Global DNA methylation: Nutritional Correlates and Child Growth

by

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"There are far better things ahead than any we leave behind."

C.S. Lewis

Dedication

To my parents, Hsiu-Hui and Chin Yuan, whose words of encouragement and push for tenacity ring in my ears. Without their unwavering love and support, my accomplishments would not have been possible.

I also dedicate this work to my brother, Powell, whose impeccable work ethic and ambition in all aspects of his life have been continued sources of inspiration.

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List of Abbreviations

%5mC	% 5-methylcytosine
AS	Satellite 2 repetitive element
*at*RA	*all trans* retinoic acid
BMI	Body Mass Index
BPA	Bisphenol A
BSCC	Bogotá School Children Cohort
CI	Confidence interval
CpG	Cytosine-guanine site
CRP	C-reactive protein
CV	Coefficient of variation
CVD	Cardiovascular disease
DFE	Daily folate equivalents
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
EBLUP	Empirical best linear unbiased predictor
EDTA	Ethylenediaminetetraacetic acid
FFQ	Food frequency questionnaire
IQR	Interquartile range
LINE-1	Long interspersed nucleotide element 1
LMS	Lambda mu sigma
MESA	Multi-Ethnic Study of Atherosclerosis
PCR	Polymerase chain reaction
PPAR	Peroxisome proliferator activated receptor
RA	Retinoic acid
RXRA	Retinoid X receptor alpha
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SES	Socioeconomic status
TSS	Transcription start site
VCAM-1	Vascular cell adhesion molecule 1
WBC	White blood cell
US	United States

Chapter 1

Introduction

Overview

DNA methylation is a modifiable epigenetic mechanism that regulates gene expression without changing the nucleotide sequence (1). As a basic biochemical process, DNA methylation directs tissue-specific transcription and suppresses expression of viral genes and transposable elements. However, inappropriate gene expression or silencing can lead to disease - the most widely studied being cancer (2). Genome-wide methylation content ('global DNA methylation') also plays a role in pathogenesis (3). Although there is prolific literature on the consequences of aberrant tissue-specific global DNA methylation in tumors (4-8), less is known regarding the value of systemic global DNA methylation measured from peripheral leukocytes as a biomarker of disease risk.

Epidemiologic studies in adults indicate that alterations in peripheral leukocyte global DNA methylation are related to risk of obesity-related chronic diseases (9-12). Yet, the association of DNA methylation with body size remains unclear, and there is hardly any research in pediatric populations. Considering that the childhood obesity epidemic represents one of the greatest current public health challenges, it is critical to elucidate biological mechanisms involved in excess weight gain for effective interventions. Identifying modifiable predictors of global DNA methylation, such as diet, and understanding its relation with weight status would have important ramifications for intervention programs aimed at improving long term cardiometabolic health.

Specific Aims

This work aims to identify nutritional and sociodemographic correlates of global DNA methylation, and to investigate its relation with early life weight gain.

Aim 1: To evaluate whether dietary intake of methyl-donor (folate) and methylation cofactor (vitamin B12, vitamin B6, zinc, and methionine) micronutrients is associated with two measures of global DNA methylation (LINE-1 and Alu repetitive elements), and to assess the relation of total plasma homocysteine with global DNA methylation in a cohort of healthy middle-aged adult participants in Multi-Ethnic Study of Atherosclerosis (MESA) Stress Study.

Aim 2: To examine whether micronutrient biomarkers (erythrocyte folate; plasma vitamin B12, vitamin A, and ferritin; and serum zinc) are associated with global DNA methylation, quantified by methylation of LINE-1 repetitive elements, in a representative group of low- and middle-income school children from Bogotá, Colombia (the Bogotá School Children Cohort; BSCC). A secondary endpoint is to assess the relations of maternal and child sociodemographic characteristics with LINE-1 methylation.

*Aim 3**:*** To investigate the prospective association of global LINE-1 DNA methylation at time of recruitment into the BSCC with three indicators of adiposity (age- and sex-standardized body mass index (BMI), an indicator of overall adiposity; age- and sex-standardized abdominal circumference, a measure of central visceral adiposity; and age- and sex- standardized subscapular-to-triceps skinfold thickness ratio, an index of subcutaneous truncal adiposity) and linear growth (height-for-age Z-score) during a median of 2.5 years of follow-up.

DNA methylation in health and disease

Human epidemiologic and animal model data indicate that susceptibility to non-communicable adult-onset diseases is influenced by the prenatal and early postnatal environment (13-16). The underlying biological mechanisms were unclear until recent research in the field of epigenetics shed light on ways in which the epigenome and environment interact to induce lifelong phenotypic changes. DNA methylation, the covalent addition of a methyl group to the fifth carbon of cytosine at cytosine-guanine dinucleotides ('CpG sites'), is a stable and well-characterized epigenetic mechanism that plays a critical role in health and disease. As a fundamental biochemical process of mammalian development, DNA methylation regulates gene expression by physically blocking transcriptional proteins from binding to a gene (17), or by influencing chromatin configuration through associations with methyl-CpG-binding domain proteins (MBDs) (18). In addition to directing tissue-specific cell differentiation during embryogenesis, DNA methylation is involved in a number of other key processes including genomic imprinting (19), X-chromosome inactivation (20), oncogene silencing (21), and suppression of endogenous retroviruses (22).

Because DNA methylation is a highly regulated process, there is little inter-individual variability in methylation patterns across differentiated cells and tissues. Accordingly, sources of systemic between-person variation in DNA methylation, such as metastable epialleles (23) and transposable element insertion sites (14), provide a unique opportunity to link epigenetic dysregulation to disease. The stochastic nature of *de novo* DNA methylation at these regions during embryogenesis makes the epigenome particularly vulnerable to environmental and metabolic factors (23, 24). After cell

differentiation, DNA methylation patterns are preserved and maintained in the germ layer, resulting in marked inter-individual variation. Animal models (25-27) and human studies (28-30) indicate that the methylation profile in these regions may reflect environmental influences *in utero* and throughout life, providing evidence that DNA methylation could serve as a mechanistic link between environmental factors and health outcomes.

Inappropriate losses of (hypomethylation) or increases in (hypermethylation) gene-specific methylation are established causes of pathogenesis. Global DNA methylation, estimated by methylation of repetitive and transposable elements such as long interspersed nucleotide element (LINE)-1 and Alu (31), has recently received attention as a measure of genomic stability (32) and a potential biomarker of disease risk. Transposable elements are endogenous and ubiquitous remnants of ancestral infections fixed in germ-line DNA that comprise approximately 45% of the human genome (33). LINE-1 and Alu have the ability to amplify and propagate to new genomic locations through a cut-and-paste mechanism, inducing chromosomal strand breaks and mutations (34). Due to the deleterious consequences of their activity, transposable element sites are typically silenced through heavy methylation. Loss of methylation in these regions contributes to genomic instability (4) and could be indicative of decreased genome-wide methylation content (32). Studies have consistently demonstrated that global DNA hypomethylation of peripheral leukocytes is a risk factor for human cancers (35), and recent findings indicate that aberrant changes in leukocyte DNA methylation is related to risk of several other prominent chronic diseases (10, 11, 36), underscoring the value in understanding the role of this modifiable epigenetic mark in disease etiology.

Predictors of DNA methylation: The role of nutrition

Although DNA methylation occurs mainly during intrauterine development, it is responsive to environmental modifications and can change sporadically throughout life (37). Researchers found that peripheral leukocyte global DNA methylation decreased after exposure to environmental agents including black carbon traffic particles (38) and a low-dose of benzene (39). Similarly, plasma concentrations of several persistent organic pollutants were inversely related to global DNA methylation in a study of Greenlandic Inuit (40). Global DNA methylation is also associated with various epidemiologic risk factors such as age (41), sex (42, 43), race/ethnicity (42, 43), dietary patterns (44), body size (10, 11), physical activity (45), and alcohol and tobacco use (42). While the mechanisms that underlie these associations remain unclear, dietary intake of certain micronutrients, including methyl-donors and retinoids, play a direct role in DNA methylation pathways.

One-carbon metabolism is a cyclical physiological process that ultimately provides the methyl group in all mammalian DNA methylation reactions. The transfer of the methyl group from the universal methyl-donor, S-adenosylmethionine (SAM), to the 5th carbon of the cytosine ring is catalyzed by the DNA methyltransferases (DNMTs) in the presence of methyl-donor micronutrients such as folate and choline, and methylation cofactors including vitamin B12, vitamin B6, vitamin B2, and zinc (see **Figure 1.1** for details of DNA methylation pathways). The folate-mediated and choline-mediated methylation pathways intersect at the formation of methionine, the precursor of SAM, from homocysteine. Successful recycling of methionine from homocysteine is essential to the procurement of SAM for subsequent methylation reactions. Because methyl-donors

and methylation cofactors are obtained from the diet, an imbalance or deficiency can lead to elevated plasma homocysteine (46, 47), which is a cause of oxidative stress and an established marker of cardiovascular disease risk (48). Additionally, high homocysteine concentrations could adversely influence kinetics of the methylation reaction (49, 50).

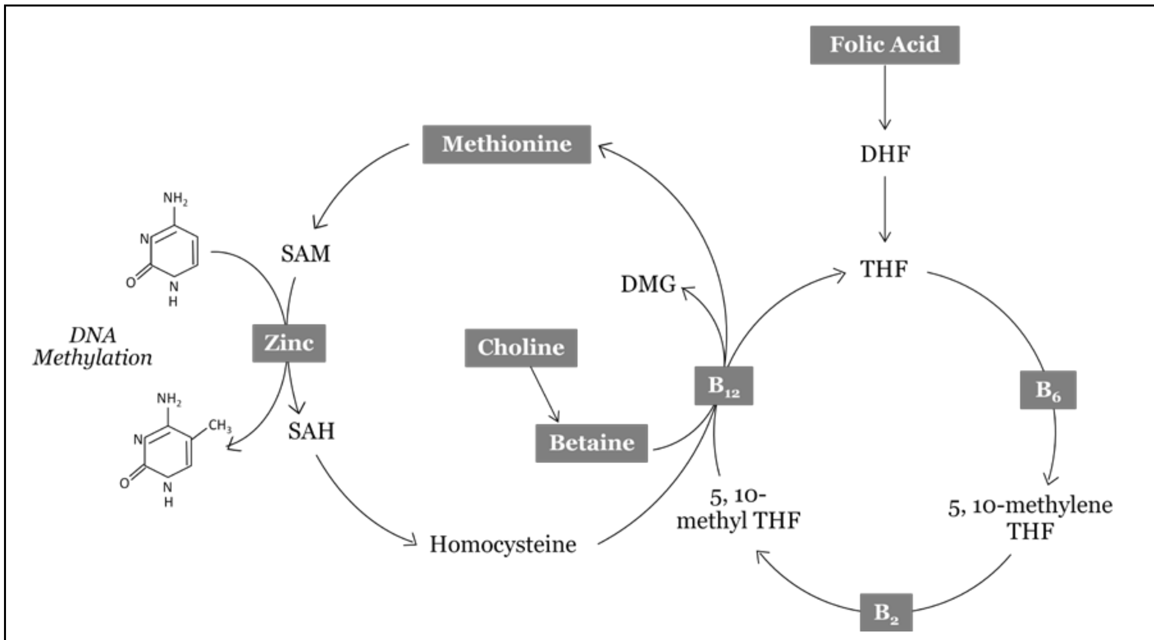

Figure 1.1 Involvement of dietary micronutrients in one-carbon metabolism. Substrates obtained from dietary sources are shaded in gray. Folic acid enters one-carbon metabolism as tetrahydrofolate (THF). The conversion of THF to 5,10-methylene THF is catalyzed by cofactor vitamin B6 and serine hydroxy-methyltransferase. Vitamin B2, precursor to flavin adenine dinucleotide (FAD), is a cofactor to methylenetetrahydrofolate reductase (MTHFR) in the conversion of 5,10-methylene THF to 5-methyl THF. Vitamin B12 is a precursor to methionine synthase, which is involved in the production of methionine and dimethylglycine (DMG) from homocysteine and betaine. Zinc is a cofactor to the DNA methyltransferases (DNMT) in the transfer of the methyl group from S-adenosylmethionine (SAM) to the the 5th carbon of cytosine. Demethylated SAM becomes S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed to homocysteine by adenosylhomocysteinase. Homocysteine can be recycled back to methionine with adequate methyl-donor (folate and choline) and methylation cofactor (vitamin B12, vitamin B6, vitamin B2, and zinc) micronutrients. Figure and text adapted from Anderson et al 2012 (51).

However, the literature regarding the relation of micronutrient status with global DNA methylation is inconsistent. Some controlled-feeding trials in adults have found changes in global DNA methylation following folate supplementation and depletion (52, 53), while others showed no effect (54-57). Findings from cross-sectional studies are also discordant. Intake of methyl-donor micronutrients was not related to global DNA methylation in one study of healthy middle-aged adults (44), while another study reported

a positive correlation between folate intake from fortified foods and global DNA methylation (58). Two perinatal studies conducted to examine the relations of maternal nutrient intake with DNA methylation during early life (59, 60) reported no relation between micronutrient status and cord blood DNA methylation. The discrepancies in study findings could be due to differences in study design, potential recall bias of dietary intake in observational studies, and variability in study population characteristics. There is also considerable variation in the methodology used to determine global DNA methylation, ranging from the [(3)H]-methyl group acceptance assay, which is inherently limited by its *in vitro* stability (61), to various pyrosequencing-based assays.

In addition to the micronutrients involved in one-carbon metabolism, retinoids can also influence DNA methylation by affecting DNMT activity. For example, treatment of breast cancer cells with *all trans* retinoic acid (*at*RA), the most biologically active metabolite of vitamin A, inhibited cell growth by reducing DNMT expression in a highly differentiated cancer cell line (62). A reduction in DNMT activity could lead to decreased DNA methylation since the DNMTs catalyze the methylation reaction. Unfortunately, the DNMT-inhibitory effects of retinoic acid treatments have only been examined in the context of chemoprevention and cancer therapies, and little is known regarding its influence on global DNA methylation.

The limited and discordant findings point toward the need to improve understanding of how dietary factors influence global DNA methylation in healthy populations. Additionally, use of reliable and reproducible laboratory techniques to quantify global DNA methylation is necessary to facilitate comparability across epigenetic studies.

Global DNA methylation and obesity

Animal models provide evidence that changes in DNA methylation patterns can influence risk of obesity and obesity-related diseases (25, 26, 63). In the yellow Agouti mouse, the protective effect of methyl-donor micronutrient intake against obesity was specifically due to increased methylation of the A^{vy} metastable epiallele (25, 26). In humans, researchers have identified alterations in gene-specific methylation that were related to weight change (64, 65) and weight status (66, 67) in obese patients. While gene-specific changes provide insight into regulatory pathways, changes at the global level are also important to consider because global DNA methylation is responsive to environmental modifications (38), and because genomic stability has health implications beyond the function of a specific gene.

Despite findings that global DNA methylation is related to several obesity-related diseases including cardiovascular disease (9-11), metabolic syndrome (68), and diabetes (12), little is known regarding the direct relation between global DNA methylation and body size. Current knowledge consists mostly of mixed findings from cross-sectional studies in adult populations (10, 11, 42, 43). Some studies from maternal-infant dyads have been conducted to investigate relations of perinatal characteristics, including birth weight, with cord blood DNA methylation (59, 60, 69). One study reported that high and low birth weight, as well as premature birth were associated with significantly lower cord blood DNA methylation (69). However, inference from perinatal studies is limited, as it is not known whether functional consequences of these associations contribute to obesity in later life. To date, the largest gap in literature is the lack of longitudinal studies to assess the temporal relation between global DNA methylation and subsequent changes in

anthropometry. While cross-sectional studies provide a preliminary idea of how global DNA methylation and body size are related, they cannot overcome the potential for reverse causation bias. Furthermore, little is known regarding global DNA methylation and weight status in children. Pediatric populations are an ideal setting to examine this relation, as the childhood epigenome has endured fewer environmental exposures that could obscure the associations of interest. Furthermore, animal models indicate that DNA methylation profiles are sensitive to external stimuli, such as dietary intake, during specific postnatal periods, and that the epigenetic changes can influence body composition (27). Because adipocyte quantity is not set until early adolescence (70), the childhood years represent a particularly important time frame to elucidate molecular mechanisms involved in adipogenesis for obesity intervention efforts. Considering the compelling evidence that excess weight gain in early life is related to increased cardiometabolic morbidity in adulthood (71, 72), it is critical to understand biological processes that underlie childhood obesity for effective early interventions.

Summary of Chapters

This dissertation expands the existing knowledge surrounding predictors of global DNA methylation by elucidating its association with modifiable nutritional and sociodemographic characteristics in adults and children. This work also addresses the chasm in epigenetic pediatric research, and the need for longitudinal studies to clarify the temporal relation of global DNA methylation with excess weight gain. The associations examined are depicted in the conceptual framework shown in **Figure 1.2**.

The first two analytic chapters are focused on micronutrients and global DNA methylation, quantified using a highly reproducible pyrosequencing technique (31). Chapter 2 uses data from the Multi-Ethnic Study of Atherosclerosis (MESA) Stress Study to assess the relations of methyl-donor/methylation cofactor micronutrient intake and total plasma homocysteine with two measures of global DNA methylation in an ethnically diverse population of healthy middle-aged adults. Chapter 3 examines associations of micronutrient biomarkers and sociodemographic characteristics with global DNA methylation in a representative sample of low- and middle-income school-age children from the Bogotá School Children Cohort (BSCC). In Chapter 4, we used prospectively collected data to investigate the relation of global DNA methylation at time of recruitment into the BSCC with changes in adiposity and linear growth during a median of 2.5 years of follow-up. Finally, a summary of the dissertation's main findings, public health implications, and suggestions for future research are discussed in Chapter 5.

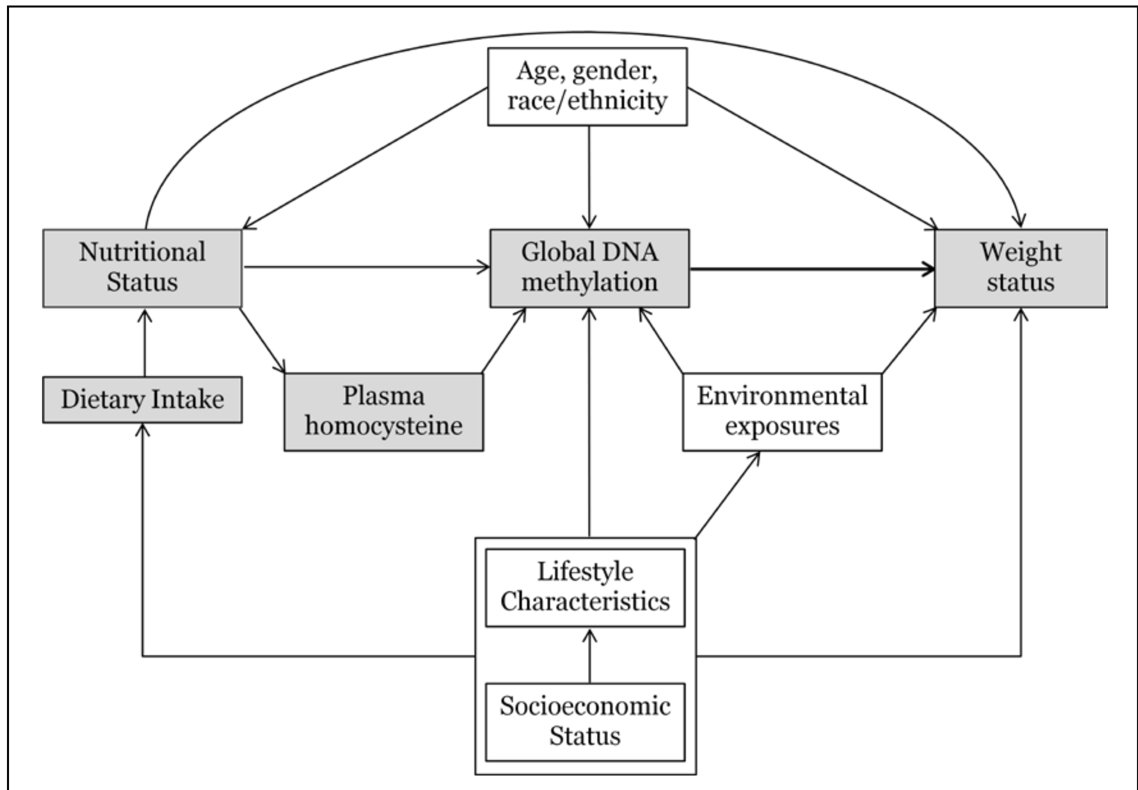

Figure 1.2 Conceptual framework of how dietary intake, nutritional status, global DNA methylation and weight status are related. Shaded gray boxes are the main exposures and outcomes examined in the three dissertation aims.

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Chapter 2

Dietary intake, plasma homocysteine, and global DNA methylation in the Multi-Ethnic Study of Atherosclerosis (MESA)

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Introduction

Epigenetic mechanisms, such as DNA methylation, are heritable changes in gene expression made over the nucleotide sequence. Genome-wide methylation content, or global DNA methylation, is a measure of genomic stability that is related to risk of adult onset diseases such as cancer (1). Recent findings from epidemiologic studies indicate that aberrations in global DNA methylation could serve as a biomarker of cardiovascular disease (CVD) risk (2, 3). Global DNA hypomethylation may play a role in CVD etiology through its relation with hyperhomocysteniemia (4), a prominent risk factor for endothelial injury and vascular disorders. Considering that CVD is the leading cause of death in most countries (6), it is crucial to identify correlates of modifiable risk factors such as DNA methylation for effective interventions.

Nutrition plays a key role in DNA methylation pathways. One-carbon metabolism is an essential process that provides the methyl group in all mammalian DNA methylation reactions. The reaction is catalyzed by several endogenous enzymes in the presence of methyl-donor micronutrients such as folate and choline, and methylation

cofactors including vitamin B12, vitamin B6, and zinc. The folate- and choline-dependent methylation pathways intersect at the formation of methionine, precursor to the universal methyl-donor S-adenosylmethionine (SAM), from homocysteine. Successful metabolic cycling of methionine from homocysteine ensures provision of SAM for subsequent methylation reactions. Because methyl-donor and methylation cofactor micronutrients are obtained from the diet, an imbalance or deficiency in these micronutrients can result in elevated plasma homocysteine concentrations, which is a marker of CVD risk (7).

Although it is well-established that a deficiency in methyl-donor micronutrients can lead to hyperhomocysteinemia (8, 9), the relations of methyl-donor micronutrient intake and plasma homocysteine concentrations with global DNA methylation remain unclear. Some controlled-feeding trials in adults have found changes in global DNA methylation following folate supplementation and depletion (10, 11), while others showed no effect (12-15). Findings from cross-sectional studies have also been inconsistent. Intake of methyl-donor micronutrients was not related to global DNA methylation among healthy adults in Texas (16), while another study of cancer-free adults in New York reported a positive correlation between folate intake from fortified foods and global DNA methylation (17). In a recent study of Colombian schoolchildren, neither erythrocyte folate nor serum vitamin B12 concentrations were associated with global DNA methylation (18). Two perinatal studies have also been conducted to examine the relations of maternal nutrient intake with DNA methylation during early life (19, 20). Although prenatal intake of methyl-donor micronutrients was not related to global DNA methylation in either study, Fryer et al. noted an inverse association between

homocysteine and DNA methylation in cord blood (20). This was expected since elevated homocysteine may reflect a reduced systemic methylation capacity (21); yet, some animal (22) and human (23) studies have also reported no association between homocysteine and global DNA methylation. The conflicting literature underscores the need to elucidate how micronutrients involved in one-carbon metabolic pathways and plasma homocysteine levels are related to global DNA methylation in a population at risk of CVD.

We examined the associations of daily folate, vitamin B12, vitamin B6, methionine, and zinc intake, and total plasma homocysteine with global DNA methylation using data from the MESA Stress Study, a subsample of healthy adults aged 45-84 participating in the Multi-Ethnic Study of Atherosclerosis (MESA).

Methods

Subjects

This cross-sectional investigation uses data collected by the MESA Stress Study, and ancillary study to the Multi-Ethnic Study of Atherosclerosis (MESA). MESA is a longitudinal investigation initiated in July 2000 in six U.S. cities to investigate the prevalence and progression of subclinical CVD. Details of the MESA sampling and recruitment procedures have been previously reported (24). The MESA Stress Study sampled 1002 MESA participants enrolled at the New York and Los Angeles sites. Participants were recruited in conjunction with the third and fourth follow-up exams of the full MESA; participant enrollment proceeded in the order in which they attended the follow-up exam. At each field center, enrollment continued until approximately 500 participants were enrolled. All data used in these analyses was obtained from the MESA baseline examination conducted between 2000 and 2002. Anthropometric measurements including height and weight were obtained as part of the baseline examination. Participants also completed a set of subclinical cardiovascular disease measurements, as well as a comprehensive questionnaire to obtain information on sociodemographic characteristics, standard cardiovascular disease risk factors, lifestyle, and psychosocial factors. Physical activity was measured using a detailed, semiquantitative questionnaire adapted from the Cross-Cultural Activity Participation Study (B. Ainsworth, University of South Carolina, personal communication, 2000). All procedures were carried out with written consent of the subjects.

Sociodemographic characteristics of the MESA Stress participants were similar to the large MESA cohort at the two participating sites with the exceptions that the

subsample had fewer individuals aged 75-84 (12.1% vs. 18.2%), more men (47.6% vs. 44.7%) and more college-educated participants (29.7% vs. 23.9%).

Dietary Assessment

At the baseline examination, each participant completed a 120-item food-frequency questionnaire (FFQ) (25) that was modified to include typical Chinese and Hispanic ethnic foods to accommodate the MESA subject population. The FFQ inquired about serving size (small, medium, or large) and frequency of intake (times per day) for selected foods and beverages; nine frequency options were given that ranged from “rare or never” to a maximum of “≥ 2 times/day” for foods and a maximum of “≥ 6 times/day” for beverages. A section of the questionnaire included information on frequency, dosage, and duration of supplement use, allowing quantification of nutrient intake from supplements. Daily nutrient intakes from foods were estimated by multiplying the reported amount of food or beverage consumed (frequency x serving size) by its nutrient content (Nutrition Data Systems for Research [NDS-R]; University of Minnesota; Minneapolis). For folate, nutrient content from foods was converted to dietary folate equivalent (DFE) units to account for differences in absorption of naturally occurring dietary folate and the more bioavailable synthetic folic acid. All nutrients were adjusted for total energy intake using the residual method (26). In the analyses, we considered total nutrient intake, intake from foods alone, and intake from supplements alone. Values of extreme nutrient intake values (> 13,000 μg/d of folate, > 7000 μg/d of vitamin B12, > 180,000 mg/day of vitamin B6, or > 2000 mg/d of zinc), or extreme total energy intake (> 6000 or < 500 kcal/d) were excluded from the analyses.

Laboratory Methods

Phlebotomists obtained approximately 80 mL of blood from all participants in the fasting state at the baseline examination. Standardized methods were used to process and ship samples to a central laboratory (Laboratory for Clinical Biochemistry Research, University of Vermont, Burlington, Vermont). Plasma was separated in an aliquot for homocysteine determinations. Total plasma homocysteine concentration was measured with a fluorescence polarization immunoassay (IMx Homocysteine Assay, Axis Biochemicals ASA, Oslo, Norway) with use of the the IMx analyzer (Abbott Diagnostics, Abbott Park, Illinois). The method is based on the enzymatic conversion of free homocysteine to S-adenosyl-L-homocysteine, which is subsequently detected by a competitive immunoassay. The laboratory analytical coefficient of variation (CV) range was 4.5%. The detection range for this assay is 0.5 – 50.0 μmol/L.

LINE-1 and Alu DNA Methylation Determinations

We quantified methylation of two repetitive and dispersed elements, LINE-1 and Alu, as measures of global DNA methylation. Together, LINE-1 and Alu comprise roughly 30% of the human genome and are valid measures of genomic methylation content (27, 28). High-molecular-weight DNA was extracted with commercially available PureGene Kits (Gentra Systems, Minneapolis, MN) from the leukocytes of blood collected at the baseline exam. Approximately 200 ng of DNA at 10 ng/μl were bisulfite-treated using the EZ-96 DNA Methylation Kit™ (Zymo Research, Orange, CA). Bisulfite conversion of DNA deaminates unmethylated cytosine to uracil, which is read as thymidine during polymerase chain reaction (PCR). Methylated cytosines (5-

methylcytosine) are protected from bisulfite conversion and thus remain unchanged, resulting in genome-wide methylation-dependent differences in DNA sequences.

Pyrosequencing-based methylation analysis was used to quantify methylation at four genomic LINE-1 sites and three genomic Alu sites using previously described methods (29). Global DNA methylation was assessed through simultaneous PCR of LINE-1 and Alu elements, using primers designated towards consensus LINE-1 and Alu sequences to allow for amplification of a representative pool of repetitive elements. The percentage of 5-methylated cytosines (%5-methylcytosine; %5mC) for each CpG target region was quantified using the Pyro Q-CpG Software. This software assigns quality scores for each measurement and internal quality controls to assess the efficiency of bisulfite conversion. The interassay CV for LINE-1 and Alu was 2.10% and 5.73%, respectively.

Statistical Analysis

Of the 1002 participants in the MESA Stress study, information on LINE-1 and Alu methylation was available for 961 and 987 individuals, respectively. All participants had data on at least one exposure of interest (daily intake of folate, vitamin B12, vitamin B6, zinc, or methionine; or total plasma homocysteine concentrations) and were included in the analyses.

We first evaluated the distributions and correlations of %5mC for the four genomic LINE-1 sites and the three genomic Alu sites. The mean ± SD %5mC for the four LINE-1 sites was 79.91 ± 2.81, 81.91 ± 1.62, 76.93 ± 2.48, and 84.14 ± 2.48. For the three Alu sites, mean ± SD %5mC was 31.82 ± 1.55, 26.40 ± 1.61, and 15.07 ± 1.03. The

Spearman's correlation coefficient ranged from 0.06 to 0.56 for LINE-1 sites and from 0.44 to 0.66 for the Alu sites. Because the distributions and correlations of %5mC differed by site for both measures of genomic methylation, mixed effects linear regression models were used to derive a single estimate of LINE-1 and Alu for each individual. In these models, LINE-1 or Alu at each site were the dependent variables. Models included a random intercept for each site that was allowed to vary from person to person. The empirical best linear unbiased predictors (EBLUPs) obtained from the random effects represent the between-person variation in DNA methylation. The final person-specific LINE-1 and Alu methylation variables were calculated by adding these EBLUPS to the raw average %5mC across the four sites for LINE-1, and across the three sites for Alu for the entire population. This method enabled us to incorporate the between-person variability of the underlying means for each LINE-1 and Alu site.

Next, we examined the distribution of LINE-1 and Alu methylation separately across quartiles of homocysteine, as well as by categories of potential confounding variables that included sociodemographic, anthropometric, and lifestyle characteristics. Categories of body mass index (BMI) were created according to the World Health Organization (WHO) international classification of adult weight status (30). Alcohol and cigarette use were categorized as "never user", "former user", and "current user." A four-level income/wealth index was used as an indicator of socioeconomic status (SES). The variable was created using total family income and a 5-point wealth index used in prior MESA studies (31). The income component of the index was based on a 5-level income variable created from continuous family income of the participants. The wealth index is a 5-point variable based on ownership of assets including a car, a home, land,

and investments. Thus, the income/wealth index is a sum of the income and wealth components that ranges from 0 to 8. In this analysis, the income/wealth index was categorized into 4 levels: 0-2 (lowest), 3-4, 5-6, and 7-8 (highest). Daily physical activity level was categorized into quartiles based on total hours of physical activity per week. We also compared DNA methylation with respect to cancer status, a dichotomous variable defined as "Yes" if the participant had ever been informed by a physician that they had cancer. We assessed the statistical significance of differences in DNA methylation across categories of these variables with the use of linear regression models in which LINE-1 or Alu methylation was the outcome and predictors included indicator variables for each characteristic. For ordinal characteristics, we obtained a test of linear trend.

Next, we compared the distribution of LINE-1 and Alu methylation by energy-adjusted quartiles of total nutrient intake, nutrient intake from foods only, and nutrient intake from supplements only for folate, vitamin B12, vitamin B6, and zinc (for intake category cut points, see **Table 1.1**). Quartiles of micronutrient intake from foods only were estimated in the subpopulation of participants who did not use supplements because effects of nutrients from foods may be overpowered by the relatively high levels of intakes from supplements (26). A test for linear trend was obtained for each intake variable.

Finally, we conducted multivariable linear regression with the micronutrient intake and sociodemographic, anthropometric, and lifestyle variables that were related to DNA methylation based on prior knowledge, or were significantly related to LINE-1 or Alu methylation in the univariate analysis at $P < 0.10$. The estimates for micronutrient

intake were not adjusted for homocysteine, as it could be on the causal pathway between nutrient intake and DNA methylation. A test for linear trend was obtained for ordinal characteristics by introducing into the model a continuous variable representing the ordinal categories of the predictor. For continuous variables, estimates were obtained for a difference in %5mC associated with a one standard deviation difference in the variable of interest (per 5 kg/m^2 BMI, per 10 cm height, and per 3 μmol/L homocysteine). To determine whether the associations varied by sex, we tested for interactions with use of the likelihood ratio test. We found no evidence that associations with LINE-1 or Alu methylation differed by sex; thus, results reported pool men and women and are adjusted for sex.

All analyses were carried out with the use of the Statistical Analyses System software (version 9.2; SAS Institute Inc).

Results

Mean ± SD age of the 987 participants was 61.4 ± 9.9 years; 47.5% were men. The overall mean ± SD DNA methylation was 82.73 ± 1.07 %5mC for LINE-1 and 24.42 ± 0.85 %5mC for Alu. The correlation (Spearman's ρ) between LINE-1 and Alu methylation was 0.15.

Men had an average 0.40 %5mC higher LINE-1 methylation than women ($P <$ 0.0001), whereas Alu methylation did not differ by sex (**Table 1.2**). Age was positively associated with LINE-1 methylation (*P* trend = 0.07). DNA methylation differed significantly by race; compared to Whites, African Americans and Hispanics had 0.34 %5mC and 0.24 %5mC higher LINE-1 methylation, respectively. On the other hand, Alu methylation for African Americans and Hispanics was 0.20 %5mC and 0.23 %5mC lower than Whites, respectively. A higher income/wealth index score was related to lower LINE-1 methylation (*P* trend = 0.04) and higher Alu methylation (*P* trend = 0.002). Height was positively correlated with both LINE-1 (*P* trend = 0.001) and Alu methylation (*P* trend = 0.12). Higher BMI and homocysteine were both associated with higher LINE-1 methylation (*P* trend = 0.06 and *P* trend = 0.0009, respectively). Never and former alcohol users had lower LINE-1 methylation than current alcohol users (*P* = 0.06). Physical activity was positively associated with Alu methylation (*P* trend = 0.06). Individuals who reported a history of cancer had 0.20 %5mC lower LINE-1 methylation than those who did not report cancer (*P* = 0.09).

We did not find statistically significant associations between micronutrient intake and LINE-1 methylation (**Table 1.3)**. However, there was a positive association of total vitamin B12 intake and total zinc intake with Alu methylation (**Table 1.4**). Men and

women in the highest quartile of total vitamin B12 had 0.21 %5mC higher Alu methylation than those in the first quartile (*P* = 0.008). Similarly, those in the highest quartile of total zinc intake had 0.16 %5mC higher Alu methylation than those in the lowest quartile (*P* = 0.05).

We next examined the adjusted associations of homocysteine with LINE-1 methylation using multivariable linear regression (**Table 1.5**). After adjustment for covariates including sex, age, height, BMI, race, alcohol use, self-reported history of cancer, and income/wealth index, BMI remained positively associated with LINE-1 methylation; each 5 unit difference in BMI was associated with a 0.06 %5mC higher LINE-1 methylation (*P* = 0.04). We also observed a marginally statistically significant positive association between homocysteine and LINE-1 methylation. Each 3 umol/L difference in homocysteine was related to a 0.06 %5mC higher LINE-1 methylation (*P* = 0.07).

Finally, we examined the associations of vitamin B12 intake, total zinc intake, and homocysteine with Alu methylation with use of a multivariable model that also included age, sex, height, race, height, income/wealth index; the models for vitamin B12 and zinc did not include homocysteine because it may be on the causal pathway of micronutrient intake and DNA methylation (**Table 1.6**). The positive associations of zinc and vitamin B12 intake with Alu methylation were attenuated and became statistically insignificant after multivariable adjustment. Height remained positively associated with Alu methylation; each 10 cm difference in height was associated with a 0.10 %5mC higher Alu methylation (*P* = 0.02).

Discussion

In this study of healthy adults aged 45-84 y, intake of methyl-donor and methylation cofactor micronutrients was not associated with global DNA methylation. However, we found positive associations of BMI and total plasma homocysteine with LINE-1methylation. We also observed a positive relation between height and Alu methylation. Although the differences in LINE-1 and Alu methylation were small, they represent changes at a global level that likely reflect larger differences in the context of the entire genome.

Our finding of a positive relation between BMI and LINE-1 methylation contributes to the ongoing discussion regarding the role of DNA methylation in obesity-related disease etiology. Studies from the Dutch Winter Famine Cohort reported that periconceptional famine exposure was related to persistent changes in methylation of genes involved in cardiometabolic diseases (32, 33), as well as higher BMI and waist circumference in middle age (34). While such findings suggest that aberrant DNA methylation is related to excess weight, current evidence on the association of BMI with global DNA methylation from cross-sectional studies in adults have been mixed (35-37). A recent cohort study conducted among 286 Singaporean-Chinese adults reported a positive association between global DNA methylation and BMI at baseline among both men and women (2). Additionally, global DNA hypermethylation was associated with higher incidence of CVD (myocardial infarction, stroke) and its predisposing conditions (hypertension, diabetes) over the follow-up period among men only. In the same study, DNA methylation did not differ by intake of folate or B vitamins, plasma folate levels, or folate-metabolizing genotypes (2), leading the investigators to postulate that the

unexpected positive relations were driven by a mechanism independent of the DNA methylation pathway, such as systemic inflammation. This hypothesis is lent support from a case-control study which showed that elevated C-reactive protein, an inflammation biomarker, was associated with global DNA hypermethylation in chronic kidney disease patients (38); yet, the opposite trend was observed in healthy school-age children (18). While we cannot rule out the possibility that systematic inflammation may underlie the association observed in our study, it remains important to consider that global DNA hypermethylation could be a biomarker of adverse metabolic changes related to excess weight. For example, researchers have identified differential methylation of several genes associated with weight-loss responsiveness to dieting, as well as changes in methylation in certain genes after weight loss (39). Whether gene-specific changes manifest at the global level is not known; however, there is some evidence that prenatal exposures associated with gene-specific methylation are also related to global DNA methylation (40). Further research is warranted to confirm the direction of the association between BMI and global DNA methylation, and longitudinal investigations are required to disentangle whether aberrations in DNA methylation patterns are a cause or consequence of weight gain.

We also found a positive association between height and Alu methylation. This is a salient finding, as height is a sensitive indicator of early life exposures that otherwise may not be quantifiable. Although some studies have examined associations of BMI with DNA methylation, the literature regarding height is scant. A recent study used data from two prospective birth cohorts to identify genes associated with high BMI, and to examine relations of gene-specific methylation in cord blood with BMI and its components at 9 y

(41). After adjustment for multiple comparisons, the only association that remained apparent was a 0.15% lower height at 9 y for every 1 %5mC difference in DNA methylation of *ALPL* (41). The *ALPL* gene encodes the alkaline phosphatase enzyme, which plays a critical role in bone mineralization; thus methylation silencing of *ALPL* could hinder skeletal growth. Although this finding links gene-specific methylation to linear growth, changes at the global level are also important, as genomic methylation content has health implications beyond the function of one specific gene. Smaller stature may be associated with lower global DNA methylation through exposure to adverse early life conditions, such as low socioeconomic status, which is related to shorter adult height (42), as well as lower DNA methylation (18). Considering that height has been consistently inversely related to CVD risk (43, 44), understanding the biological mechanisms associated with poor linear growth would enhance knowledge of disease etiology and identify avenues for intervention.

The marginally significant positive association between homocysteine and LINE-1 methylation was unexpected. During the DNA methylation cycle, the universal methyl-donor SAM is de-methylated to S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed to homocysteine. Under optimal physiological conditions, homocysteine is re-methylated to methionine, which is then converted to SAM to provide the methyl group for subsequent methylation reactions. Accordingly, a deficiency in methyl-donor micronutrients leads to increased plasma homocysteine and reversal of the SAH hydrolase reaction, resulting in accumulation of intra-cellular SAH, which was reported to decrease DNA methylation (45) since SAH is an inhibitor of the DNA methyl-transferases (DNMTs) (46). However, two human cases of SAH hydrolase deficiency

exhibited hypermethylated leukocyte DNA in comparison to controls, despite high levels of plasma SAH (47, 48). Although the biological mechanism through which higher SAH would lead to increased global DNA methylation is unknown, it is important to note that the magnitude of the association we observed is quite small, and that only 3.7% (N = 35) of our study population is considered hyperhomocysteinemic according to the American Heart Association advisory statement (49). It is possible that the expected inverse association between plasma homocysteine and DNA methylation would be observed if homocysteine levels were higher than those of our study population. Future studies are required to better understand the kinetics of the methylation reaction in a population with a broader range of plasma homocysteine concentrations.

Of note, we did not observe any associations of dietary micronutrient intake with LINE-1 or Alu methylation. Although unexpected, the null findings are consistent with some studies in adults (12-14). There are a few potential explanations for the lack of association between micronutrient intake and global DNA methylation in this study. First, due to folic acid fortification of the U.S. food supply, it is likely that few MESA participants were deficient in methyl-donor micronutrients; this speculation is further supported by the small proportion of hyperhomocysteinemic individuals in our study population. A positive association of folate intake with global DNA methylation might be detectable in populations with a higher prevalence of methyl-donor micronutrient deficiencies. Second, we only examined consumption of specific micronutrients, which does not account for the combinations of foods and nutrients that characterize the human diet. In a study of healthy adults, a prudent dietary pattern and consumption of green leafy vegetables was related to higher global DNA methylation, yet there were no

differences in DNA methylation by methyl-donor nutrient intake (16). Such findings suggest the involvement and interactions of multiple micronutrients and antioxidants in DNA methylation. Finally, although folate status (50, 51) and intake (52) have been positively related to DNA methylation, erythrocyte folate was not associated with global DNA methylation among school-age children (18), and folate intake was actually inversely related to DNA methylation in two other human studies (53, 54). The folate pathway is not the only source of methyl groups for DNA methylation. Approximately 60% of methyl groups are derived from choline in the liver (55); thus choline-mediated homocysteine re-methylation may play a more important role in providing methyl groups for DNA methylation reactions. Because we only had information on folate intake this population, we were not able to consider associations with choline. Animal and human studies both indicate that the folate and choline transmethylation pathways are interrelated (56, 57); thus, it will be important to examine associations of both micronutrients with DNA methylation in future research.

The discrepancies in associations of LINE-1 and Alu methylation with sociodemographic and dietary factors are also noteworthy. Repetitive elements are interspersed throughout the genome and are sensitive to environmental exposures, making them attractive surrogates of global DNA methylation in epidemiologic studies. Yet, there is considerable study-to-study variation with respect to the degree of correlation between the two assays, and the accuracy with which each measure approximates total genomic methylation content. A recent study that compared methylation of LINE-1 and Alu with three other representative and dispersed sequences (strong CpG islands, weak CpG islands, and non-islands) in a set of placental chorionic

villi at three gestational ages (1st trimester, 2nd trimester, and at term) and in four somatic tissues (fetal brain, fetal kidney, fetal muscle and adult blood) showed that LINE-1 and Alu followed distinct methylation patterns from each other (58). While LINE-1 methylation varied across the different tissue types, there were no significant differences in Alu methylation. Furthermore, the methylation pattern of LINE-1 was most similar to those of weak CpG island and non-island sequences, which are highly methylated CpG regions with no active role in transcriptional regulation (59). LINE-1 sequences are sites of *de novo* DNA methylation during embryogenesis and are typically concentrated in low guanine-cytosine (GC) content regions. Gene silencing through DNA methylation spreads from LINE-1 sequences to transcription start sites (TSS) (60), a phenomenon that is thought to be buffered by Alu elements, which tend to cluster around TSS associated with CpG islands (61). Taken together, the evidence suggests that LINE-1 and Alu are functionally different, in addition to exhibiting unique dispersion patterns. There is need to characterize shared and exclusive environmental predictors of LINE-1 and Alu methylation, how modification of these predictors affects methylation of repetitive sequences, and the degree to which these changes influences disease risk.

Our study had several strengths. We were able to examine LINE-1 and Alu methylation from circulating leukocytes in a large and ethnically diverse population of healthy adults. LINE-1 and Alu methylation were determined using pyrosequencing technology, a highly reproducible and accurate method to quantify DNA methylation. Furthermore, quantifying DNA methylation from peripheral white blood cells is of high intrinsic value in epidemiologic studies, as it is easily obtained and reflects systemic interindividual variation in germ-layer cells (62). We also used a culturally-tailored FFQ

to ascertain nutrient intake, which is the most appropriate method of dietary assessment in epidemiologic studies (26). While use of a FFQ does not preclude the possibility of recall bias, it is an efficient and inexpensive measure of long-term dietary habits, which is more relevant to disease risk than short-term intake. Because the FFQ ranks individuals within a population by their usual intake, it also is useful for nutrients with substantial day-to-day variation, including micronutrients. This study is limited by its cross-sectional design, which hampers the possibility of making causal inference on the predictors of global DNA methylation.

In summary, we conclude that higher BMI and homocysteine levels are associated with higher LINE-1 methylation, whereas height is positively related to Alu methylation. The value of LINE-1 and Alu methylation as biomarkers of health outcomes requires further examination in prospective studies.

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Table 1.1 Quartiles of nutrient intake in 987 MESA participants

	Quartiles of energy-adjusted nutrient intake[1]			
	Q1	**Q2**	**Q3**	**Q4**
Folate, µg				
	n = 219	***n = 221***	***n = 220***	***n = 219***
Total Intake	< 299.9	299.9 - 406.6	406.7 - 974.9	> 974.9
	n = 147	***n = 146***	***n = 147***	***n = 146***
From foods only	< 278.1	278.1 - 331.7	331.8 - 401.1	> 401.1
	n = 690	***n = 98***	***n = 98***	***n = 100***
From supplements only	0	> 0, < 768.5	768.5 - 832.9	> 832.9
Vitamin B12, µg				
	n = 219	***n = 220***	***n = 221***	***n = 219***
Total	< 2.4	2.4 - 3.9	4.0 - 12.8	> 12.8
	n = 139	***n = 139***	***n = 139***	***n = 139***
From foods only	< 1.94	1.94 - 2.72	2.73 - 3.65	> 3.65
	n = 660	***n = 109***	***n = 108***	***n = 110***
From supplements	0	> 0, < 9.18	9.18 - 31.17	> 31.17
Vitamin B6, mg				
	n = 220	***n = 220***	***n = 220***	***n = 220***
Total	< 1.36	1.36 - 2.01	2.02 - 1982.24	> 1982.24
	n = 139	***n = 139***	***n = 139***	***n = 140***
From foods only	< 1.2	1.2 - 1.4	1.5 - 1.8	> 1.8
	n = 661	***n = 107***	***n = 110***	***n = 109***
From supplements	0	>0, < 2007.7	2007.7 - 3952.9	> 3952.9
Zinc, mg				
	n = 221	***n = 221***	***n = 221***	***n = 220***
Total	< 6.9	6.9 - 8.7	8.8 - 19.3	> 19.3
	n = 144	***n = 144***	***n = 144***	***n = 144***
From foods only	< 6.4	6.4 - 7.3	7.4 - 8.5	> 8.5
	n = 680	***n = 101***	***n = 104***	***n = 102***
From supplements	0	> 0, < 14.8	14.8 - 15.6	> 15.6
Methionine, g				
	n = 221	***n = 221***	***n = 220***	***n = 221***
Total Intake[1]	< 1.03	1.03 - 1.23	1.24 - 1.43	> 1.43

1 Intake from foods.

Table 1.2 Mean LINE-1 and Alu DNA methylation according to characteristics of MESA participants

	N[2]	**LINE-1**[1] N = 961	*P*[3]	N[2]	**Alu**[1] N = 987	*P*[3]
Sex						
M	457	80.94 (1.03)	<0.0001	469	24.45 (0.81)	0.74
F	504	80.54 (1.06)		518	24.42 (0.89)	
Age, *years*						
45 - 54	290	80.66 (1.07)	0.07	298	24.43 (0.84)	0.34
55 - 64	263	80.68 (1.09)		272	24.44 (0.86)	
65 - 74	292	80.81 (1.01)		299	24.31 (0.83)	
75 - 84	116	80.80 (1.15)		118	24.69 (0.83)	
Race						
White, Caucasian	180	80.50 (1.21)	0.008	185	24.61 (0.96)	0.01
Black, African-American	271	80.84 (0.98)		277	24.40 (0.79)	
Hispanic	510	80.75 (1.05)		525	24.38 (0.83)	
Education						
Less than high school	257	80.72(1.09)	0.41	268	24.34 (0.85)	0.29
High school	196	80.87 (1.07)		200	24.50 (0.83)	
Some college	287	80.65 (0.98)		293	24.46 (0.85)	
Bachelor's degree or higher	221	80.71 (1.13)		226	24.43 (0.86)	
Income/Wealth Index						
0-2 (lowest)	311	80.82 (1.14)	0.04	324	24.33 (0.86)	0.002
3-4	319	80.71 (0.96)		323	24.42 (0.81)	
5-6	202	80.71 (1.09)		207	24.51 (0.88)	
7-8 (highest)	126	80.57 (1.09)		130	24.56 (0.83)	
Height, *cm*						
Q1: <162	239	80.57 (1.03)	0.001	243	24.41 (0.90)	0.12
Q2: 162-165	241	80.65 (0.97)		250	24.34 (0.89)	
Q3: 165-172	240	80.85 (1.12)		246	24.45 (0.83)	
Q4: ≥ 172	241	80.84 (1.12)		248	24.50 (0.77)	
BMI, kg/m^2						
< 25.0	220	80.67 (1.13)	0.06	231	24.37 (0.84)	0.19
25.0-29.0	382	80.71 (1.07)		392	24.42 (0.82)	
30.0-34.9	234	80.72 (1.05)		237	24.45 (0.90)	
35.0-39.9	79	80.86 (0.94)		81	24.62 (0.96)	
≥ 40.0	46	80.96 (1.01)		46	24.35 (0.67)	
Homocysteine, *μmol/L*						
Q1: < 7.2	231	80.53 (1.09)	0.0009	240	24.53 (0.93)	0.17
Q2: 7.2 - 8.5	231	80.75 (1.09)		235	24.40 (0.82)	
Q3: 8.6 - 10.2	256	80.75 (0.97)		263	24.39 (0.84)	
Q4: ≥ 10.3	242	80.88 (1.10)		248	24.41 (0.79)	
History of cancer						
No	895	80.74 (1.08)	0.09	920	24.43 (0.85)	0.36
Yes	63	80.54 (0.91)		64	24.34 (0.79)	
Cigarette Use						
Never	504	80.66 (1.06)	0.13	520	24.45 (0.91)	0.31
Current	346	80.81 (1.05)		354	24.43 (0.78)	
Former	111	80.75 (1.11)		112	24.33 (0.76)	
Alcohol Use						
Never	201	80.60 (0.99)	0.08	191	24.44 (0.91)	0.52
Current	242	80.81 (1.12)		225	24.38 (0.81)	
Former	517	80.74 (1.06)		465	24.45 (0.84)	
Daily Physical Activity Level						
1 (lowest)	238	80.85 (1.08)	0.19	244	24.36 (0.83)	0.06
2	231	80.69 (1.05)		239	24.36 (0.84)	
3	250	80.66 (0.99)		256	24.53 (0.85)	
4 (highest)	241	80.72 (1.13)		247	24.47 (0.88)	

1 From mixed effects linear regression models where site was treated as a random effect.

2 Totals may be < 961 for LINE-1 and < 987 for Alu due to missing values.

3 Test for linear trend for all variables except for sex, race, self-report cancer, alcohol use, and cigarette use (ANOVA).

Table 1.3 Mean percent LINE-1 methylation of leukocyte DNA by quartiles of micronutrient intake

	LINE-1 DNA Methylation by quartiles of energy-adjusted micronutrient intake Mean (SD) %5mC				
	Q1	**Q2**	**Q3**	**Q4**	***P*[2]**
Folate					
Total Intake	80.78 (1.13)	80.57 (1.00)	80.90 (1.06)	80.74 (1.05)	0.53
From foods only[2]	80.80 (1.12)	80.68 (1.04)	80.69 (1.10)	80.79 (1.04)	0.97
From supplements only	80.74 (1.07)	80.75 (1.06)	80.84 (1.03)	80.69 (1.09)	0.95
Vitamin B12					
Total Intake	80.70 (1.04)	80.81 (0.92)	80.74 (1.15)	80.74 (1.15)	0.90
From foods only[2]	80.70 (0.93)	80.80 (0.97)	80.81 (1.01)	80.80 (1.19)	0.46
From supplements only	80.78 (1.03)	80.60 (1.08)	80.89 (1.02)	80.59 (1.25)	0.33
Vitamin B6					
Total Intake	80.70 (1.07)	80.73 (1.05)	80.79 (1.10)	80.78 (1.06)	0.38
From foods only[2]	80.75 (0.98)	80.68 (1.07)	80.75 (1.07)	80.84 (1.04)	0.41
From supplements only	80.76 (1.04)	80.64 (1.21)	80.91 (1.04)	80.63 (1.06)	0.74
Zinc					
Total Intake	80.74 (0.99)	80.65 (1.07)	80.89 (1.09)	80.71 (1.10)	0.71
From foods only[2]	80.80 (1.02)	80.70 (0.92)	80.73 (1.13)	80.77 (1.12)	0.91
From supplements only	80.75 (1.05)	80.75 (1.03)	80.75 (1.11)	80.71 (1.17)	0.79
Methionine					
Total Intake[3]	80.72 (1.09)	80.75 (1.05)	80.71 (1.05)	80.81 (1.08)	0.49

1 Adjusted for total energy intake using the residual method.

2 Represents a test for linear trend from univariate linear regression models where an ordinal variable for quartiles of the micronutrient was entered into the model as a continuous variable.

3 From non-supplement users; n = 551 for folate, n = 522 for vitamin B12, n = 524 for vitamin B6, n = 542 for zinc.

4 From foods only.

Table 1.4 Mean percent Alu methylation of leukocyte DNA by quartiles of micronutrient intake

	Alu DNA Methylation by quartiles of energy-adjusted micronutrient intake Mean (SD) %5mC				
	Q1	**Q2**	**Q3**	**Q4**	***P*[1]**
Folate					
Total Intake	24.48 (0.87)	24.42 (0.92)	24.42 (0.77)	24.48 (0.83)	0.98
From foods only[2]	24.41 (0.89)	24.46 (0.90)	24.53 (0.84)	24.36 (0.82)	0.81
From supplements only	24.41 (0.86)	24.58 (0.86)	24.50 (0.81)	24.32 (0.77)	0.86
Vitamin B12					
Total Intake	24.33 (0.80)	24.49 (0.89)	24.44 (0.88)	24.53 (0.81)	0.02
From foods only[2]	24.31 (0.82)	24.50 (0.81)	24.35 (0.85)	24.49 (0.85)	0.27
From supplements only	24.39 (0.85)	24.46 (0.87)	24.58 (0.79)	24.47 (0.85)	0.09
Vitamin B6					
Total Intake	24.50 (0.88)	24.36 (0.87)	24.46 (0.83)	24.47 (0.81)	0.91
From foods only[2]	24.54 (0.97)	24.31 (0.84)	24.43 (0.80)	24.43 (0.81)	0.56
From supplements only	24.40 (0.85)	24.50 (0.92)	24.53 (0.78)	24.41 (0.80)	0.38
Zinc					
Total Intake	24.37 (0.84)	24.44 (0.90)	24.44 (0.81)	24.54 (0.85)	0.06
From foods only[2]	24.40 (0.88)	24.37 (0.85)	24.42 (0.85)	24.44 (0.84)	0.61
From supplements only	24.39 (0.85)	24.43 (0.81)	24.61 (0.82)	24.50 (0.88)	0.01
Methionine					
Total Intake[3]	24.44 (0.80)	24.42 (0.87)	24.50 (0.84)	24.43 (0.89)	0.88

1 Test for linear trend from univariate linear regression models where an ordinal variable for quartiles of the micronutrient was entered into the model as a continuous variable.
2 From non-supplement users; n = 566 for folate, n = 538 for vitamin B12, n = 538 for vitamin B6, n = 557 for zinc.
3 From foods only.

Table 1.5 Nutritional correlates of LINE-1 DNA methylation

	Mean %5mC difference (95% CI)	
	Unadjusted	**Adjusted**[1]
Height, per 10 cm	0.13 (0.06, 0.20)	0.02 (-0.08, 0.12)
BMI, per 5 units	0.05 (0.00, 0.11)	0.06 (0.00, 0.12)
Homocysteine, per 3 μmol/L	0.13 (0.07, 0.20)	0.06 (-0.01, 0.13)

1 Multivariable model included sex, age, race, height, BMI, total plasma homocysteine, alcohol use, self-report cancer, and income/wealth index.

Table 1.6 Nutritional correlates of Alu DNA methylation

	Mean %5mC Difference (95% CI)	
	Unadjusted	**Adjusted**[1]
Height, per 10 cm	0.04 (-0.02, 0.10)	0.10 (0.02, 0.19)
Homocysteine, per 3 μmol/L	-0.04 (-0.09, 0.01)	-0.04 (-0.09, 0.02)
Total vitamin B12 intake, μg[3]		
Q1: < 2.40	Reference	Reference
Q2: 2.40 - 4.02	0.16 (0.00, 0.33)	0.14 (-0.02, 0.31)
Q3: 4.03 - 12.77	0.12 (-0.04, 0.28)	0.04 (-0.14, 0.22)
Q4: ≥ 12.78	0.21 (0.05, 0.36)	0.10 (-0.10, 0.30)
P[2]	0.02	0.44
Total zinc intake, mg[3]		
Q1: < 6.89	Reference	Reference
Q2: 6.89 - 8.67	0.07 (-0.10, 0.23)	0.03 (-0.14, 0.20)
Q3: 8.68 - 19.30	0.07 (-0.09, 0.23)	0.04 (-0.14, 0.21)
Q4: ≥ 19.30	0.16 (0.00, 0.32)	0.09 (-0.12, 0.29)
P[2]	0.06	0.42

1 Multivariable model includes sex, age, height, total plasma homocysteine, total vitamin B12 intake, total zinc intake, race, physical activity level, and income/wealth index. Model for vitamin B12 and zinc intake does not include homocysteine

2 Test for trend from a linear regression model where the outcome was Alu methylation and an ordinal indicator for the variable was entered as a continuous variable.

3 Total micronutrient intake (foods + supplements); adjusted for total energy intake using the residuals method.

Chapter 3

Micronutrient status and global DNA methylation in school-age children

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Introduction

DNA methylation is a modifiable epigenetic mechanism that alters gene expression without changing the nucleotide sequence. Aberrations in global DNA methylation patterns, as measured by methylation of long interspersed nucleotide element (LINE)-1 in peripheral white blood cells (WBC) (1, 2), have been related to risk of non-communicable diseases including cancer (3, 4) and cardiovascular disease (5, 6); however, the mechanisms remain unclear.

Methylation of LINE-1 repetitive elements is responsive to external cues including diet (7), prenatal exposures (8), and environmental agents (9). Nutrition plays an important role in DNA methylation, as many dietary micronutrients are directly involved in DNA methylation pathways. One-carbon metabolism, an essential metabolic process that ultimately provides the methyl group for DNA methylation reactions, requires adequate intake of methyl-donor nutrients such as folate, and methylation cofactors including vitamin B12 and zinc. Although animal studies provide unequivocal evidence of the positive association between methyl-donor nutrient status and DNA methylation (10, 11), the evidence in humans is inconsistent and limited to adult

populations. Some controlled-feeding trials showed changes in global DNA methylation in response to folate depletion (12, 13) and repletion (12), while other studies reported no difference in methylation after folate restriction or supplementation (14, 15). A recent prospective study of maternal-infant dyads found no relations of periconceptional or 2nd trimester methyl-donor nutrient intake with cord blood LINE-1 methylation (16). On the other hand, intake of folate-fortified foods was positively associated with LINE-1 methylation in 165 cancer-free adults 18-78 years of age (17). In another study of healthy adults, adherence to a prudent dietary pattern was related to lower prevalence of LINE-1 hypomethylation (7). Furthermore, although the investigators found no difference in DNA methylation by methyl-donor nutrient intake, there was a positive correlation between consumption of dark green leafy vegetables and LINE-1 methylation (7). This suggests that multiple micronutrients present in those vegetables, including folate and vitamins A, C, and K, could be involved in DNA methylation. For example, *in vitro* treatment of human embryonic stem cells with retinoic acid (RA), a bioactive metabolite of vitamin A, influenced both global and gene-specific DNA methylation (18); yet, these associations have not been examined in epidemiologic studies.

To date, there have not been any studies evaluating micronutrient status and global DNA methylation in pediatric populations. In spite of current evidence that altered LINE-1 methylation is related to cardiometabolic risk factors that begin in early life such as atherosclerosis (19) and obesity (17), few factors are known to predict DNA methylation in children. DNA methylation is fundamentally stable yet responsive to environmental exposures in the short term (9), thus identifying early correlates of global

DNA methylation would provide insight on disease etiology and inform preventive intervention efforts.

In this study, we examined associations of micronutrient status biomarkers including erythrocyte folate; plasma vitamin B12, vitamin A, and ferritin, an indicator of iron status; and serum zinc concentrations with WBC LINE-1 methylation in 568 children randomly selected from the Bogotá School Children Cohort (BSCC), an ongoing longitudinal study of children from low- to-middle income families in Bogotá, Colombia.

Methods

This study was conducted in the context of the Bogotá School Children Cohort (BSCC), a longitudinal investigation of nutrition and health among children from public schools in Bogotá, Colombia, ongoing since 2006. Details of the study design have been previously reported (20). Briefly, we recruited a representative sample of 3,202 school children aged 5-12 years in February of 2006 from public schools in Bogotá, with use of a cluster sampling strategy. The sample represents families from low- and middle-income socioeconomic backgrounds in the city, as the public school system enrolls the majority of children from these groups (21).

At the time of enrollment, comprehensive self-administered questionnaires were sent to parents and returned by 82% of households. The questionnaires inquired about sociodemographic characteristics (including age, marital status, education level and socioeconomic level) as well as anthropometric measures of the mother (self-reported height and weight) and information about physical activity and sedentary habits of the child. In the proceeding weeks, trained research assistants visited the schools to obtain anthropometric measurements and a fasting blood sample from the children. Height was measured without shoes to the nearest 1 mm using a wall-mounted portable Seca 202 stadiometer, and weight was measured in light clothing to the nearest 0.1 kg on Tanita HS301 solar-powered electronic scales according to standard protocols (22). The parents or primary caregivers of all children gave written informed consent prior to enrollment into the study. The study protocol was approved by the Ethics Committee of the National University of Colombia Medical School; the Institutional Review Board at the University of Michigan approved the use of data and samples from the study.

Laboratory Methods

At the baseline assessment, phlebotomists obtained a blood sample from the children's antecubital vein after an overnight fast. Samples were collected in EDTA tubes and transported the same day on ice and protected from sunlight to the National Institute of Health in Bogotá. A complete blood count was carried out and plasma was separated into an aliquot for vitamin B12, C-reactive protein (CRP), and retinol determinations. Vitamin B12 concentrations were measured using a competitive chemiluminescent immunoassay in an ADVIA Centaur analyzer (Bayer Diagnostics). CRP was measured with the use of a turbidimetric immunoassay on an ACS180 analyzer (Bayer Diagnostics). Retinol was measured using high-performance liquid chromatography on a Waters 600 System. Another aliquot was collected on a metal-free polypropylene BD tube without anticoagulant for determination of zinc concentrations according to the atomic absorption technique described by Makino and Takahara (23) on a Shimadzu AA6300 spectrophotometer. Erythrocyte folate was measured on red blood cell lysates with the use of chemiluminescent immunoassay after the packed red cell volume was hemolyzed by dilution in a hypotonic aqueous solution of 1% ascorbic acid. All samples were measured in duplicate. DNA was isolated from the buffy coat using the QIAmp DNA Blood Mini Kit (Qiagen, catalogue #: 51104, 51106) and cryopreserved until transportation to the University of Michigan for analyses.

LINE-1 DNA Methylation Determinations

Pyrosequencing-based DNA methylation analysis was carried out according to previously described methods (24). Approximately 500 ng of DNA was bisulfite

converted using the EpiTect Bisulfite Kit (Qiagen, catalogue #: 59110, 59104). Bisulfite conversion of DNA deaminates unmethylated cytosine to uracil, which is read as a thymidine during polymerase chain reaction (PCR). Methylated cytosines (5-methylcytosine) are protected from bisulfite conversion and thus remain unchanged, resulting in genome-wide methylation-dependent differences in DNA sequence. Global DNA methylation was assessed through simultaneous PCR of the DNA LINE-1 elements, using primers designed towards consensus LINE-1 sequences that allow for the amplification of a representative pool of repetitive elements. PyroQ-CpG software (Qiagen) was used to estimate the degree of methylation as the percentage of 5-methylcytosine (%5mC) computed over the sum of methylated and unmethylated cytosines of four LINE-1 CpG sites. All assays, starting with the bisulfite conversion, were run in duplicate. %5mC site measurements that were more than 5 standard deviations above or below the raw mean LINE-1 methylation (< 69 or > 91 %5mC) were excluded from the analyses.

Data Analyses

Specimens were collected in 2816 (88%) of cohort participants. We selected a random sample of 600 children for LINE-1 methylation determinations. Of them, 568 children had adequate DNA concentrations and constituted the final study population. These children did not differ from the rest of the BSCC in terms of nutritional status or sociodemographic characteristics.

We first evaluated whether LINE-1 methylation means, variances, and correlations differed significantly by site and run. Within-site correlations of duplicate

runs were high, thus the average %5mC for each site was obtained across duplicate runs. We then used mixed effects linear regression models to estimate overall LINE-1 DNA methylation assuming that each site's estimate represented an independent underlying distribution. In these models, individual intercepts for site were random effects. The final LINE-1 methylation variable was calculated by adding these random effects (effectively, the between-subject variation in LINE-1 methylation) to the average %5mC across the four sites. This method enables us to incorporate the between-person variability of the underlying means for each LINE-1 site.

Next, we examined the distribution of LINE-1 methylation across categories of potential confounding characteristics for all children and separately by sex. Predictors included sociodemographic and maternal characteristics, child's anthropometric status, and CRP concentrations, a biomarker of inflammation. Maternal body mass index (BMI) was calculated from measured height and weight in 26% of the mothers and from self-reported data otherwise. Maternal weight status was classified according to BMI categories as underweight (<18.5), adequate (18.5-24.9), overweight (25.0-29.9), or obese (≥30) (25). Household socioeconomic stratum corresponded to the local government's classification assigned to each household for planning and tax purposes. Children's BMI-for-age and height-for-age Z-scores were calculated with use of the sex-specific growth references for children 5-19 years from the World Health Organization (26). CRP was dichotomized at the median value (<1.0 mg/L and ≥1.0 mg/L). The statistical significance of these associations was tested with use of univariate linear regression models in which LINE-1 methylation was the outcome, while predictors included indicator variables for each characteristic. For ordinal predictors, we obtained a

test of trend. Robust estimates of variance were included in all models to overcome potential deviations from the multivariate normal.

Next, we examined the associations of micronutrient status biomarkers and LINE-1 methylation for all children and separately by sex. The micronutrient biomarkers were categorized into quartiles, with the exception of vitamin A (categorized as < 0.700 μmol/L, 0.700 – 1.049 μmol/L, or ≥ 1.050 μmol/L) (27). We estimated differences and 95% confidence intervals (95% CI) in %5mC by categories of each micronutrient biomarker using linear regression models.

Finally, we conducted multivariable linear regression with the micronutrient biomarkers and predictors that were significantly related to LINE-1 methylation in the univariate analysis at $P < 0.10$. Due to potential threshold effects of maternal BMI and household socioeconomic stratum observed in the univariate analysis, we also considered dichotomous indicators of these variables (maternal BMI < 18.5 kg/m^2 vs ≥ 18.5 kg/m^2, and household socioeconomic strata 1-3 vs 4). Variables that remained significantly associated with the outcome at $P < 0.05$ were retained in the final model. A test for linear trend was obtained for ordinal characteristics by introducing into the model a continuous variable representing the ordinal categories of the predictor. To determine whether the associations varied by sex, we tested for interactions with use of the likelihood ratio test. We found no evidence that associations with LINE-1 methylation differed by sex; thus, the final model is presented for both boys and girls.

All analyses were carried out with the use of the Statistical Analyses System software (version 9.2; SAS Institute Inc).

Results

Mean ± SD age of children was 8.8 ± 1.7 years; 46.3% were boys. Overall mean ± SD LINE-1 DNA methylation was 80.25 ± 0.65 %5mC. We assessed LINE-1 methylation at four genomic sites in duplicate. The duplicate runs within site were highly correlated, with Spearman's ρ of 0.71, 0.74, 0.66, and 0.64 for sites 1 through 4, respectively (**Table 2.1**). Average %5mC of duplicate runs within site were 81.74 ± 2.72, 81.70 ± 2.99, 80.10 ± 2.99, and 77.43 ± 2.90 for sites 1 through 4, respectively.

In bivariate analyses (**Table 2.2**), boys had a 0.22 %5mC higher DNA methylation than girls on average ($P < 0.0001$). There was an inverse association between age and LINE-1 methylation in boys; however, it was only marginally significant ($P = 0.08$). Higher plasma CRP was related to lower LINE-1 methylation ($P = 0.01$), although the association was stronger in girls than boys. Maternal education was positively associated with LINE-1 methylation in boys only (P trend = 0.06). Although no monotonic trend was observed between maternal BMI and LINE-1 methylation (Table 2), children in the lowest category of maternal BMI had notably lower DNA methylation than those in the other three categories ($P = 0.01$). Similarly, we did not observe a significant linear trend between household socioeconomic stratum and LINE-1 methylation, yet there appeared to be a threshold effect; children in the highest stratum had higher LINE-1 methylation than those in the lower three strata ($P = 0.0002$).

We next examined the associations of micronutrient biomarkers with DNA methylation (**Table 2.3**). Retinol concentrations were inversely related to LINE-1 methylation (P trend = 0.002), especially among girls (P trend = 0.006). DNA

methylation was not related to erythrocyte folate, serum zinc, plasma vitamin B12, or ferritin.

Finally, we examined the independent associations of these factors with LINE-1 methylation with the use of a multivariable linear regression model. The variables retained in the model as predictors included sex, plasma vitamin A, CRP, maternal BMI, and household socioeconomic stratum (**Table 2.4**). In the multivariable analysis, LINE-1 methylation was 0.21 %5mC lower in girls than boys ($P = 0.0007$). Plasma vitamin A and CRP were each inversely related to LINE-1 methylation, while maternal BMI and household socioeconomic stratum were both positively associated with LINE-1 methylation. Children with ≥ 1.05 µmol/L plasma vitamin A had 0.19 %5mC lower LINE-1 methylation than those with < 0.70 µmol/L plasma vitamin A ($P = 0.03$). Likewise, children with plasma CRP ≥ 1 mg/L had a 0.12 %5mC lower LINE-1 methylation than those with CRP < 1 mg/L ($P = 0.04$). Children of mothers with BMI ≥ 18.5 had an average 0.31 %5mC higher LINE-1 methylation than those of mothers with BMI < 18.5 ($P = 0.04$). Similarly, those in the highest stratum of household socioeconomic status have a mean LINE-1 methylation 0.29 %5mC higher than those in the lower three strata ($P = 0.01$). These associations did not differ significantly by sex.

Discussion

We examined associations of micronutrient status biomarkers with WBC LINE-1 DNA methylation in 568 children randomly selected from the *BSCC*, a representative cohort of low- to middle-income school-age children from Bogotá, Colombia. In addition, we ascertained associations of LINE-1 methylation with child and maternal sociodemographic and anthropometric characteristics. As previously reported in adults (28), boys had higher global DNA methylation than girls. We also found that higher plasma levels of vitamin A and CRP were each related to lower LINE-1 methylation, while higher maternal BMI and household socioeconomic status were each related to higher DNA methylation. Although the differences in LINE-1 methylation were small, they represent changes at a global level that likely reflect larger differences in the context of the entire genome.

The inverse association we observed between plasma vitamin A and LINE-1 methylation could be related to retinoid-mediated changes in the expression or activity of DNA methyltransferase (DNMT), the endogenous enzyme that catalyzes the methylation reaction. Treatment of breast cancer cells with *all trans* retinoic acid (*at*RA), the most biologically active metabolite of vitamin A, and with a synthetic retinoid X receptor-selective retinoid (*9cUAB30*) down-regulated DNMT gene expression and telomerase activity when administered individually and in combination (29). The RA treatments also suppressed expression of *hTERT*, the catalytic component of telomerase that is paradoxically hypermethylated and highly expressed in cancer cells (30). Because inhibition of DNA methylation in cancer cells down-regulated expression of *hTERT*, the authors postulated that the retinoid-induced reduction of DNMT gene expression and

subsequent decrease in *hTERT* promoter methylation is a likely mechanism for decreased telomerase activity in human breast cancer cells. In another study, the ability of *at*RA to incite cellular senescence in a broad range of human cell lines was strongly correlated with its ability to activate tumor suppressor genes p17 and p21 through promoter hypomethylation (31). Although the DNMT-inhibitory effects of RA treatment have only been examined in the context of chemoprevention and cancer therapies, it is plausible that they can influence global DNA methylation as well. Further research is warranted to investigate the effects of changes in vitamin A status on global DNA methylation, and also to evaluate whether lower global DNA methylation is related to poor health outcomes in school-age children.

We also found that higher CRP was related to lower LINE-1 methylation. Low grade inflammation, characterized by elevated circulating CRP, is an established risk factor of CVD in adults (32), and global DNA methylation is increasingly recognized as a key mechanism involved in the pathogenesis of inflammation-mediated cardiovascular risk factors such as atherosclerosis (33). While a few studies in adults have examined the relation between inflammation and global DNA methylation, the findings have not been cohesive. Elevated circulating vascular cell adhesion molecule 1 (VCAM-1), an endothelial marker found in atherosclerotic lesions, was related to LINE-1 hypomethylation in a population-based study of community-dwelling elderly men, while no associations were observed with CRP (34). Similarly, a recent study of 165 cancer-free adults found no association between LINE-1 methylation and inflammation biomarkers including CRP (17). However, high sensitivity CRP (*hs*CRP) was related to global DNA hypermethylation in chronic kidney disease patients (35). Although there is

need to better understand the nature of this association in more diverse populations, our finding that higher CRP was related to lower LINE-1 methylation in school-age children has important implications for identifying the relation of DNA methylation with other early CVD risk factors.

We observed a positive relation of maternal BMI with LINE-1 methylation. Specifically, children of underweight mothers (BMI < 18.5) had significantly lower global DNA methylation than those whose mothers were not underweight. This is a salient finding, assuming that maternal BMI is consistent with pre-pregnancy BMI in this population. A low pre-pregnancy BMI is related to low birth weight (36), which has been associated with decreased cord blood DNA methylation (37). The periconceptional period represents a critical window in ontogenic development, and is characterized by responsiveness of DNA methylation patterns to nutritional and environmental exposures (38). Studies using data from the Dutch famine cohort reported that periconceptional exposure to famine was related to an unfavorable cardiometabolic risk profile in adulthood (39), and persistent changes in methylation of genes involved in growth and metabolism (40, 41). While such findings suggest that aberrant DNA methylation could be a mechanistic link between maternal malnutrition and an adverse metabolic phenotype, it is not possible to parse out specific exposures due to the retrospective nature of the data. Currently, the literature regarding the relation of maternal BMI with child global DNA methylation is limited. However, two studies conducted in maternal-child dyads have included data on pre-pregnancy BMI and cord blood LINE-1 methylation (16, 42). Although the associations were not statistically significant, higher pre-pregnancy BMI was related to higher cord blood DNA methylation in both studies.

The implications of the association observed in our study are contingent upon longitudinal studies to verify the correlation between cord blood and childhood DNA methylation. Furthermore, whether associations of maternal BMI with child DNA methylation represent epigenetic "programming" related to later-life health outcomes requires further investigation.

Finally, we found a positive association between household socioeconomic stratum and LINE-1 methylation. Lower socioeconomic status is related to adverse prenatal exposures such as maternal cigarette smoking (43), as well as unhealthy lifestyle characteristics during childhood including decreased physical activity levels (44) and a tendency to consume a diet high in fats and sugars (45). The trend we observed was in accordance with expectations, as each of the above factors has been related to lower global DNA methylation in adults (7, 8, 46).

Of note, we did not find significant associations between LINE-1 methylation and erythrocyte folate. A potential explanation for the lack of association could be that the BSCC is a folate-replete population, with less than 1% prevalence of folate deficiency (47). Associations between folate status and LINE-1 methylation might be observable in populations with erythrocyte folate levels lower than those of our study population. It is also possible that effects of methyl-donor nutrients on DNA methylation occur during intrauterine life; however, a perinatal study did not find any associations between maternal intake of methyl-donor nutrients, including folate, periconceptionally or during the 2nd trimester with cord blood LINE-1 methylation (16).

Our study has several strengths. Many studies of diet and LINE-1 methylation had a small sample size and were underpowered to detect small differences in LINE-1 methylation. We were able to examine global DNA methylation in a large and representative sample of children from a setting where the increasing prevalence of cardiovascular risk factors, such as child overweight, is becoming a serious problem. We determined LINE-1 methylation using pyrosequencing technology, a highly reproducible and accurate method to quantify DNA methylation. Furthermore, we used DNA from peripheral WBC, which is of high intrinsic value in epidemiologic studies, as it is easily obtained and reflects systemic interindividual variation in germ-layer cells (48). We also used valid biochemical indicators of micronutrient status, which is the most accurate method of ascertaining micronutrient intake. In addition, all assays were run in duplicate to minimize variability and enhance accuracy. Limitations of the study include its cross-sectional design, which restricts the possibility of making causal inference on the predictors of global DNA methylation, and its generalizability to other ethnicities, as there is some evidence that Hispanics may have lower LINE-1 methylation than non-Hispanic whites (28).

In summary, global DNA methylation in school-age children was inversely related to female sex, plasma retinol, and CRP concentrations, and positively associated with maternal BMI and household socioeconomic stratum. The value of LINE-1 DNA methylation as a biomarker of health outcomes in children requires further examination in prospective studies.

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Table 2.1 LINE-1 DNA methylation at four genomic LINE-1 sites in 568 school-age children from Bogotá, Colombia

%5MeC	Site 1		Site 2		Site 3		Site 4	
	Run 1 N=555	**Run 2** N=506	**Run 1** N=561	**Run 2** N=505	**Run 1** N=559	**Run 2** N=499	**Run 1** N=559	**Run 2** N=504
Mean ± SD	80.73 ± 3.08	82.53 ± 2.67	81.04 ± 3.46	82.95 ± 2.09	79.23 ± 3.34	81.22 ± 2.50	76.72 ± 3.02	77.70 ± 2.57
Median (Range)	80.63 (70.45-90.49)	82.48 (75.08-90.45)	82.09 (69.44-86.29)	83.11 (70.03-90.11)	79.94 (69.17-87.04)	81.43 (69.17-90.24)	76.47 (69.21-90.69)	77.32 (70.13-90.67)
Spearman's ρ	0.71		0.74		0.66		0.64	
ICC[1]	0.48		0.56		0.52		0.55	

1 Intraclass correlation coefficient.

Table 2.2 LINE-1 DNA Methylation according to background characteristics of 568 school-age children from Bogotá, Colombia

	LINE-1 DNA Methylation (%5mC)[2]					
	N[1]	All	N[1]	Males	N[1]	Females
Overall	568	80.25 (0.65)				
Child's Sex						
F	305	80.15 (0.65)				
M	263	80.37 (0.64)				
P[3]		< 0.0001				
Age, years						
5-6	96	80.19 (0.64)	43	80.42 (0.62)	53	80.01 (0.60)
7-8	183	80.29 (0.58)	85	80.43 (0.55)	98	80.17 (0.58)
9-10	238	80.27 (0.71)	108	80.36 (0.71)	130	80.19 (0.70)
11-12	50	80.15 (0.65)	27	80.13 (0.63)	23	80.18 (0.69)
P trend[4]		0.93		0.08		0.17
Child was born in Bogotá						
Yes	467	80.26 (0.65)	212	80.39 (0.63)	255	80.15 (0.65)
No	51	80.27 (0.66)	23	80.34 (0.66)	28	80.22 (0.66)
P[3]		0.89		0.70		0.59
Birth weight, *g*						
<2500	44	80.17 (0.77)	20	80.20 (0.61)	24	80.14 (0.90)
2500-2999	110	80.28 (0.67)	41	80.49 (0.73)	69	80.16 (0.60)
3000-3499	128	80.26 (0.66)	57	80.41 (0.57)	71	80.14 (0.70)
>3500	147	80.22 (0.62)	73	80.30 (0.64)	74	80.13 (0.59)
P trend[4]		0.90		0.72		0.87
Height-for-age Z score[5]						
Less than -2.0	55	80.27 (0.69)	22	80.27 (0.64)	33	80.27 (0.73)
-2.0 to < -1.0	176	80.29 (0.62)	90	80.40 (0.57)	86	80.17 (0.65)
-1.0 to < 1.0	299	80.22 (0.69)	133	80.37 (0.70)	166	80.11 (0.65)
≥1.0	20	80.24 (0.33)	6	80.34 (0.31)	14	80.20 (0.34)
P trend[4]		0.41		0.75		0.25
BMI-for-age Z-score[5]						
Less than -2.0	10	80.41 (0.51)	5	80.39 (0.63)	5	80.43 (0.43)
-2.0 to < -1.0	63	80.34 (0.68)	30	80.36 (0.64)	33	80.32 (0.72)
-1.0 to < 1.0	371	80.22 (0.64)	160	80.36 (0.64)	211	80.11 (0.62)
1.0 to < 2.0	92	80.31 (0.73)	46	80.42 (0.70)	46	80.19 (0.75)
≥ 2.0	14	80.28 (0.55)	10	80.31 (0.57)	4	80.23 (0.58)
P trend[4]		0.79		0.90		0.42
CRP, *mg/L*						
< 1.0	279	80.32 (0.67)	143	80.42 (0.62)	136	80.21 (0.71)
≥ 1.0	285	80.18 (0.63)	119	80.32 (0.67)	166	80.08 (0.58)
P[3]		0.01		0.22		0.07
Maternal Education						
Incomplete Primary	37	80.28 (0.62)	18	80.38 (0.52)	19	80.19 (0.70)
Complete Primary	106	80.24 (0.67)	46	80.30 (0.66)	60	80.19 (0.68)
Incomplete Secondary	128	80.20 (0.65)	60	80.30 (0.63)	68	80.11 (0.65)
Complete Secondary	209	80.29 (0.65)	94	80.44 (0.65)	115	80.16 (0.63)
University	33	80.39 (0.70)	15	80.71 (0.56)	18	80.13 (0.72)
P trend[4]		0.34		0.06		0.78

Table 2.2 (Continued) LINE-1 DNA Methylation according to background characteristics of 568 school-age children from Bogotá, Colombia

	LINE-1 DNA Methylation (%5mC)[2]					
	N[1]	All	N[1]	Males	N[1]	Females
Maternal Height, *cm*						
<154	109	80.30 (0.63)	51	80.37 (0.65)	58	80.24 (0.62)
154-157	133	80.21 (0.68)	60	80.36 (0.67)	73	80.09 (0.67)
158-161	110	80.29 (0.70)	49	80.47 (0.65)	61	80.15 (0.72)
≥162	134	80.25 (0.60)	59	80.38 (0.64)	75	80.14 (0.55)
P trend[4]		0.77		0.75		0.54
Maternal BMI, *kg/m²*						
<18.5	17	79.88 (0.66)	5	80.11 (0.82)	12	79.78 (0.59)
18.5-24.9	289	80.27 (0.63)	124	80.39 (0.67)	165	80.19 (0.59)
25.0-29.9	136	80.27 (0.67)	70	80.43 (0.64)	66	80.10 (0.65)
≥30	32	80.29 (0.71)	15	80.37 (0.53)	17	80.22 (0.84)
P trend[4]		0.33		0.61		0.63
Household Socioeconomic Stratum [6]						
1 (lowest)	44	80.35 (0.48)	23	80.40 (0.55)	21	80.29 (0.38)
2	174	80.20 (0.67)	87	80.35 (0.68)	87	80.06 (0.63)
3	305	80.21 (0.64)	123	80.32 (0.64)	182	80.13 (0.64)
4 (highest)	45	80.62 (0.71)	30	80.62 (0.61)	15	80.62 (0.89)
P trend[4]		0.15		0.30		0.27

1 Totals may be <568 for all children, < 263 for males, and < 305 for females because of missing values.

2 From mixed effects linear regression models where site was a random effect.

3 From analysis of variance (ANOVA).

4 From univariate regression models in which a variable representing the ordinal predictor was introduced as continuous.

5 According to the World Health Organization 2007 Child-Growth Reference (26).

6 According to the local government classification.

Table 2.3 LINE-1 DNA Methylation according to micronutrient status in 568 school-age children from Bogotá, Colombia

	LINE-1 DNA Methylation (%5mC)[2]								
		All			Males			Females	
	N[1]	Mean (SD)	%5mC difference (95% CI)	N[1]	Mean (SD)	%5mC difference (95% CI)	N[1]	Mean (SD)	%5mC difference (95% CI)
Erythrocyte Folate, *nmol/L*									
Q1	139	80.24 (0.61)	Reference	64	80.32 (0.60)	Reference	75	80.13 (0.59)	Reference
Q2	139	80.21 (0.64)	-0.03 (-0.18, 0.11)	64	80.31 (0.65)	-0.01 (-0.23, 0.20)	74	80.17 (0.66)	0.04 (-0.16, 0.24)
Q3	139	80.25 (0.67)	0.01 (-0.14, 0.16)	65	80.41 (0.62)	0.09 (-0.11, 0.30)	75	80.11 (0.69)	-0.02 (-0.23, 0.18)
Q4	139	80.28 (0.67)	0.04 (-0.11, 0.19)	64	80.46 (0.68)	0.14 (-0.09, 0.36)	75	80.14 (0.63)	0.01 (-0.19, 0.20)
P trend[3]		0.51			0.15			0.90	
Plasma Vitamin B12, *pmol/L*									
Q1	137	80.30 (0.64)	Reference	64	80.40 (0.69)	Reference	72	80.19 (0.58)	Reference
Q2	136	80.26 (0.61)	-0.04 (-0.19, 0.11)	63	80.41 (0.63)	0.01 (-0.22, 0.24)	72	80.13 (0.53)	-0.06 (-0.24, 0.12)
Q3	134	80.24 (0.66)	-0.06 (-0.22, 0.09)	64	80.38 (0.63)	-0.02 (-0.25, 0.21)	72	80.12 (0.69)	-0.07 (-0.28, 0.14)
Q4	136	80.18 (0.72)	-0.12 (-0.28, 0.04)	64	80.26 (0.64)	-0.15 (-0.38, 0.08)	72	80.11 (0.78)	-0.07 (-0.30, 0.15)
P trend[3]		0.15			0.20			0.52	
Serum Zinc, *μmol/L*									
Q1	140	80.22 (0.66)	Reference	66	80.33 (0.65)	Reference	75	80.13 (0.64)	Reference
Q2	142	80.23 (0.64)	0.01 (-0.14, 0.16)	65	80.36 (0.65)	0.03 (-0.19, 0.25)	76	80.12 (0.63)	-0.01 (-0.22, 0.19)
Q3	141	80.30 (0.64)	0.07 (-0.08, 0.23)	65	80.43 (0.62)	0.10 (-0.11, 0.32)	75	80.16 (0.62)	0.03 (-0.17, 0.23)
Q4	141	80.24 (0.68)	0.02 (-0.14, 0.18)	66	80.36 (0.67)	0.04 (-0.19, 0.26)	76	80.15 (0.69)	0.01 (-0.20, 0.22)
P trend[3]		0.60			0.61			0.82	
Plasma ferritin, *μg/L*									
Q1	141	80.34 (0.66)	Reference	66	80.49 (0.65)	Reference	75	80.17 (0.62)	Reference
Q2	139	80.18 (0.63)	-0.16 (-0.31, -0.01)	66	80.35 (0.63)	-0.14 (-0.35, 0.08)	76	80.10 (0.65)	-0.07 (-0.27, 0.14)
Q3	143	80.26 (0.64)	-0.08 (-0.24, 0.07)	64	80.31 (0.61)	-0.17 (-0.39, 0.04)	75	80.15 (0.63)	-0.01 (-0.21, 0.19)
Q4	141	80.21 (0.68)	-0.13 (-0.28, 0.03)	66	80.33 (0.69)	-0.16 (-0.38, 0.07)	76	80.14 (0.69)	-0.02 (-0.23, 0.19)
P trend[4]		0.22			0.16			0.96	
Plasma vitamin A, *μmol/L*									
< 0.700	72	80.37 (0.63)	Reference	34	80.40 (0.59)	Reference	38	80.35 (0.67)	Reference
0.700 - 1.049	235	80.32 (0.63)	-0.06 (-0.22, 0.11)	111	80.45 (0.67)	0.05 (-0.18, 0.29)	124	80.20 (0.57)	-0.16 (-0.39, 0.07)
≥ 1.050	260	80.16 (0.67)	-0.22 (-0.38, -0.05)	118	80.29 (0.63)	-0.10 (-0.33, 0.12)	142	80.04 (0.69)	-0.31 (-0.55, -0.07)
P trend[3]		0.002			0.13			0.006	

1 Totals may be <568 for all children, < 263 for males, and < 305 for females because of missing values.
2 From mixed effects linear regression models where site was a random effect.
3 From univariate regression models in which a variable representing the ordinal predictor was introduced as continuous.

Table 2.4 Correlates of LINE-1 DNA methylation in 568 school-age children from Bogotá, Colombia

	Adjusted %5mC difference[1] **β (95% CI)**
Sex	
Male	Reference
Female	-0.21 (-0.32, -0.09)
P	0.0007
Plasma vitamin A, *μmol/L*	
< 0.700	Reference
0.700 - 1.049	-0.07 (-0.24, 0.10)
≥ 1.050	-0.19 (-0.36, -0.02)
P trend[2]	0.006
C-reactive Protein, *mg/L*	
< 1.0	Reference
≥ 1.0	-0.12 (-0.24, -0.01)
P	0.04
Maternal BMI, kg/m^2	
< 18.5	Reference
≥ 18.5	0.31 (0.01, 0.60)
P	0.04
Household Socioeconomic Stratum[3]	
1-3 (lower)	Reference
4 (highest)	0.29 (0.07, 0.51)
P	0.01

1 From a linear regression model with LINE-1 methylation as the outcome and predictors that included sex, vitamin A, CRP, maternal BMI, and household socioeconomic stratum.

2 Test for linear trend from a linear regression model where an ordinal indicator for the variable was entered as a continuous predictor.

3 According to the local government classification.

Chapter 4

A prospective study of global DNA methylation and development of adiposity in school-age children

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Introduction

Childhood obesity has reached epidemic proportions worldwide. Of special concern are countries undergoing the nutrition transition, including Latin America, that have experienced a marked increase in pediatric obesity rates in the last two decades (1). Although researchers have identified dietary and lifestyle factors accountable for the rapid increase in childhood obesity, the biological mechanisms remain unclear. Epigenetic animal models indicate that maternal intake of methyl-donor micronutrients is protective against offspring obesity through increased DNA methylation in genomic regions that regulate satiety and metabolism (2-4). Recent epidemiologic evidence suggests that aberrant changes in global DNA methylation quantified from peripheral white blood cells (WBC) are associated with prominent obesity-related diseases including cancer (5), diabetes (6), and cardiovascular disease (7-9). Yet, there is little research on the relation between global DNA methylation and weight status.

Current evidence on global DNA methylation and body size consists mostly of mixed findings from cross-sectional studies in adult populations (8, 10-14). Some studies of maternal-infant dyads have been conducted to investigate relations of perinatal

characteristics, including birth weight, with cord blood DNA methylation (15-17). One study reported that high and low birth weight, as well as premature birth were associated with significantly lower cord blood DNA methylation (16). However, inference from perinatal studies is limited because it is not known whether functional consequences of these associations contribute to obesity in later life. A recent study that used data from two independent birth cohorts identified nine differentially methylated genes quantified from cord blood at birth that were associated with body composition at 9 y (18). While such findings shed light on regulatory pathways involved in weight gain, gene-specific methylation does not provide a global picture of methylation changes within the entire genome. Genome-wide methylation is important to understand because it is responsive to environmental modifications (19), and because genomic stability has profound health implications beyond the specific function of a gene. Considering that early life weight status influences cardiometabolic morbidity in adulthood (20), it is critical to identify molecular mechanisms involved in the etiology of childhood obesity.

In this study, we examined the prospective relation of global DNA methylation at recruitment into a cohort, as measured by methylation of long interspersed nucleotide element (LINE)-1, with changes in adiposity and linear growth indicators in a representative group of low- and middle-income children from Bogotá, Colombia, a country in the early stages of the nutrition transition.

Methods

This study was conducted in the context of the Bogotá School Children Cohort (BSCC), a longitudinal investigation of nutrition and health among children from public schools in Bogotá, Colombia, ongoing since 2006. Details of the study design have been previously reported (21). Briefly, we recruited a representative sample of 3,202 school children aged 5-12 y in February 2006 from public schools in Bogotá, using a random sampling strategy. The sample represents families from low- and middle-income socioeconomic backgrounds in the city, as the public school system enrolls the majority of children from these groups (22).

At the time of enrollment, comprehensive self-administered questionnaires were sent to parents and returned by 82% of households. The questionnaires inquired about sociodemographic characteristics (including age, marital status, education, and socioeconomic level) as well as anthropometric measures of the mother (self-reported height and weight) and information about physical activity and sedentary habits of the child. In the proceeding weeks, trained research assistants visited the schools to obtain anthropometric measurements and a fasting blood sample from the children. Height was measured without shoes to the nearest 1 mm using a wall-mounted portable Seca 202 stadiometer, and weight was measured in light clothing to the nearest 0.1 kg on Tanita HS301 solar-powered electronic scales. The subscapular and tricipital skinfold thicknesses were measured to the nearest 0.5 mm using Slim Guide Skinfold Calipers (Creative Health Products, Inc. Plymouth, MI) according to standard protocols (23). Follow-up anthropometric measurements were obtained in June and November 2006 and once yearly thereafter by visiting the schools or homes of the children when they were

absent from school on the day of assessment. At these follow-up visits, we also measured waist circumference using a non-extensible measuring tape at the level of the umbilicus (23).

The parents or primary caregivers of all children gave written informed consent prior to enrollment into the study. The study protocol was approved by the Ethics Committee of the National University of Colombia Medical School. The Institutional Review Board at the University of Michigan approved the use of data and samples from the study.

Laboratory Methods

At the baseline assessment, phlebotomists obtained a blood sample from the children's antecubital vein after an overnight fast. Samples were collected in EDTA tubes and transported the same day on dry ice and protected from sunlight to the National Institute of Health in Bogotá. A complete blood count was carried out and plasma was separated into an aliquot for micronutrient and inflammation biomarker determinations; details are described elsewhere (24). DNA was isolated from the buffy coat using the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and cryopreserved until transportation to the University of Michigan for analyses.

LINE-1 DNA Methylation Determinations

Pyrosequencing-based DNA methylation analysis was carried out according to methods described by Tost and Gut (25). Approximately 500 ng of DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen). Bisulfite conversion of DNA deaminates unmethylated cytosine to uracil, which is read as a thymidine during polymerase chain reaction (PCR). Methylated cytosines (5-methylcytosine) are protected

from bisulfite conversion and thus remain unchanged, resulting in genome-wide methylation-dependent differences in DNA sequence. Global DNA methylation was assessed through simultaneous PCR of the DNA LINE-1 elements, using primers designed towards consensus LINE-1 sequences that allow for the amplification of a representative pool of repetitive elements. PyroQ-CpG software (Qiagen) was used to estimate the degree of methylation as the percentage of 5-methylcytosine (%5mC) computed over the sum of methylated and unmethylated cytosines of four LINE-1 CpG sites. All assays, starting with the bisulfite conversion, were run in duplicate. %5mC site measurements that were more than 5 standard deviations above or below the raw mean LINE-1 methylation (< 69 or > 91 %5mC) were excluded from the analyses.

Statistical Analysis

Specimens were collected in 2816 (88%) of cohort participants. We selected a random sample of 600 children for LINE-1 methylation determinations; adequate DNA concentrations were available for 568 children. Of them, 553 children who had valid anthropometric measurements at baseline and at least one additional follow-up measurement constituted the final study population. These children did not differ from the rest of the BSCC in terms of nutritional status or sociodemographic characteristics at the baseline assessment.

Because the distribution of %5mC at each LINE-1 site differed from individual to individual, we used a mixed effects linear regression model to create the final person-specific LINE-1 methylation variable. First, we took the average %5mC for each site across the duplicate runs since within-site correlations were high (24). Next, we fit a mixed effects model that included a random intercept for %5mC methylation at each site

that was allowed to vary from person to person. Accordingly, the empirical best linear unbiased predictors (EBLUPs) from the random effects represent the between-person variation in DNA methylation. The EBLUPS were added to the population raw average %5mC across the four LINE-1 sites. This method enabled us to incorporate the between-person variability of the underlying means for each LINE-1 site.

Next, we examined the relations of LINE-1 methylation with change in three indicators of adiposity: BMI-for-age Z-score (BMIZ) as an indicator of overall adiposity;(26) waist circumference Z-score as an indicator of central adiposity (27); and subscapular-to-triceps skinfold thickness ratio Z-score as an indicator of truncal adiposity (28) over the 2.5 years of follow-up, separately for boys and girls. We also examined associations with height-for-age Z-score. Children's BMI-for-age and height-for-age Z-scores were calculated with use of the sex-specific growth references for children 5-19 years from the World Health Organization (29). We age-standardized waist circumference and the subscapular-to-triceps skinfold thickness ratio with the LMS method (30) using data from children 5-16 y of age in the third National Health and Nutrition Examination Survey (NHANES III) (31). The LMS method summarizes the distribution of each anthropometric measurement by its median (M) and coefficient of variation (S), plus a measure of skewness based on the Box-Cox power (L) to normalize data. This approach is ideal for age-related standardization because it accounts for differential degrees of asymmetry in the distribution of an anthropometric measure within each stratum of age, rather than assuming the same skewness across age groups. We estimated mean changes in each of the adiposity indicators and height-for-age Z-score during follow-up and compared them across quartiles LINE-1 methylation with the use of

mixed effects linear regression models for repeated measures. In each model, the anthropometric measurement was the outcome and the predictors included indicator variables for quartiles of LINE-1 methylation, age in decimal years, and interaction terms between the LINE-1 methylation indicator and age. These mixed models included random effects for the intercept and slope; an unstructured variance-covariance matrix was specified to account for within-child correlations of anthropometric measurements. For the waist circumference model, random effects for the slope were not included because measurements were only obtained in the second and third years of follow-up; the change in waist circumference represented the change between the two measurements taken during follow-up. These methods do not require an even number of observations or that measurements be collected at exactly the same time in all subjects, thus all measurements available for every child were included in the analyses. Because non-linear associations seemed apparent in preliminary analyses, we also examined associations with a dichotomous indicator that represented the 1st vs. $2\text{-}4^{th}$ quartiles of LINE-1 methylation.

In the multivariable models, we considered adjustment by nutritional and sociodemographic covariates that are associated with LINE-1 methylation in this population (24), as well as known predictors of child growth. Empirical estimates of variance were used in all models to overcome deviations from the multivariate normality assumption.

All analyses were carried out with the use of the Statistical Analyses System software (version 9.3; SAS Institute Inc).

Results

Mean ± SD age of children was 8.8 ± 1.7 years; 45.9% were boys. Each child contributed a median of 4 measurements for BMI, height, and the skinfold thicknesses, and a median of 2 measurements for waist circumference over a median of 30 months of follow-up (IQR = 29, 31). Prevalence of overweight/obesity at baseline was 9.8%. Boys had significantly higher LINE-1 methylation than girls ($P < 0.0001$; **Table 3.1**). Additional correlates of LINE-1 methylation in this population have been reported before (24).

Among boys, LINE-1 methylation was not related to baseline BMI-for-age Z-score or skinfold thickness ratio Z-score (*P* trend = 0.73 and *P* trend = 0.60, respectively). However, DNA methylation was inversely related to change in these indicators during follow-up in a non-linear manner (**Table 3.2**). Boys in the lowest quartile of LINE-1 methylation experienced annual gains in BMI-for-age Z-score and skinfold thickness ratio Z-score that were 0.06 Z/y (95% CI = -0.11, 0.00; $P = 0.04$) and 0.07 Z/y (95% CI = -0.13, -0.01; $P = 0.03$), respectively, higher than those in the upper three quartiles after adjustment for baseline age and socioeconomic status. Further adjustment for maternal BMI and plasma vitamin A did not change the direction, magnitude, or significance of the associations (data not shown). In addition, LINE-1 DNA methylation was inversely associated with change in waist circumference Z-score in a linear fashion (*P* trend = 0.02). Boys in the lowest quartile of LINE-1 methylation experienced a 0.09 Z/y greater annual gain in waist circumference than those in the highest quartile ($P = 0.01$).

There were no significant associations between LINE-1 methylation and any of the adiposity indicators at baseline, or over the follow-up period among girls (**Table 3.3**). Global DNA methylation was not related to linear growth in boys or girls (**Table 3.4**).

Discussion

In this study of 553 school children from low- and middle-income families in Bogotá, Colombia, we found inverse associations of global DNA methylation at time of recruitment into the cohort with annual change in three age- and sex-standardized measures of adiposity (BMI-for-age Z-score, waist circumference-for-age Z-score, and subscapular-to-triceps skinfold thickness ratio-for-age Z-score) among boys. Because mean BMI of this population at baseline was higher than the international growth reference (29), these associations likely reflect unhealthy gains in adiposity.

These findings contribute to the understanding of biological mechanisms that underlie childhood obesity. Early life environmental stimuli could induce long-lasting changes in DNA methylation profiles that are related to obesity and cardiometabolic disease. For example, middle-aged adults who were conceived during the Dutch Winter Famine exhibited persistent changes in methylation of genes involved in cardiometabolic diseases (32, 33) and had a higher BMI and waist circumference than their unexposed same-sex siblings (34). The direct relation between DNA methylation and obesity has mostly been examined in cross-sectional studies of adults. A study conducted among middle-aged Samoan islanders reported that LINE-1 DNA methylation was positively correlated with BMI among women (8). In a cohort of Singaporean Chinese adults, hypermethylation of the satellite repetitive element (AS) was associated with higher BMI at baseline in both men and women (11). In the same study, higher AS methylation was related to incidence of cardiovascular disease over follow-up among men only (11). The authors postulated that global DNA hypermethylation could serve as a biomarker of cardiovascular disease risk and obesity; yet, the relation between AS methylation and

BMI was assessed only at baseline, making it impossible to determine whether higher DNA methylation preceded high BMI or vice versa. Other cross-sectional studies have not found an association between DNA methylation and measures of adiposity in adults (10, 12-14, 35). To date, the largest gap in this area of research is the lack of longitudinal investigations to assess the temporal relation of global DNA methylation with changes in anthropometry. We were able to examine prospective changes in three indicators of adiposity with respect to global DNA methylation quantified from blood samples collected at baseline from a large and representative sample of school-age children. Our results indicate that global DNA hypomethylation is related to adverse weight gain patterns during the school years. The fact that change in waist circumference was strongly associated with LINE-1 methylation is especially important because abdominal fat mass is an independent predictor of morbidity (36) and mortality (37) in adults. Furthermore, there is prospective evidence that accrual of central fat during childhood is related to adverse metabolic consequences in later life (38). Our findings also provide a basis for to further investigate molecular mechanisms involved in early life weight gain. Considering that adipocyte quantity is established sometime between late childhood and early adolescence (39), identifying modifiable pathways involved in adipogenesis during the school-age years would be particularly valuable to interventions efforts aimed at improving long term cardiometabolic health.

The trend between LINE-1 methylation and the adiposity indicators was apparent among boys only. It is uncertain whether this dichotomy is due to copy number variation in LINE-1 on the X and Y chromosomes, a sex hormone effect on methylation, or sex-specific weight gain patterns. We also noted that the association between LINE-1 DNA

methylation and change in anthropometry was in the opposite direction of what was observed in adults (8, 11). This discrepancy could be attributed to differences in study design, as the adult studies were cross-sectional, which restricted inference on causation. There may also be inherent age-specific differences in epigenetic associations. For example, although studies in adults have reported inverse relations between age and global DNA methylation (10, 40), DNA methylation was not associated with age in our study population (24), or in a pilot study of girls 6-17 y in the US (41). Because the epigenome reflects perinatal (42) and lifetime exposures (19, 43, 44), findings from children may not be directly comparable to adult studies. Nevertheless, longitudinal studies in both adults and children will be useful to disentangle the nature of the relation between global DNA methylation and weight gain.

There are a few pathways that may explain the associations observed in boys. The variability in methylation at the global level may reflect concomitant differences in gene-specific methylation, as there is evidence that some prenatal exposures are associated with both gene-specific and global DNA methylation (45). Two recent prospective studies found significant associations between methylation of candidate genes quantified from cord blood at birth and childhood body size (18, 46). Godfrey et al. reported that hypermethylation of the retinoid X receptor alpha (*RXRA*) promoter region was correlated with greater adiposity at age 9 in two independent birth cohorts (46). Because *RXRA* regulates transcriptional activity through heterodimerization with peroxisome proliferator-activated receptors (*PPARs*) directly involved in regulating insulin sensitivity and fat metabolism (47), hypermethylation silencing of *RXRA* could lead to weight gain. This hypothesis is consistent with the observation that *RXRA* expression in adipose tissue

of obese mice and humans is diminished (48). There is also increasing evidence from animal models that exposure to ubiquitous obesogenic chemicals, such as Bisphenol-A (BPA), can influence DNA methylation (3) and promote adipogenesis (49). An *in vitro* study that examined omental adipose tissue biopsies from children demonstrated that exposure to environmentally relevant levels of BPA increased expression of *PPAR*-γ (50), a nuclear hormone receptor that stimulates fat cell differentiation. Taken together, these findings support the notion that DNA methylation and gene expression changes could precede alterations in body composition. Whether modification of DNA methylation profiles through dietary interventions influences body composition deserves further investigation.

We did not find any associations between LINE-1 methylation and linear growth. The lack of association could be related to the age distribution of the study population. Preliminary results in a U.S. birth cohort suggested that higher cord blood LINE-1 methylation was related to greater childhood height up to 7 y among boys (51). However, the school-age years coincide with the pre-pubertal nadir, where linear growth velocity decelerates and reaches a minimum approximately two years prior to the adolescent height spurt, which occurs around 14 y in boys and 12 y in girls (52). Given that mean age at baseline in our cohort was 8.8 y, the follow-up period may not have been sufficient to capture substantial changes in height. Long-term cohort studies examining the relation of global DNA methylation with linear growth beyond adolescences are warranted.

Our study has several strengths. We were able to examine global DNA methylation in a large and representative sample of children from a setting where the

increasing prevalence of childhood obesity is becoming a serious public health problem. The prospective design and use of repeated anthropometric measures enhanced our ability to explore the temporal relation between LINE-1 methylation and changes in body weight. We also adjusted the estimates of association for key potential confounders, including baseline age and socioeconomic status. LINE-1 methylation was determined using pyrosequencing technology, a highly reproducible and accurate method to quantify DNA methylation, with all assays run in duplicate to minimize variability and enhance accuracy. Additionally, we used DNA from peripheral WBC, which is of high intrinsic value in epidemiologic studies as it is easily obtained and reflects systemic interindividual variation in germ-layer cells (53). Limitations of the study include reliance on only one measurement of LINE-1 methylation at the time of enrollment, potential random measurement error in anthropometry, and generalizability to other ethnicities as there is some evidence that Hispanics may have lower LINE-1 methylation than non-Hispanic whites (12).

We conclude that lower global DNA methylation is related to development of adiposity in school-age boys. Because global DNA methylation is responsive to external cues such as diet (54, 55), these findings are of particular relevance to nutrition intervention efforts that are feasibly delivered at the school level.

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Table 3.1 Characteristics of 553 school-age children from Bogotá, Colombia[1]

Characteristic[2]	**All Children**	**Boys**	**Girls**
Sex, (N)	553	254	299
Age, y	8.8 ± 1.7	8.9 ± 1.7	8.8 ± 1.7
LINE-1 DNA methylation, %5mC	80.25 ± 1.55	80.53 ± 1.52	80.01 ± 1.54
Child was born in Bogota, % (N)	90.5 (458)	90.8 (207)	90.3 (251)
Birthweight, g	3202 ± 849	3240 ± 825	3172 ± 868
Height-for-age Z-score[3]	-0.78 ± 0.98	-0.85 ± 0.91	-0.72 ± 1.02
BMI-for-age Z-score[3]	0.10 ± 0.99	0.15 ± 1.06	0.05 ± 0.92
C-reactive protein, mg/L	1.49 ± 3.25	1.22 ± 1.83	1.73 ± 4.08
Plasma vitamin A, µmol/L	1.04 ± 0.33	1.04 ± 0.33	1.04 ± 0.33
Maternal Education, y	8.7 ± 3.2	8.6 ± 3.2	8.7 ± 3.2
Maternal Height, cm	157.8 ± 6.0	157.6 ± 5.9	158.1 ± 6.1
Maternal BMI, kg/m^2	24.2 ± 3.8	24.5 ± 3.9	24.0 ± 3.7
Lowest Socioeconomic Status,[4] % (N)	7.8 (43)	8.7 (22)	7.0 (21)

1 Totals may be < 553 for all children, < 254 for boys, and < 299 for girls due to missing values.

2 Values are Mean (SD) unless otherwise noted.

3 According to the World Health Organization 2007 growth reference for children 5-19 y.

4 Stratum 1 of a maximum of 4, according to the local government classification for tax and planning purposes.

Table 3.2 Change in adiposity indicators in 254 school-age boys from Bogotá, Colombia, according to quartiles of LINE-1 DNA methylation

	Quartiles of LINE-1 DNA methylation				
	Q1 **N = 64**	**Q2** **N = 64**	**Q3** **N = 63**	**Q4** **N = 63**	***P***[1]
Median (Range) %5mC	79.05 (76.26, 79.62)	80.05 (79.63, 80.59)	80.90 (80.59, 81.40)	82.20 (81.41, 85.24)	
BMI-for-age Z-score[2]					
Baseline[3]	0.18 ± 1.09	0.20 ± 1.05	0.08 ± 0.99	0.14 ± 1.13	
Change (/y)[4]	0.04 ± 0.02	-0.04 ± 0.03	0.00 ± 0.03	-0.02 ± 0.02	0.24
Adjusted difference (95% CI)[5]	Reference	-0.08 (-0.16, 0.00)	-0.04 (-0.11, 0.03)	-0.06 (-0.12, 0.01)	0.21
Waist Circumference Z-score[6]					
Change (/y)[4]	0.05 ± 0.03	0.00 ± 0.03	0.00 ± 0.03	-0.03 ± 0.02	0.03
Adjusted difference (95% CI)[5]	Reference	-0.05 (-0.14, 0.04)	-0.06 (-0.14, 0.02)	-0.09 (-0.17, -0.02)	0.02
Skinfold Thickness Ratio Z-score[6]					
Baseline[3]	-0.03 ± 0.91	0.13 ± 0.77	0.28 ± 0.84	0.03 ± 0.95	
Change (/y)[4]	0.01 ± 0.03	-0.07 ± 0.03	-0.08 ± 0.03	-0.03 ± 0.02	0.32
Adjusted difference (95% CI)[5]	Reference	-0.08 (-0.16, 0.00)	-0.09 (-0.17,-0.01)	-0.04 (-0.12, 0.03)	0.28

1 For a test of linear trend when a variable that represented the median value of each quartile was introduced into a linear regression model as a continuous predictor (Wald test).

2 According to the World Health Organization growth reference for children 5-19 y (29).

3 Values are means ± SD.

4 Values are means ± SE.

5 Adjusted for baseline age and socioeconomic status.

6 Age-standardized using the LMS method (30) with data for boys 5-16 y of age in NHANES III.

Table 3.3 Change in adiposity indicators in 299 school-age girls from Bogotá, Colombia, according to quartiles of LINE-1 DNA methylation

	Quartiles of LINE-1 DNA methylation				
	Q1 **N = 75**	**Q2** **N = 74**	**Q3** **N = 75**	**Q4** **N = 75**	***P*[1]**
Median (Range) %5mC	78.43 (75.16, 79.23)	79.65 (79.23, 79.97)	80.32 (79.97, 80.89)	81.52 (80.91, 85.69)	
BMI-for-age Z-score[2]					
Baseline[3]	0.09 ± 0.83	0.06 ± 0.95	-0.04 ± 0.91	0.09 ± 0.99	
Change (/y)[4]	0.00 ± 0.02	0.05 ± 0.02	0.01 ± 0.02	0.00 ± 0.02	0.65
Adjusted difference (95% CI)[5]	Reference	0.05 (-0.02, 0.11)	0.00 (-0.06, 0.07)	-0.01 (-0.07, 0.06)	0.65
Waist Circumference Z-score[6]					
Change (/y)[4]	0.00 ± 0.02	0.00 ± 0.03	0.03 ± 0.03	-0.01 ± 0.03	0.84
Adjusted difference (95% CI)[5]	Reference	0.01 (-0.06, 0.08)	0.04 (-0.03, 0.11)	-0.01 (-0.08, 0.06)	0.99
Skinfold Thickness Ratio Z-score[6]					
Baseline[3]	0.03 ± 0.72	-0.01 ± 0.82	-0.09 ± 0.61	0.02 ± 0.57	
Change (/y)[4]	0.00 ± 0.03	0.00 ± 0.03	0.01 ± 0.02	0.00 ± 0.03	0.87
Adjusted difference (95% CI)[5]	Reference	0.00 (-0.08, 0.07)	0.01 (-0.05, 0.07)	0.00 (-0.07, 0.07)	0.85

1 For a test of linear trend when a variable that represented the median value of each quartile was introduced into a linear regression model as a continuous predictor (Wald test).

2 According to the World Health Organization growth reference for children 5-19 y (29).

3 Values are means ± SD.

4 Values are means ± SE.

5 Adjusted for baseline age and socioeconomic status.

6 Age-standardized using the LMS method (30) with data for girls 5-16 y of age in NHANES III.

Table 3.4 Change in height-for-age in 553 school-age children from Bogotá, Colombia, according to quartiles of LINE-1 DNA methylation[1]

	Quartiles of LINE-1 DNA methylation				
	Q1	**Q2**	**Q3**	**Q4**	***P***[2]
Boys					
N	64	64	63	63	
Median (Range) %5mC	79.05 (76.26, 79.62)	80.05 (79.63, 80.59)	80.90 (80.59, 81.40)	82.20 (81.41, 85.24)	
Baseline height-for-age Z-score[3]	-0.84 ± 1.01	-0.96 ± 0.96	-0.81 ± 0.90	-0.79 ± 0.78	
Change (/y)[4]	0.04 ± 0.02	0.05 ± 0.02	0.04 ± 0.02	0.06 ± 0.02	0.48
Adjusted difference (95% CI)[5]	Reference	0.01 (-0.05, 0.08)	0.00 (-0.06, 0.06)	0.02 (-0.03, 0.08)	0.55
Girls					
N	75	74	75	75	
Median (Range) %5mC	78.43 (75.16, 79.23)	79.65 (79.23, 79.97)	80.32 (79.97, 80.89)	81.52 (80.91, 85.69)	
Baseline height-for-age Z-score[3]	-0.73 ± 0.90	-0.61 ± 1.19	-0.73 ± 0.88	-0.82 ± 1.11	
Change (/y)[4]	0.06 ± 0.02	0.03 ± 0.02	0.03 ± 0.02	0.08 ± 0.02	0.54
Adjusted difference (95% CI)[5]	Reference	-0.02 (-0.08, 0.03)	-0.03 (-0.08, 0.03)	0.02 (-0.04, 0.08)	0.54

1 Height-for-age Z-scores were determined using the World Health Organization growth reference for children 5-19 y (29).

2 For a test of linear trend when a variable that represented quartiles was introduced into a linear regression model as a continuous predictor (Wald test).

3 Values are mean ± SD.

4 Values are mean ± SE.

5 Adjusted for baseline age and socioeconomic status.

Chapter 5

Conclusions

Summary of Main Findings

This work extends current knowledge regarding nutritional and sociodemographic predictors of global DNA methylation, and contributes to the understanding of biological mechanisms that underlie early life obesity.

In Chapter 2, we found that intake of methyl-donor and methylation cofactor micronutrients was not related to global DNA methylation in a large sample of healthy middle-aged adults. However, we found a positive association between BMI and LINE-1 methylation, which is corroborated by results from a study of Samoan adults (1). Yet, other cross-sectional studies in adults have not found an association between DNA methylation and measures of adiposity (2-6), highlighting the need for longitudinal investigations to assess the causal relation of global DNA methylation with body size. We also found a positive correlation between height and Alu methylation, which is supported by preliminary findings from a U.S. birth cohort that higher cord blood global DNA methylation is related to greater height up to 7 y of age (7). This association might reflect early life conditions, as height is a sensitive indicator of environmental influences that otherwise may not be quantifiable.

In Chapter 3, we found that higher plasma levels of plasma vitamin A and CRP, and low maternal BMI (< 18.5) were each associated with lower LINE-1 methylation, whereas higher household socioeconomic status was related to higher LINE-1 methylation in healthy school-age children. The associations with vitamin A are in line with findings from studies of retinoic acid treatment in cancer cells, and could be related to diminished DNMT expression (8). Plasma CRP concentration, an inflammation biomarker, was also inversely related to LINE-1 methylation. Although the literature regarding inflammation and global DNA methylation in adults is inconsistent (9-11), this relation will be important to examine in other pediatric populations, as it sheds light on the pathogenesis atherosclerosis and other inflammation-mediated vascular disorders. The relations of maternal BMI and socioeconomic status with global DNA methylation could portray early life environmental influences on the epigenome. Assuming that maternal BMI is representative of the women's pre-pregnancy weight status, the association between low maternal BMI and global DNA hypomethylation could reflect differences in the children's early life nutrient environment. Studies from the Dutch Winter Famine cohort indicate that maternal undernourishment during the periconceptional period is associated with persistent changes in offspring DNA methylation profiles (12), and data from perinatal studies provide support for a positive correlation between pre-pregnancy BMI and cord blood DNA methylation (13, 14). Socioeconomic status also plays an important role in the early life environment. Lower socioeconomic status is associated with adverse prenatal exposures such as maternal cigarette smoking (15), and unhealthy lifestyle characteristics during childhood including poor diet and lack of exercise (16, 17) - all of which are related to lower global DNA

methylation in adults (4, 5, 18). Whether these associations represent epigenetic 'programming' related to later-life health outcomes requires further investigation. As was consistent with findings from Chapter 2, there were no significant associations between methyl-donor (erythrocyte folate) or methylation cofactor micronutrients (plasma vitamin B12) and global DNA methylation.

The null findings from Chapters 2 and 3 surrounding the methyl-donor and methylation cofactor micronutrients could be related to the nutritional status of the study populations. Considering the folic acid fortification of the U.S. food supply, and the low prevalence of folate deficiency in the Bogotá school children (19), it is likely that few participants in either study were folate-deficient. A positive association of folate intake or status with DNA methylation might be detectable in populations with a higher prevalence of methyl-donor micronutrient deficiencies. Additionally, we only examined specific micronutrients, which does not account for the combinations of foods and nutrients in the human diet that may interact to promote DNA methylation (5). Finally, it is important to acknowledge that we were not able to examine associations with choline, which also plays an important role in the metabolic cycling of homocysteine. Because the folate- and choline-mediated methylation pathways may be interrelated (20, 21), future studies should examine associations of both micronutrients with global DNA methylation.

In Chapter 4, we shifted focus to the relation of global DNA methylation with child growth. Among boys, we found inverse, non-linear relations of LINE-1 methylation with change in BMI-for-age Z-score and skinfold thickness ratio-for-age Z-score. Boys in the lowest quartile of LINE-1 methylation experienced greater annual

gains in BMI and the skinfold thickness ratio than those in the upper three quartiles during follow-up. In addition, lower LINE-1 methylation was related to greater gains in waist circumference-for-age in a linear fashion. These results indicate that lower global DNA methylation is related to development of adiposity school-age boys. On the other hand, there were no significant associations between global DNA methylation and any of the adiposity indicators among girls, and LINE-1 methylation was not related to linear growth in either sex.

This dissertation has several strengths. Many epigenetic studies had small sample sizes and were underpowered to detect small differences in global DNA methylation. We were able to examine global DNA methylation in a large sample of healthy, ethnically diverse middle-age adults, as well as in a large and representative sample of school-age children from a setting where child overweight is becoming a serious public health concern. The prospective design and use of repeated anthropometric measures in Chapter 4 enhanced our ability to assess the temporal relation between LINE-1 methylation and changes in body composition. Global DNA methylation was quantified using a highly-reproducible pyrosequencing-based technology. We also used DNA from peripheral leukocytes, which are of high intrinsic value in epidemiologic studies as it is easily obtained and reflects systemic interindividual variation in germ-layer cells (22). This work also has some limitations. We did not account for the proportion of white blood cell subtypes in the analyses. There is some evidence that global DNA methylation is negatively related to the percentage of lymphocytes (2); whether differential leukocyte counts are associated with development of adiposity is a possibility that warrants further investigation. Additionally, comparability of findings regarding global DNA methylation

is limited due to inconsistencies in laboratory techniques and assays used to quantify genomic methylation content in the literature. Finally, because global DNA methylation may serve only as a biomarker of concomitant molecular mechanisms, these findings provide a basis for further investigation of mechanistic pathways, such as gene-specific DNA methylation, involved in the etiology of excess weight gain. Future studies could examine methylation of genes implicated in adipogenesis, such as the retinoid X receptor alpha (*RXRA*) (23) and the peroxisome proliferator-activated receptors (*PPAR*s) (24), in relation to changes in body composition.

Public Health Implications

While we did not find significant associations between methyl donor/methylation cofactor micronutrients and global DNA methylation in adults or children, we identified important sociodemographic and nutritional correlates of global DNA methylation. The positive association between height and Alu methylation in the MESA population is noteworthy, since short adult stature is associated with risk of some chronic diseases, such as cardiovascular disease (25, 26). Elucidating the role of DNA methylation in linear growth would improve understanding of regulatory mechanisms involved in human development, and may also provide insight on disease etiology. In the BSCC children, vitamin A status, plasma CRP, maternal BMI, and socioeconomic status were each associated with LINE-1 methylation. Although the health consequences of altered LINE-1 methylation in children require additional research, and longitudinal investigations are necessary to determine whether modification of these exposures during the school-age years leads to changes in DNA methylation, linking modifiable characteristics, such as maternal weight status and child nutritional status, to a reversible epigenetic mechanism can unveil viable avenues for intervention opportunities.

Perhaps the most salient finding of this dissertation is the inverse relation between global DNA methylation and development of adiposity in boys. The fact that change in waist circumference was strongly associated with LINE-1 methylation is especially important because abdominal adiposity has disproportionate consequences for metabolic physiology. Central fat mass is related to increased cardiovascular morbidity (27) and mortality (28) in adults. Furthermore, recent findings indicate that accrual of abdominal

fat during childhood is a strong independent predictor of poor cardiometabolic health in later life (29).

In light of evidence that aberrations in DNA methylation are reversible via dietary changes (30), our findings point toward opportunities for public health intervention. For example, changes in child nutritional status could feasibly be accomplished through school meal programs. Additionally, the fact that children of underweight mothers exhibited lower global DNA methylation than their counterparts suggests that the documented relation between gestational nutrient restriction and risk of adult chronic disease (31) may be mediated by epigenetic mechanisms. Primary healthcare settings are a practical venue to target underweight reproductive-aged women for nutrition education. Such interventions aimed at modifiable epigenetic mechanisms are relatively inexpensive, readily available, and could become a public health priority.

As epigenetic research continues to evolve and progress, we anticipate that studies evaluating the influence of dietary interventions on DNA methylation profiles and subsequent changes in body composition will improve knowledge of biological pathways that underlie excess weight gain. Findings will have important implications for the development of effective intervention programs, as well as clinical recommendations aimed at improving cardiometabolic health and preventing adult chronic disease.

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