking during pregnancy, maternal age at delivery. Comparisons are for low and medium diet adherence, compared with high adherence, and low and high methylation compared with moderate methylation (assessed in tertiles).

monounsaturated fatty acids consumed in Mediterranean populations. However, in other populations, e.g. the USA, a large proportion of monounsaturated fat consumption comes from animal fats [61], which may not confer the same health benefits linked to olive oil.

A limitation of this study was its small sample size. This likely resulted in 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. 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 further insights related to 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 is associated with a modest, sex-specific change in offspring DNA methylation at the *MEG3-IG* DMR. Associations between maternal diet in early pregnancy and methylation changes at the *MEG3*, *H19*, *NNAT*, and *PLAGL1* regions may also exist; however, a larger study may be needed to uncover these associations. Although 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 [62], adiposity [63], and chronic disease [12, 41] at the CpG 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. Some DNA methylation and other epigenetic markers can be viewed as a way in which adverse environmental exposures are recorded in accessible cells in the body, and are stable over the life course. Researchers should look to maternal diet as a modifiable risk factor that may help mitigate the damage done by environmental exposures.

Materials and Methods

Study Participants 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 leukocytes as part of the Newborn Epigenetic Study (NEST). Recruitment and enrollment strategies have been described in detail elsewhere [64]. Briefly, between 2009 and 2011, women were recruited from five prenatal clinics and obstetrics 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, lifestyle, and dietary characteristics. At delivery, birth outcomes were abstracted from medical records and infant cord blood specimens were obtained to assess offspring DNA 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, illiteracy, being underage, refusing further participation, or who could no longer be found. Given the malleability of DNA methylation patterns in very early gestation, we included 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 the FFQ with respect to race, education, age, BMI, and smoking status. These factors were accounted for in statistical analysis. Women with extreme energy intakes, defined in our study as an intake of <500 or >7000 kcal/d, 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 $>$ or $< \pm 2$ SD [65]). 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 leukocytes 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 first trimester or preconception FFQ were part of the subsample with analyzed DNA methylation. The present analysis includes the 390 women who completed a first trimester or preconception FFQ, who did not have extreme high or low reported caloric intakes, and whose infants had DNA methylation data available.

Dietary data were collected through a Block FFQ [66] 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: (i) the periconception period, (ii) the first trimester, and (iii) the second and third trimesters. For this study, only peri-conceptional and first trimester FFQs were used, as DMRs of imprinted genes may be vulnerable due to widespread methylation reprogramming (to which these regions are normally resistant) 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.

Specimen Handling

Infant cord blood specimens 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).

DNA Methylation

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 [67]. 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 >2 SDs 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. Multiple CpGs were identified and methylation was quantitatively measured for each DMR. The number of CpGs sites analyzed at each DMR is as follows: *MEG3-IG*: 4 CpGs, *MEG3*: 8 CpGs, *IGF2*: 3 CpGs, *H19*: 4 CpGs, *PLAGL1*: 6 CpGs, *MEST*: 4 CpGs, *NNAT*: 3 CpGs, *PEG3*: 10 CpGs, *SGCE/PEG10*: 6 CpGs. Pyrosequencing assay validation data for these DMRs, and the results of sensitivity tests showing that pyrosequencing can distinguish methylation differences as low as 0.5% have been previously described in [67, 68].

Diet Assessment

Pregnant women's 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 MDS [69]. 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 or 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, or 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 (or 0 otherwise). For this study, the MDS was modified as follows:

i. Fruits and nuts were separated into two groups.
ii. The grains category was refined to reflect only whole grain intake.
iii. 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.
iv. 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:

a. Fruit (including fresh, dried, and frozen fruit, but excluding fruit juice)
b. Vegetables (excluding vegetable juice and white potatoes)
c. Nuts and seeds (including nut butters)
d. Beans and legumes (including soy beans)
e. Whole grains and whole grain products
f. Dairy (including full fat dairy, but excluding dairy desserts)
g. Fish
h. The ratio of mono-unsaturated fat to saturated fat intake
i. 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 used 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 MDSs were categorized to reflect three groups of roughly the same size, with scores between 0 and 3 considered 'low adherence', scores of 4–5 considered 'medium adherence', and scores of 6–9 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 [64]. Normality of the percent methylation of each DMR was assessed using the Kolmogorov-Smirnov test. As five of the nine 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 'moderate' category of DNA methylation was used as the referent. Likelihood ratio tests were performed to test for the interaction of association between an infant's sex and maternal diet on infant DNA methylation at birth ($\alpha = 0.20$). The addition of an interaction term for infant's sex in four of the nine DMRs assessed (*MEG3*, *MEG3-IG*, *H19*, *NNAT*) 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 were added one at-a-time into the original, unadjusted model. Those that changed the estimates by >10% or 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. All statistical analyses were conducted using SAS 9.4 (SAS Institute, Inc.).

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Author contributions

CH and SKM conceived and designed the study, SGN analyzed the data, SGN, MM, CH, SKM wrote the manuscript. All authors read, edited, and approved the manuscript prior to submission.

Conflict of interest statement. None declared.

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