RESEARCH PAPER

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Micronutrient status and global DNA methylation in school-age children

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Abbreviations: LINE-1, long interspersed nucleotide element 1; %5-mC, percentage of 5-methyl-cytosine; WBC, white blood cell; CRP, C-reactive protein; DNMT, DNA methyltransferase; RA, retinoic acid; CVD, cardiovascular disease; BMI, body mass index

Aberrations in global LINE-1 DNA methylation have been related to risk of cancer and cardiovascular disease. Micronutrients including methyl-donors and retinoids are involved in DNA methylation pathways. We investigated associations of micronutrient status and LINE-1 methylation in a cross-sectional study of school-age children from Bogotá, Colombia. Methylation of LINE-1 repetitive elements was quantified in 568 children 5–12 years of age using pyrosequencing technology. We examined the association of LINE-1 methylation with erythrocyte folate, plasma vitamin B12, vitamin A ferritin (an indicator of iron status) and serum zinc concentrations using multivariable linear regression. We also considered associations of LINE-1 methylation with socio-demographic and anthropometric characteristics. Mean (± SD) LINE-1 methylation was 80.25 (± 0.65) percentage of 5-mC (%5-mC). LINE-1 methylation was inversely related to plasma vitamin A. After adjustment for potential confounders, children with retinol levels higher than or equal to 1.05 μmol/L showed 0.19% 5-mC lower LINE-1 methylation than children with retinol levels lower than 0.70 μmol/L. LINE-1 methylation was also inversely associated with C-reactive protein, a marker of chronic inflammation, and female sex. We identified positive associations of maternal body mass index and socioeconomic status with LINE-1 methylation. These associations were not significantly different by sex. Whether modification of these exposures during school-age years leads to changes in global DNA methylation warrants further investigation.

Introduction

DNA methylation is a modifiable epigenetic modification 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

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

%5-mC	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[a]	0.48		0.56		0.52		0.55	

[a]Intraclass correlation coefficient.

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, 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.

Results

Mean ± SD age of children was 8.8 ± 1.7 y; 46.3% were boys. Overall mean ± SD LINE-1 DNA methylation was 80.25 ± 0.65% 5-mC. 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 1**). Average %5-mC 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**), boys had a 0.22% 5-mC 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 trend = 0.08). Higher plasma c-reactive protein (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 body mass index (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 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 4**). In the multivariable analysis, LINE-1 methylation was 0.21% 5-mC 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% 5-mC 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% 5-mC 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% 5-mC 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% 5-mC 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 socio-demographic and anthropometric characteristics. As previously reported in adults,[20] boys had higher global

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Table 2. LINE-1 DNA methylation according to background characteristics of 568 school-age children from Bogotá, Colombia

		LINE-1 DNA Methylation (%5-mC)[b]				
	N[a]	All	N[a]	Males	N[a]	Females
Overall	568	80.25 (0.65)				
Child's sex						
F	305	80.15 (0.65)				
M	263	80.37 (0.64)				
p[c]		< 0.0001				
Age, years						
5–6	96	80.19 (0.64)	43	80.41 (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[d]		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[c]		0.89		0.70		0.59
Birth weight, g						
< 2,500	44	80.17 (0.77)	20	80.20 (0.61)	24	80.14 (0.90)
2,500–2,999	110	80.28 (0.67)	41	80.49 (0.73)	69	80.16 (0.60)
3,000–3,499	128	80.26 (0.66)	57	80.41 (0.57)	71	80.14 (0.70)
> 3,500	147	80.22 (0.62)	73	80.30 (0.64)	74	80.13 (0.59)
p trend[d]		0.90		0.72		0.87
Height-for-age Z score[e]						
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[d]		0.41		0.75		0.25
BMI-for-age Z-score[e]						
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[d]		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[c]		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)

[a]Totals may be < 568 for all children, < 263 for males and < 305 for females because of missing values. [b]From mixed effects linear regression models where site was a random effect. [c]From analysis of variance (ANOVA). [d]From univariate regression models in which a variable representing the ordinal predictor was introduced as continuous. [e]According to the World Health Organization 2007 Child-Growth Reference. [f]According to the local government classification.

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Table 2. LINE-1 DNA methylation according to background characteristics of 568 school-age children from Bogotá, Colombia (continued)

		LINE-1 DNA Methylation (%5-mC)[2b]				
	N[a]	All	N[a]	Males	N[a]	Females
Maternal education						
University	33	80.39 (0.70)	15	80.71 (0.56)	18	80.13 (0.72)
p trend[d]		0.34		0.06		0.78
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[d]		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[d]		0.33		0.61		0.63
Household socioeconomic stratum[f]						
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[d]		0.15		0.30		0.27

[a]Totals may be < 568 for all children, < 263 for males and < 305 for females because of missing values. [b]From mixed effects linear regression models where site was a random effect. [c]From analysis of variance (ANOVA). [d]From univariate regression models in which a variable representing the ordinal predictor was introduced as continuous. [e]According to the World Health Organization 2007 Child-Growth Reference. [f]According to the local government classification.

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 the DNA methyltransferases(DNMTs), endogenous enzymes that catalyze 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*) downregulated DNMT gene expression and telomerase activity when administered individually and in combination.[21] The RA treatments also suppressed expression of *hTERT*, the catalytic component of telomerase that is paradoxically hypermethylated and highly expressed in cancer cells.[22] Because inhibition of DNA methylation in cancer cells downregulated 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.[23] 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 DNMT expression and 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,[24] and global DNA methylation is increasingly recognized as a key mechanism involved in the pathogenesis of inflammation-mediated cardiovascular risk factors such as atherosclerosis.[25] 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.[26] Similarly, a recent study of 165 cancer-free adults found no association between LINE-1

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Table 3. LINE-1 DNA Methylation according to micronutrient status in 568 school-age children from Bogotá, Colombia

	LINE-1 DNA Methylation (%5mC)[b]								
	All			Males			Females		
	N[a]	Mean (SD)	%5-mC difference β (95% CI)	N[a]	Mean (SD)	%5-mC difference β (95% CI)	N[a]	Mean (SD)	%5-mC difference β (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[c]		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[c]		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[c]		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[c]		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[c]		0.002			0.13			0.006	

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

methylation and inflammation biomarkers including CRP.[17] However, high sensitivity CRP (*hs*CRP) was related to global DNA hypermethylation in chronic kidney disease patients.[27] 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,[28] which has been associated with decreased cord blood DNA methylation.[29] The periconceptional period represents a critical window in ontogenic development, and is characterized by responsiveness of DNA methylation patterns to nutritional and environmental exposures.[30] Studies using data from the Dutch famine cohort reported that periconceptional exposure to famine was related to an unfavorable cardiometabolic risk profile in adulthood[31] and persistent changes in methylation of genes involved in growth and metabolism.[32,33] While such findings suggest that aberrant DNA methylation

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Table 4. Correlates of LINE-1 DNA methylation in 568 school-age children from Bogotá, Colombia

	Adjusted %5-mC difference[a] 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[b]	0.006
C-reactive Protein, mg/L	
< 1.0	Reference
≥ 1.0	-0.12 (-0.24, -0.01)
p	0.04
Maternal BMI, kg/m²	
< 18.5	Reference
≥ 18.5	0.31 (0.01, 0.60)
p	0.04
Household socioeconomic stratum[c]	
1–3 (lower)	Reference
4 (highest)	0.29 (0.07, 0.51)
p	0.01

[a]From a linear regression model with LINE-1 DNA methylation as the outcome and predictors that included sex, vitamin A, CRP, maternal BMI and household socioeconomic stratum. [b]Test for linear trend from a linear regression model where an ordinal indicator for the variable was entered as a continuous predictor. [c]According to the local government classification.

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.[16,34] 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,[35] as well as unhealthy lifestyle characteristics during childhood including decreased physical activity levels[36] and a tendency to consume a diet high in fats and sugars.[37] 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.[7,8,38]

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.[39] 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.[40] 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.[20]

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.

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.[41] Briefly, we recruited a representative sample of 3,202 school children aged 5–12 y 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.[42]

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,

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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.[43] 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 performed 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[44] 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, catalog #: 51104, 51106) and cryopreserved until transportation to the University of Michigan for analyses.

LINE-1 DNA methylation determinations. Pyrosequencing-based DNA methylation analysis was performed according to previously described methods.[45] Approximately 500 ng of DNA was bisulfite converted using the EpiTect Bisulfite Kit (Qiagen, catalog #: 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-methyl-cytosine) are protected from bisulfite conversion and thus remain unchanged, resulting in genome-wide methylation-dependent differences in the DNA sequence. Global DNA methylation was assessed through simultaneous PCR of the DNA LINE-1 elements, using primers designed toward 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-methyl-cytosine (%5-mC) 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. Percent of 5-mC site measurements that were more than five standard deviations above or below the raw mean LINE-1 methylation (< 69% or > 91% 5-mC) were excluded from the analyses.

Data analyses. Specimens were collected in 2,816 (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 socio-demographic 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 %5-mC 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 and slopes 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 %5-mC 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 socio-demographic 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).[46] 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 y from the World Health Organization.[47] 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).[48] We estimated differences and 95% confidence intervals (95%

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CI) in %5-mC 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 performed with the use of the Statistical Analyses System software (version 9.2; SAS Institute Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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