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Study on Prevalence of Global DNA Methylation Preceded Due to Malnutrition in School-age-Children of Bhopal and Adjoining Areas

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Abstract: ***Abnormality in global LINE-1 DNA methylation is directly related to risk of many serious disease. The DNA methylation pathways are affected by micronutrients which have methyl-donors and retinoids. Here we have investigated associations of malnutrition status and cross-sectional study to findout LINE-1 methylation in school-age children from Bhopal and adjoining areas. we have chosen a group of 600 school age children between age of 5-12 years. Global LINE-1 DNA methylation repetitive elements were quantified by using pyrosequencing technology. Furthermore, the investigation have been done to see the association of global LINE-1 DNA methylation with folic acid concentration, plasma vitamin B12, vitamin A ferritin and serum zinc concentrations using multivariable linear regression. 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. These associations were not found significantly different by sex. Modification of these factors during school-age years leads to changes in global DNA methylation warrants further investigation.***

Keywords: LINE-1: long interspersed nucleotide element 1; %5-mC: percentage of 5-methyl-cytosine; CRP: C-reactive protein; DNMT: DNA methyltransferase; RA: retinoic acid; CVD: cardiovascular disease

1. 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] folate - vitamin B9- or its synthetic analogue folic acid is essential for numerous metabolic functions such as biosynthesis of RNA and DNA, repair of DNA and methylation of DNA processes that are central in the maintenance of the integrity of the genome and the cells in the body.[14] There is great interest in assessing the potential for changes in folate intake to modulate DNA methylation both as a biomarker for folate status and as a mechanistic link to developmental disorders and chronic diseases including cancer,[15] while other studies reported no difference in methylation after folate restriction or supplementation.[16] The suggests that multiple micronutrients present in green/leafy 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[17] 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 India. In spite of current evidence that altered LINE-1 methylation is related to cardiometabolic risk factors that begin in early life, such as atherosclerosis,[18] and obesity, 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 600 children randomly selected from the slum area of Bhopal and Mandideep (Raisen District) Madhya Pradesh, India, an ongoing longitudinal study of children from low-income families in Bhopal and Mandideep.

2. Methodology

This study was conducted at slum area of Bhopal and Mandideep, Madhya Pradesh, India, a longitudinal investigation of nutrition and health among children during 2015-2017. We recruited a 1000 representative sample of school age children aged 5–12 year in April, 2015, of which 600 from Bhopal and 400 from Mandideep with use of a cluster sampling strategy. The sample represents families from low income socioeconomic backgrounds in the city. A survey study have already been done by door to door visit with the help of questionnaire as per the Anthropometric Survey Manual.[19] The questionnaires was based on sociodemographic characteristics (including age, marital status, education level and socioeconomic level) as well as anthropometric measures and information about physical activity and sedentary habits of the child. For collection of samples, we have conducted free health check-up camp with the help of Doctors (MBBS), qualified nursing staff with one pathologist (DMLT) for the surveyed children to obtain anthropometric measurements as per the WHO manual [20] and a fasting blood sample from the children as per the WHO guidelines.[21] Height was measured without shoes to the nearest 1 mm using a wall-mounted stadiometer, and weight was measured in light clothing by electronic balance machine. The parents or primary caregivers of all children gave written informed consent prior to enrollment into the study.

At the baseline assessment, nursing staff 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 pathology lab for further investigation. 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 and CRP concentrations were measured using a competitive chemiluminescent immunoassay in an ADVIA Centaur CP Immunoassay System (Siemens Healthineers India). 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 on a Shimadzu AA6300 spectrophotometer.[22] 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 RKDF University for analyses of LINE-1 DNA methylation determinations.

Pyrosequencing based DNA methylation analysis was performed.[23] 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.

3. Results

In the study 63% were males and rest of are females and mean ± SD age of children was 7.9 ± 1.5 y. The LINE-1 DNA methylation's overall mean ±SD 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),** females had a 0.22% 5-mC higher DNA methylation than males on average ($p < 0.0001$). There was an inverse association between age and LINE-1 methylation in females; 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 males than females. 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 males (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, household socioeconomic stratum **(Table 4).** In the multivariable analysis, LINE-1 methylation was 0.21% 5-mC lower in males than females ($p = 0.0007$). Plasma vitamin A and CRP were each inversely related to LINE-1 methylation, household socioeconomic stratum were 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 ($p = 0.04$). 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.

4. Data Analysis

We selected a random sample of 1000 children for LINE-1 methylation determinations. Of which, 600 children had adequate DNA concentrations and constituted the final study population. These children did not differ from the rest of the target group 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 to the average %5-mC across the both sites. 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. 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, child's anthropometric status, and CRP concentrations. 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[20] 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). We estimated differences and 95% confidence intervals (95% 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 $p < 0.10$. 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; downloaded from https://www.sas.com/en_in/software/ university-edition/download-software.html).

Table 1: LINE-1 DNA methylation at four genomic LINE-1 sites in 600 school-age children of Bhopal and adjoining areas

%5-mC	Genomic Site 1		Genomic Site 2		Genomic Site 3		Genomic Site 4	
	N = 578	N = 549	N = 521	N = 550	N = 552	N = 523	N = 575	N = 535
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	
Intraclass correlation coefficient	0.48		0.56		0.52		0.55	

Table 2: LINE-1 DNA methylation according to background characteristics of 600 school-age children of Bhopal and adjoining areas

LINE-1 DNA Methylation (%5-mC)						
Overall			**Male**		**Female**	
	N	**Methylation**	**N**	**Methylation**	**N**	**Methylation**
Overall	**600**	80.25 (0.65)	378	80.15 (0.65)	222	80.37 (0.64)
p value		<0.0001				
Age, years						
5–6	112	80.19 (0.64)	70	80.41 (0.62)	42	80.01 (0.60)
7–8	201	80.29 (0.58)	125	80.17 (0.58)	76	80.43 (0.55)
9–10	227	80.27 (0.71)	147	80.19 (0.70)	80	80.36 (0.71)
11–12	60	80.15 (0.65)	36	80.18 (0.69)	24	80.13 (0.63)
p value		0.93		0.17		0.08
Child was born in Madhya Pradesh						
Yes	485	80.26 (0.65)	294	80.15 (0.65)	191	80.39 (0.63)
No	115	80.27 (0.66)	84	80.22 (0.66)	31	80.34 (0.66)
p value		0.89		0.59		0.70
Height-for-age Z score						
Less than -2.0	123	80.27 (0.69)	80	80.27 (0.73)	43	80.27 (0.64)
-2.0 to < -1.0	184	80.29 (0.62)	124	80.17 (0.65)	60	80.40 (0.57)

-1.0 to < 1.0	272	80.22 (0.69)	165	80.11 (0.65)	107	80.37 (0.70)
≥ 1.0	21	80.24 (0.33)	9	80.20 (0.34)	12	80.34 (0.31)
p value		0.41		0.25		0.75
BMI-for-age Z-score						
Less than -2.0	12	80.41 (0.51)	7	80.43 (0.43)	5	80.39 (0.63)
-2.0 to < -1.0	79	80.34 (0.68)	54	80.32 (0.72)	25	80.36 (0.64)
-1.0 to < 1.0	389	80.22 (0.64)	238	80.36 (0.64)	151	80.36 (0.64)
1.0 to < 2.0	102	80.31 (0.73)	67	80.19 (0.75)	35	80.42 (0.70)
≥ 2.0	18	80.28 (0.55)	12	80.23 (0.58)	6	80.31 (0.57)
p value		0.79		0.42		0.90
CRP, mg/L						
< 1.0	308	80.32 (0.67)	201	80.21 (0.71)	107	80.42 (0.62)
≥ 1.0	292	80.18 (0.63)	177	80.08 (0.58)	115	80.32 (0.67)
p value		0.01		0.07		0.22
Household socioeconomic stratum						
*BPL	313	80.21 (0.64)	214	80.13 (0.64)	99	80.32 (0.64)
Non-BPL	185	80.20 (0.67)	115	80.06 (0.63)	70	80.35 (0.68)
Lower-middle	60	80.35 (0.48)	34	80.29 (0.38)	26	80.40 (0.55)
Middle	42	80.62 (0.71)	15	80.62 (0.89)	27	80.62 (0.61)
p value		0.15		0.27		0.30

*BPL: below poverty line

Table 3: LINE-1 DNA Methylation according to micronutrient status in 600 school-age children of Bhopal and adjoining areas

LINE-1 DNA Methylation (%5mC)									
All				*Males*			*Females*		
	N	*Mean (SD)*	*%5-mC difference β (95% CI)*	*N*	*Mean (SD)*	*%5-mC difference β (95% CI)*	*N*	*Mean (SD)*	*%5-mC difference β (95% CI)*
Erythrocyte Folate, nmol/L									
Q1	150	80.24 (0.61)	Reference	95	80.13 (0.59)	Reference	55	80.32 (0.60)	Reference
Q2	150	80.21 (0.64)	-0.03(-0.18, 0.11)	94	80.17 (0.66)	0.04 (-0.16, 0.24)	56	80.31 (0.65)	-0.01(-0.23, 0.20)
Q3	150	80.25 (0.67)	0.01(-0.14, 0.16)	94	80.11 (0.69)	-0.02 (-0.23, 0.18)	56	80.41 (0.62)	0.09 (-0.11, 0.30)
Q4	150	80.28 (0.67)	0.04(-0.11, 0.19)	95	80.14 (0.63)	0.01(-0.19, 0.20)	55	80.46 (0.68)	0.14 (-0.09, 0.36)
p value		0.51			0.90			0.15	
Plasma Vitamin B12, pmol/L									
Q1	150	80.30 (0.64)	Reference	94	80.19 (0.58)	Reference	56	80.40 (0.69)	Reference
Q2	148	80.26 (0.61)	-0.04(-0.19, 0.11)	93	80.13 (0.53)	-0.06 (-0.24, 0.12)	55	80.41 (0.63)	0.01 (-0.22, 0.24)
Q3	152	80.24 (0.66)	-0.06(-0.22, 0.09)	96	80.12 (0.69)	-0.07 (-0.28, 0.14)	56	80.38 (0.63)	-0.02(-0.25, 0.21)
Q4	150	80.18 (0.72)	-0.12(-0.28, 0.04)	95	80.11 (0.78)	-0.07 (-0.30, 0.15)	55	80.26 (0.64)	-0.15(-0.38, 0.08)
p value		0.15			0.52			0.20	
Serum Zinc, μmol/L									
Q1	150	80.22 (0.66)	Reference	93	80.13 (0.64)	Reference	57	80.33 (0.65)	Reference
Q2	151	80.23 (0.64)	0.01 (-0.14, 0.16)	94	80.12 (0.63)	-0.01 (-0.22, 0.19)	57	80.36 (0.65)	0.03 (-0.19, 0.25)
Q3	149	80.30 (0.64)	0.07 (-0.08, 0.23)	94	80.16 (0.62)	0.03 (-0.17, 0.23)	55	80.43 (0.62)	0.10 (-0.11, 0.32)
Q4	150	80.24 (0.68)	0.02 (-0.14, 0.18)	97	80.15 (0.69)	0.01 (-0.20, 0.22)	53	80.36 (0.67)	0.04 (-0.19, 0.26)
p value		0.60			0.82			0.61	
Plasma feritin, μg/L									
Q1	150	80.34 (0.66)	Reference	94	80.17 (0.62)	Reference	56	80.49 (0.65)	Reference
Q2	150	80.18 (0.63)	-0.16(-0.31,-0.01)	95	80.10 (0.65)	-0.07 (-0.27, 0.14)	55	80.35 (0.63)	-0.14(-0.35, 0.08)
Q3	149	80.26 (0.64)	-0.08(-0.24,0.07)	94	80.15 (0.63)	-0.01 (-0.21, 0.19)	55	80.31 (0.61)	-0.17(-0.39, 0.04)
Q4	151	80.21 (0.68)	-0.13(-0.28,0.03)	95	80.14 (0.69)	-0.02 (-0.23, 0.19)	56	80.33 (0.69)	-0.16(-0.38, 0.07)
p value		0.22			0.96			0.16	
Plasma vitamin A, μmol/L									
<0.700	74	80.37 (0.63)	Reference	46	80.35 (0.67)	Reference	28	80.40 (0.59)	Reference
0.700 - 1.049	246	80.32 (0.63)	-0.06(-0.22, 0.11)	160	80.20 (0.57)	-0.16 (-0.39, 0.07)	86	80.45 (0.67)	0.05(-0.18, 0.29)
≥1.050	280	80.16 (0.67)	-0.22(-0.38,-0.05)	172	80.04 (0.69)	-0.31(-0.55, -0.07)	108	80.29 (0.63)	-0.10(-0.33, 0.12)
p value		0.002			0.006			0.13	

Table 4: Correlations of LINE-1 DNA methylation in 600 school-age children of Bhopal and adjoining areas

Adjusted %5-mC difference β (95% CI)	
Sex	
Females	Reference
Male	-0.21 (-0.32, -0.09)
p value	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 value	0.006
C-reactive Protein, mg/L	
< 1.0	Reference
≥ 1.0 -0.12	(-0.24, -0.01)
p value	0.04
Household socioeconomic stratum	
1–3 (lower)	Reference
4 (highest)	0.29 (0.07, 0.51)
p value	0.01

5. Discussion

We examined associations of micronutrient status biomarkers with WBC LINE-1 DNA methylation in 600 children randomly selected from the slum area of Bhopal and Mandideep. In addition, we ascertained associations of LINE-1 methylation with child socio-demographic and anthropometric characteristics. We 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 retinoidmediated changes in the expression or activity of the DNA methyltransferases (DNMTs), endogenous enzymes that catalyze the methylation reaction. It is probable that they can influence global DNA methylation as well. Further research is warranted to investigate the effects of changes in vitamin A status on gene 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,[18] 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] 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, undernutrition and deficiencies of iodine, iron, and folate are also important for the development of the brain and the emergent cognitive functions, and there is some evidence to suggest that zinc, vitamin B12, and omega-3 polyunsaturated fatty acids play important role in cognitive development.[26] 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 parents having alcohal and smoking habits,[27] as well as unhealthy lifestyle characteristics[28] during childhood including decreased physical activity levels and a tendency to consume a diet high in fats and sugars.[18], [29] 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] Of note, we did not find significant associations between LINE-1 methylation and erythrocyte folate.

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 methyldonor nutrients, including folate, periconceptionally or during the 2nd trimester with cord blood LINE-1 methylation.[30] 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 many diseases, 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[25]. 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.

6. Conclusion

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

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