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Luke Montrose
Boise State University

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Is adiposity related to repeat measures of blood leukocyte DNA methylation across childhood and adolescence?

Yue Wu[1,2] | Luke Montrose[3] | Joseph K. Kochmanski[4] | Dana C. Dolinoy[2,5] | Martha M. Téllez-Rojo[6] | Alejandra Cantoral[7] | Adriana Mercado-García[6] | Karen E. Peterson[2,5] | Jaclyn M. Goodrich[5]

[1]Department of Bioinformatics and Biostatistics, Shanghai Jiao Tong University, Shanghai, China

[2]Department of Nutritional Sciences, University of Michigan, Ann Arbor, Michigan, USA

[3]Department of Community and Environmental Health, Boise State University, Boise, Idaho, USA

[4]Department of Translational Neuroscience, Michigan State University, Grand Rapids, Michigan, USA

[5]Department of Environmental Health Sciences, University of Michigan, Ann Arbor, Michigan, USA

[6]Center for Nutrition and Health Research, National Institute of Public Health, Cuernavaca, Mexico

[7]Health Department, Universidad Iberoamericana, Mexico City, Mexico

Correspondence
Karen E. Peterson, Department of Nutritional Sciences, University of Michigan, 1415 Washington Heights, I-1967, Ann Arbor, MI 48109-2029, USA.
Email: karenep@umich.edu

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Summary

Epigenetic modifications such as DNA methylation may influence gene expression and phenotypes, including obesity in childhood. The directionality of this relationship is nevertheless unclear, and some evidence suggests that adiposity modifies the epigenome, rather than the other way around. In this pilot study, we utilize data from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) study to examine whether measures of adiposity in childhood and early adolescence are associated with repeated measures of blood leukocyte DNA methylation at LINE-1 repetitive elements and two genes implicated in growth and adiposity: *H19* and *HSD11B2*. Longitudinal epigenetic data were generated from cord blood and blood from follow-up visits in early and late adolescence. We assessed interactions between age and measures of body mass index (BMI) at 5 years of age and weight, BMI and waist circumference in early adolescence to infer whether adiposity deflects age-related DNA methylation changes throughout childhood. Applying linear mixed-effects models, we found an inverse association between measures of childhood BMI (kg/m^2) and early-teen weight (kg) with repeat measures of *H19* DNA methylation. We did not observe any statistically significant associations (p-value <.05) between any anthropometric measures and DNA methylation at LINE-1 or *HSD11B2*. We did not demonstrate statistically significant evidence in support of deflection of age-related DNA methylation trajectories by adiposity-related measures (age by adiposity interaction term). Given the pilot nature of this study, the relationships between repeat measures of DNA methylation and adiposity-measures across childhood merit further exploration in larger study populations.

KEYWORDS
biomarkers, DNA methylation, epigenetics, obesity

1 | INTRODUCTION

Overweight and obesity were estimated to cause 3.4 million deaths, 3.9% of years of life lost, and 3.8% of disability adjusted life years (DALYs) globally in 2010.[1,2] The prevalence of overweight and obesity is also rising among children and adolescents significantly in both developed and developing countries.[1] Given that the aetiology of obesity is multifactorial and likely involves gene–environment interactions,[3] epigenetics has emerged as a possible molecular biomarker that will aid understanding of obesity-related phenotypes, complications, and underlying mechanisms.[4] In recent years, mounting human epidemiology and animal model evidence has identified associations between epigenetic marks and chronic inflammatory cardiometabolic conditions including obesity,[5,6] though the directionalities of these associations remain inconclusive.[7–9]

Epigenetics is a discipline that studies mitotically heritable and potentially reversible changes in gene expression that are unrelated to the DNA sequence[10]; the major epigenetic mechanisms include DNA methylation, histone modifications, and non-coding RNA.[10] DNA methylation is a relatively well-understood epigenetic modification, in mammals, it typically occurs at the 5′-carbon position of cytosine in a Cytosine-phospho-Guanine (CpG) dinucleotide.[11] DNA methylation is generally associated with decreased transcription factor binding when it occurs at promoter or enhancer regions, resulting in decreased gene transcription.[11,12]

DNA methylation patterns at functionally relevant genes have the potential to affect obesity susceptibility.[13] However, it is also possible that obesity secondarily leads to changes to DNA methylation, thereby contributing to the development of adiposity-related chronic diseases.[7] Recently, several studies have attempted to infer the direction of the relationship between DNA methylation and obesity.[7,8] For example, Mendelson et al. conducted an association analysis of BMI and blood DNA methylation for over 400 000 CpG sites using the Framingham Heart Study and the Lothian Birth Cohorts. The authors used Mendelian randomization and genetic sequence variants to show that for a subset of CpG sites associated with BMI among elderly adults (16 out of 83), BMI likely altered DNA methylation, not the other way around. However, even with Mendelian randomization the cross-sectional nature of this study still limits definitive causal inference.[7] Taking full advantage of a longitudinal study design, Richmond et al.[8] tested for replication of associations between DNA methylation at CpG sites in Hypoxia Inducible Factor 3 Subunit Alpha (*HIF3A*) and adiposity using cord and peripheral blood samples from individuals in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort. Their findings provided evidence for a causal, positive effect of childhood BMI on *HIF3A* methylation levels in adolescents. However, this study also reported positive associations between maternal pre-pregnancy BMI on offspring *HIF3A* methylation, highlighting the importance of including measures of birth DNA methylation data whenever possible to account for the strong influence of gestational epigenetic programming on baseline levels.

We proposed 'environmental deflection' as a conceptual framework by which specific internal physiological factors or external toxicant exposures could affect the rate and direction of DNA methylation changes that occur over the life-course.[14] Environmental deflection refers to an environment-mediated (endogenous and/or exogenous) shift away from the baseline rate of age-related methylation or stochastic DNA methylation drift, e.g., gradual increases or decreases at specific loci in ageing cells and tissues within an organism.[14–16] Certain DNA methylation changes with age are so reliable and reproducible that researchers including Horvath and Raj[17] have developed estimators of age that are based on DNA methylation at specific sets of genes. They observed that individuals with an epigenetic age that is older than their chronological age exhibit accelerated biological ageing of underlying tissues and cells. Horvath and Raj.[17] More intriguingly, higher BMI and weight status were found to be associated with faster extrinsic epigenetic age acceleration in participants' blood.[17] A series of additional environmental factors, such as exposures to carcinogenic substances, smoking status, nutritionally induced oxidative stress, and traumatic stress are also associated with accelerated epigenetic ageing.[18–22] Hence, since obesity predisposes individuals to a pro-inflammatory and higher oxidative stress state,[3] it could alter the rate or direction of DNA methylation change that typically occurs with ageing, yet this question has rarely been explored in children.

Environmental deflection could manifest as either (1) shifting the rate of expected DNA methylation change at a locus known to increase or decrease with age or (2) shifting the methylation pattern of a gene over time that is typically stable with age.[14] With this in mind, we selected three regions for this pilot. Long interspersed nuclear element-1 (LINE-1) is a repetitive element that makes up 17% of the human genome and is used as a broad biomarker of DNA methylation status. LINE-1 is heavily methylated to prevent retrotransposition and is hypomethylated in cancers.[23] LINE-1 methylation decreases with age in adulthood,[24] and there is evidence for very small declines between birth and 9 years of age.[25] We also quantified DNA methylation at two growth-related genes that have previously been associated with adiposity in children—imprinted maternally expressed transcript (non-coding) *H19*; and non-imprinted hydroxysteroid (11-beta) dehydrogenase 2 *HSD11B2*.[26,27] *H19* is expected to be stable over time and across tissues,[28] while the ageing-related pattern of *HSD11B2* promoter methylation in children is unknown.

This pilot study leverages the Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) birth cohort to examine the associations between adiposity at two developmental stages and repeat longitudinal measures of DNA methylation from birth through adolescence (Figure 1). We quantified DNA methylation at LINE-1, *H19*, and *HSD11B2* via pyrosequencing. We estimated the associations between childhood BMI and early adolescent BMI, weight, or waist circumference with these repeat measures of DNA methylation in early and late adolescence. We then assessed the interaction between age and each anthropometric measure as a proof-of-concept to test whether adiposity could deflect age-related DNA methylation levels.

2 | METHODS

2.1 | Study population

The study population comprised a subset of participants from the ELEMENT project, a longitudinal epidemiological study consisting of

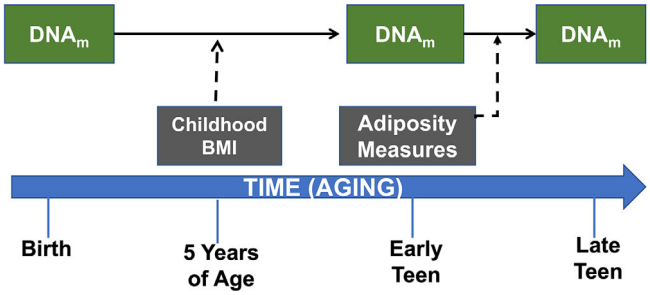

FIGURE 1 Conceptual framework and timing of measures. Baseline DNA methylation profiles at birth are shaped by a combination of the gestational environment and genetics. While DNA methylation remains stable at some genes across the life-course, others change with age. Whether environmental or physiological conditions alter or 'deflect' the rate or direction of this change with age is a new area of research. In a pilot sample from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) study, we quantified DNA methylation at LINE-1 repetitive elements and two growth and adiposity related genes (H19 and HSD11B2) via pyrosequencing at three developmental time periods. We assessed interactions between age and measures of children's adiposity (represented by the dashed arrows) to infer whether adiposity deflects age-related DNA methylation patterns. Adiposity measures, BMI, weight, and waist circumference; BMI, body mass index; DNAm, DNA methylation

three sequentially enrolled birth cohorts.[29] ELEMENT was originally designed to focus primarily on lead exposure and its impact on cognitive performance, as well as analyses of other metals and chemicals.[30,31] Epigenetics data were then added over time using archived samples.[29] As a brief overview, participants were recruited at three maternity hospitals (Instituto Mexicano del Seguro Social, Hospital Manuel Gea Gonzalez, and the National Institute of Perinatology) and clinics of the Instituto Mexicano del Seguro Social, representing low- to moderate-income populations in Mexico City from 1994 to 2005. Prior to participation, study procedures were explained to mothers and children. Mothers provided written consent upon enrolment in the study, and children also provided assent during the childhood and adolescent study visits. The research protocol was approved by the Human Subjects Committee of the National Institute of Public Health of Mexico, participating hospitals, and the Internal Review Board at all participating institutions including the University of Michigan.

The study subjects in this project were a subset of 1079 mother–child pairs from the second and third birth cohorts who had archived samples from birth and at least one other time point in mid-childhood/adolescence available for epigenetic analysis. Umbilical cord blood samples were collected shortly after birth and stored frozen at −20 to −24°C until analysis for a subset of these families. At the clinic visit after the child was born, mothers provided household and demographic information, including age, education, and previous numbers of pregnancies. The child's birth weight and gestational age were also obtained from medical records. Mother–child pairs were followed up at multiple timepoints throughout early childhood, mid-childhood, and adolescence.[29] Briefly, offspring were followed every 3 to 6 months from birth until 5 years of age. Starting in 2011, we re-contacted a subset of the offspring ($n = 250$) and brought them in for a follow-up visit, referred to as the 'early-teen' visit. Three to five years later, one additional follow-up visit ('late-teen' visit) was completed among 549 children, of whom 223 had participated in the 'early-teen' visit. Anthropometry information was measured at all visits, and fasting blood samples were collected at both teen visits. The time periods utilized in this study are depicted in Figure 1.

2.2 | Assessment of DNA methylation

DNA was isolated from umbilical cord blood nucleated cells and blood leukocytes from the 'late-teen' visit using Qiagen kits following standard protocols (Qiagen, Valencia, CA). For the 'early-teen' visit, DNA was isolated from blood leukocytes using the PaxGene Blood DNA kit (PreAnalytiX, Switzerland). All samples were bisulfite converted via the Epitect kit (Qiagen, Valencia, CA) or the EZ DNA Methylation kit (Zymo Research, Irvine, CA) as previously described.[32,33] Percent of DNA methylation was quantified at LINE-1, *H19*, and *HSD11B2*.[34] Percent DNA methylation was quantified via the pyrosequencing platform[35] using previously described assays (locations and primers described in Wu et al.[34]). Briefly, sequences were amplified from approximately 50 ng bisulfite-converted DNA using HotStartTaq Master Mix (Qiagen). Each PCR batch (experimental plate) contained at least two controls of known methylation status (0% and 100%). For pyrosequencing, the Pyro Q-CpG software was used to compute percent DNA methylation for 4–5 CpG sites per gene from the PyroMark ID Pyrosequencer (Qiagen), and this software incorporates internal quality control checks (e.g., bisulfite conversion control). To minimize the influence of batch effects on the accuracy of DNA methylation level quantifications, matched birth and 'early-teen' samples from each individual were included in the same pyrosequencing batch; meanwhile, all 'late-teen' samples were pyrosequenced at a later time period together in their own batch. Technical replicates had to pass a 10% coefficient of variance test or the samples were repeated.

2.3 | Anthropometry

Weight (kg) and height (cm) were measured at 5 years of age and at 2 adolescent visits and waist circumference at adolescent visits following Lohman standardized protocols.[36–38] BMI was calculated as weight over height squared (kg/m^2). BMI is commonly recommended as a practical estimate of obesity in children and adolescents. Abundant evidence has shown the main limitations of BMI include it measures excess weight rather than excess fat, and it does not provide information on fat distribution (Health Technology Assessment). Waist circumference assesses central adiposity, which is more closely associated with cardiometabolic risk.

2.4 | Covariates

Based on a priori knowledge, the study objective and the sample size limitation, covariates included in all final models were sex and age.

Since our focus is on the potential associations of adiposity with the rate of DNA methylation fluctuations with age,[14,39] we adjusted each participant's age by coding baseline age (the age when the predictor variable was collected) as 0 in each model. Age at the year-5 follow-up visit was considered the baseline age when using childhood BMI (kg/m^2) as the predictor; while 'early-teen' visit age was considered the baseline age when using early-teen BMI (kg/m^2), weight (kg), and waist circumference (cm) as predictors.

2.5 | Statistical methods

We first examined the distribution of age, sex, childhood BMI (calculated based on their measured weight and height) at age 5 years, as well as BMI, waist circumference, weight and height from the 'early-teen' visit, across subgroups with subjects who had cord, 'early-teen' or 'late-teen' DNA methylation levels available. For LINE-1, *H19*, and *HSD11B2*, we calculated descriptive statistics for DNA methylation at

TABLE 1 Demographic characteristics of ELEMENT participants included in the analyses

	ELEMENT participants			
Sample size of individual group	**Cord ($N = 113$)**	**Early-teen ($N = 79$)**	**Late-teen ($N = 96$)**	**ANOVA test (p value)**
Age (years)	$n = 113$	$n = 79$	$n = 96$	
	N/A	11.16 (1.90)	14.85 (2.10)	**<.0001**
Boys, *n* (%)	$n = 113$	$n = 79$	$n = 96$	
	60 (53.10)	41 (51.8)	54 (56.25)	.77
Predictors				
Childhood BMI (kg/m^2)	$n = 81$	$n = 66$	$n = 67$	
	16.68 (6.16)	16.94 (6.78)	16.71 (6.66)	.97
Early-teen BMI (kg/m^2)		$n = 79$		
		19.98 (4.24)		
Early-teen waist circumference (cm)		$n = 79$		
		72.46 (12.35)		
Early-teen weight (kg)		$n = 79$		
		42.75 (14.01)		
Repeat measures of DNA methylation loci				
LINE-1	$n = 78$	$n = 75$	$n = 94$	
Averaged Methylation of LINE-1	68.49 (3.90)	68.09 (3.74)	77.65 (3.72)	**<.0001**
Methylation of LINE-1, CpG 1	65.54 (4.46)	64.79 (5.03)	75.43 (5.64)	**<.0001**
Methylation of LINE-1, CpG 2	67.91 (3.27)	68.04 (2.71)	77.43 (2.65)	**<.0001**
Methylation of LINE-1, CpG 3	63.18 (5.78)	63.04 (5.05)	74.81 (4.73)	**<.0001**
Methylation of LINE-1, CpG 4	77.35 (5.67)	76.73 (5.06)[a]	83.33 (3.40)[a]	**<.0001**
H19	$n = 106$	$n = 79$	$n = 92$	
Averaged Methylation of *H19*	50.61 (2.84)	49.45 (2.52)	50.37 (3.45)	**.03**
Methylation of *H19*, CpG 1	53.14 (5.67)	51.81 (4.51)	52.00 (5.23)	.16
Methylation of *H19*, CpG 2	49.54 (2.16)	48.55 (2.13)	49.28 (3.55)[a]	**.04**
Methylation of *H19*, CpG 3	49.55 (2.80)	48.19 (2.62)	49.16 (4.52)[a]	**.03**
Methylation of *H19*, CpG 4	50.21 (2.62)	49.26 (2.55)	50.82 (3.21)[a]	**.002**
HSD11B2	$n = 86$	$n = 79$	$n = 93$	
Averaged Methylation of *HSD11B2*	2.24 (2.58)[a]	2.13 (2.49)[a]	2.74 (1.42)	.14
Methylation of *HSD11B2*, CpG 1	3.15 (3.21)	3.24 (2.90)	3.84 (1.24)	.15
Methylation of *HSD11B2*, CpG 2	1.05 (1.75)	0.80 (1.44)	2.24 (1.53)	**<.0001**
Methylation of *HSD11B2*, CpG 3	2.90 (4.05)	2.97 (3.81)	3.01 (1.66)[a]	.98
Methylation of *HSD11B2*, CpG 4	0.46 (1.17)	0.47 (1.22)	1.24 (1.37)[a]	**<.0001**
Methylation of *HSD11B2*, CpG 5	3.11 (3.72)[a]	2.99 (3.44)[a]	3.43 (2.02)[a]	.64

Note: p value <.05 are in bold.

[a]Specific sample size variations at certain CpG sites. This occurs from CpG sites near the end of the sequencing runs failing quality control in some samples. The fourth CpG site for LINE-1 failed in 2 early-teen and 5 late-teen samples. The fourth CpG site for *H19* failed in 2 late-teen samples. The last three CpG sites in *HSD11B2* failed in 1–4 samples per time point.

TABLE 2 Associations between early childhood BMI as well as early-teen BMI with repeated measures of cord, early-teen, and late-teen blood DNA methylation levels using linear mixed-effects models

		Model 1[a]		Model 2[b]			Model 3[c]		Model 4[d]	
		Childhood BMI					Early-teen BMI			
N	% of Methylation	$ß_3$ (95% CI)	*p* value	$ß_3$ (95% CI)	*p* value	*N*	$ß_3$ (95% CI)	*p* value	$ß_{33}$ (95% CI)	*p* value
LINE1										
78	Avg	0.02 (0.01, 0.03)	.65	0.02 (0.01, 0.03)	.67	78	0.05 (0.03, 0.07)	.53	0.05 (0.04, 0.07)	.48
78	CpG 1	0.07 (0.05, 0.08)	.31	0.06 (0.05, 0.07)	.24	78	0.08 (0.06, 0.11)	.50	0.07 (0.05, 0.10)	.51
78	CpG 2	0.00 (−0.01, 0.01)	.96	0.00 (−0.01, 0.00)	.95	78	0.05 (0.03, 0.06)	.45	0.03 (0.02, 0.04)	.53
78	CpG 3	0.01 (−0.01, 0.03)	.90	0.00 (−0.02, 0.01)	.98	78	0.04 (0.02, 0.07)	.72	0.07 (0.05, 0.09)	.50
78	CpG 4	0.02 (0.01, 0.04)	.74	0.02 (0.00, 0.03)	.77	78	0.08 (0.06, 0.11)	.46	0.08 (0.06, 0.11)	.46
H19										
81	Avg	−0.02 (−0.02, −0.01)	.66	−0.02 (−0.02, −0.01)	.66	79	−0.03 (−0.04, −0.01)	.71	−0.03 (−0.04, −0.01)	.71
81	CpG 1	−0.01 (−0.03, 0.00)	.81	0.01 (−0.02, 0.01)	.93	79	0.07 (0.04, 0.09)	.52	0.07 (0.04, 0.09)	.52
81	CpG 2	0.02 (0.01, 0.02)	.56	0.02 (0.01, 0.02)	.57	79	−0.08 (−0.09, −0.06)	.27	−0.08 (−0.09, −0.06)	.22
81	CpG 3	−0.07 (−0.08, −0.06)	**.04**	−0.06 (−0.07, −0.05)	**.06***	79	−0.02 (−0.04, −0.01)	.75	−0.03 (−0.04, −0.01)	.69
81	CpG 4	0.00 (−0.01, 0.00)	.94	−0.01 (0.00, 0.02)	.87	79	−0.07 (−0.09, −0.06)	.34	−0.07 (−0.09, −0.06)	.34
HSD11B2										
79	Avg	−0.01 (−0.02, −0.01)	.66	−0.01 (−0.01, 0.00)	.81	62	0.00 (−0.01, 0.01)	.97	0.00 (−0.01, 0.01)	.98
79	CpG 1	0.00 (−0.01, 0.01)	.95	0.00 (−0.01, 0.01)	.99	62	−0.01 (−0.02, 0.01)	.91	−0.01 (−0.02, 0.01)	.91
79	CpG 2	−0.02 (−0.03, −0.02)	.24	−0.01 (−0.02, −0.01)	.46	62	−0.01 (−0.02, −0.01)	.65	−0.02 (−0.02, −0.01)	.53
79	CpG 3	−0.02 (−0.03, −0.01)	.66	−0.01 (−0.02, 0.00)	.79	62	0.03 (0.01, 0.04)	.70	0.03 (0.01, 0.04)	.71
79	CpG 4	−0.02 (−0.02, −0.01)	.22	−0.01 (−0.01, 0.00)	.34	62	−0.01 (−0.02, −0.01)	.61	−0.02 (−0.02, −0.01)	.55
79	CpG 5	0.00 (0.00, 0.01)	.98	0.01 (0.00, 0.02)	.87	62	0.03 (0.01, 0.05)	.66	0.03 (0.02, 0.05)	.62

Note: *p* value <.05 are in bold.

[a]Model 1: % of Methylation $= ß_0 + ß_1 \times (\text{Age}) + ß_2 \times (\text{Sex}) + ß_3 \times (\text{Childhood BMI}) + (1|\text{Subject ID})$.

[b]Model 2: % of Methylation $= ß_0 + ß_1 \times (\text{Age}) + ß_2 \times (\text{Sex}) + ß_3 \times (\text{Childhood BMI}) + (1|\text{Subject ID}) + (1|\text{Batch ID})$.

[c]Model 3: % of Methylation $= ß_0 + ß_1 \times (\text{Age}) + ß_2 \times (\text{Sex}) + ß_3 \times (\text{Early-teen BMI}) + (1|\text{Subject ID})$.

[d]Model 4: % of Methylation $= ß_0 + ß_1 \times (\text{Age}) + ß_2 \times (\text{Sex}) + ß_3 \times (\text{Early-teen BMI}) + (1|\text{Subject ID}) + (1|\text{Batch ID})$.

*p value <0.1.

TABLE 3 Associations between early-teen waist circumference and weight status with repeat measures of cord, early-teen and late-teen, blood DNA methylation levels using linear mixed-effects model

		Model 1[a] Waist circumference		Model 2[b] Weight	
N	% of Methylation	ß (95% CI)	*p* value	ß (95% CI)	*p* value
LINE-1					
78	Avg	0.02 (0.01, 0.02)	.55	0.01 (0.00, 0.01)	.80
78	CpG 1	0.01 (0.00, 0.02)	.79	−0.00 (−0.01, 0.01)	.95
78	CpG 2	0.01 (0.01, 0.02)	.42	0.00 (0.00, 0.01)	.85
78	CpG 3	0.01 (0.00, 0.02)	.85	0.00 (−0.01, 0.00)	.92
78	CpG 4	0.05 (0.04, 0.06)	.24	0.02 (0.01, 0.02)	.64
H19					
79	Avg	−0.01 (−0.01, 0.00)	.70	−0.03 (−0.04, −0.03)	.11
79	CpG 1	0.02 (0.01, 0.03)	.56	−0.01 (−0.01, 0.00)	.84
79	CpG 2	−0.02 (−0.02, −0.01)	.40	−0.05 (−0.05, −0.04)	**.02**
79	CpG 3	−0.00 (−0.01, 0.00)	.85	−0.04 (−0.04, −0.03)	.12
79	CpG 4	−0.03 (−0.03, −0.02)	.31	−0.04 (−0.05, −0.03)	**.09***
HSD11B2					
62	Avg	0.00 (−0.00, 0.00)	.96	−0.00 (−0.01, 0.00)	.77
62	CpG 1	−0.00 (−0.01, 0.00)	.91	−0.01 (−0.01, −0.00)	.67
62	CpG 2	−0.01 (−0.01, −0.00)	.61	−0.01 (−0.01, −0.00)	.43
62	CpG 3	0.01 (0.00, 0.02)	.66	0.00 (−0.00, 0.01)	.97
62	CpG 4	−0.00 (−0.01, −0.00)	.62	−0.00 (−0.01, −0.00)	.73
62	CpG 5	0.01 (0.00, 0.31)	.74	0.00 (0.00, 0.01)	.93

Note: *p* value <.05 are in bold.

[a]Model 1: % of Methylation = $ß_0 + ß_1 \times$ (Age) + $ß_2 \times$ (Sex) + $ß_3 \times$ (Early-teen waist circumference) + (1|Subject ID) + (1|Batch ID).

[b]Model 2: % of Methylation = $ß_0 + ß_1 \times$ (Age) + $ß_2 \times$ (Sex) + $ß_3 \times$ (Early-teen weight) + (1|Subject ID) + (1|Batch ID).

*p value <.1.

individual CpG sites, as well as the average values of all sites in each region. The distribution comparisons of these variables across subgroups were performed using ANOVA tests.

We first assessed the main effect of childhood BMI and early adolescent BMI, weight, or waist circumference on repeat measures of DNA methylation. As such, we measured DNA methylation from three timepoints—birth (to account for baseline levels that participants were born with), 'early-teen', and 'late-teen'. To maximize the sample size as much as possible while keeping the same baseline measurement of DNA methylation for each participant, we included subjects that had either DNA methylation information from all three timepoints, or who had umbilical cord blood DNA methylation and one additional time point ('early-teen' or 'late-teen') in the analysis. Based upon previous literature evidence,[40] BMI at 5 years of age was selected as a proxy of the early childhood adiposity status. Moreover, due to limited total sample size and few participants categorized as obese, all weight-related outcomes were included as continuous variables, instead of creating categorical variables. We used linear mixed-effects models to examine the associations of BMI, weight, or waist circumference with repeat measures of DNA methylation at each loci, while accounting for age and sex (fixed effects) and random effects for intra-person variability and between-batch variability. While there is ample evidence that gestational age and maternal smoking impact offspring DNA methylation levels,[18–21] we did not adjust for these variables as they would be expected to impact 'baseline' birth DNA methylation which is one of the included repeat measures of DNA methylation in our study.

To examine evidence for environmental deflection of age-related DNA methylation trajectories by adiposity-related measures at key stages in childhood (5-years of age and early-teen), we ran a linear mixed-effects model with an interaction term between weight-related measures and age, including fixed effects for age, sex, and random effects for individual and batch. Coefficients with *p*-values smaller than .05 were considered statistically significant. All analyses were conducted using R software version 3.5.1 (cran.r-project.org). The lme4 package was used for modelling.[41]

3 | RESULTS

The ELEMENT cohort included 113 subjects who had DNA samples for epigenetic analysis at birth and at least one other time point (boys: 60 [53.1%], girls: 53 [46.9%]). Among those, 62 subjects had repeated measurements from all three time points; 17 subjects had DNA methylation measurements obtained from cord and early-teen blood

samples; and 34 subjects had DNA methylation measurements from cord and late-teen blood samples (Table 1). We observed some statistically significant differences in DNA methylation at CpG sites between age groups (Table 1). However, this effect did not remain statistically significant when adjusting for batch in linear mixed-effects models.

When averaging across all CpG sites and at CpG site 3, we observed consistent inverse associations between childhood or early-teen BMI and weight-related measures with *H19* DNA methylation. Most of these associations were not statistically significant via a standard *p* value cut-off of .05, though some demonstrated suggestive associations with *p* values <.1. For instance, for each kg/m^2 increase of early childhood BMI, we observed a 0.07%-point decrease on average across three repeat measures of *H19* methylation at CpG site 3, adjusting for age, sex and intra-person effect (*p* value = .04), or a 0.06% decrease when also adjusting for batch effects (*p* value = .06) (Table 2). We also observed suggestive evidence showing an association between early-teen weight and DNA methylation of *H19*. Specifically, for each 1 kg increase of early-teen weight, *H19* methylation at CpG site 2 and 3 decreased by 0.02% (*p* value = .07) and 0.03% (*p* value = .09) respectively, adjusting for age, sex, intra-person, and batch effects (Table 3). We did not observe any statistically significant associations of early-teen BMI and waist circumference with repeat measures of DNA methylation at any other sites of *H19* (Table 3). Our analysis results did not identify any evidence of associations between anthropometric outcomes and repeat measures of LINE-1 or *HSD11B2* DNA methylation.

We next modelled the interaction between adiposity measures and time to the last measure of DNA methylation (age) on DNA methylation to investigate evidence for environmental deflection of age-related DNA methylation by adiposity (represented by the dashed lines in Figure 1). None of the interaction terms were statistically significant (*p* value >.05; Table S1). However, this pilot study was underpowered to detect interactions, and several interactions had *p* values less than .2. There were positive interactions between age and early teen adiposity measures (BMI and waist circumference) in models of DNA methylation at *H19* CpG site 3. Effect estimates for the interaction terms were 0.013 ± 0.008 (*p* = .12) and 0.004 ± 0.003 (*p* = .20) for age × BMI and age × waist circumference, respectively. There were interaction terms with similar magnitude in models of *H19* CpG site 1 and also average of all *H19* sites. There was suggestive evidence that age and adiposity at the early teen visit interact to influence DNA methylation of *HSD11B2* CpG site 5. Effect estimates for the interaction with age were −0.013 ± 0.010 (*p* = .18), −0.004 ± 0.003 (*p* = .20), and −0.006 ± 0.003 (*p* = .10) for BMI, weight, and waist circumference, respectively. Future studies with appropriate power to detect interaction should follow up on these preliminary results.

4 | DISCUSSION

Few population-based longitudinal cohort studies have examined the association of childhood BMI and early adolescent BMI, weight, or waist circumference with levels of DNA methylation using repeat measures. Compared to cross-sectional studies, longitudinal cohorts with repeated epigenetic assessments enable higher statistical reliability and potentially provide information regarding directionality of disease-epigenome relationships. This is especially important in studies of epigenetics and adiposity given that epigenetic regulation of some genes can impact adiposity risk but the reverse is true for other genes.[7–9,13,26,42] There is a small but growing set of cohort studies that examined associations between environmental factors and age-related DNA methylation trajectories over time using repeat measures,[21,22] but they mainly focused on elderly participants. Here, we expanded on this existing literature by conducting a pilot analysis in a sample of adolescents with archived blood DNA from birth (cord blood) and two follow-up visits in adolescence. We observed an inverse association between measures of childhood BMI and repeat measures of *H19* DNA methylation, an imprinted gene that is expected to remain fairly stable over time. We also observed suggestive associations in the same, inverse direction between early-teen weight and repeat measures of *H19* DNA methylation. We do not report any statistical evidence for deflection of age-related DNA methylation by adiposity, though interactions between age and early teen adiposity on *H19* and *HSD11B2* with *p* value <.2 merit further investigation in larger studies.

We conducted this study because the epigenome can change with age, sometimes in a predictable way,[17] yet the gene–environment interactions that contribute to these changes are only now beginning to be identified. We previously used a mouse model to examine whether developmental bisphenol A (BPA) exposure, high-fat diet, and/or physical activity-related energy expenditure would lead to environmental deflection of age-related methylation.[43] We observed that western high fat diet (WHFD) as well as WHFD with BPA exposure had statistically significant impacts on trajectories of age-related DNA methylation at the Oestrogen Receptor 1 (*Esr1*) locus and at two repeat regions, Intracisternal A Particle (IAP), and LINE-1. The results from the present cohort study suggest that increased early childhood BMI and early-teen weight, which could be related to WHFD intake[44] and an altered cardiometabolic state, were associated with decreased repeat measures of *H19* DNA methylation from birth to adolescence on average. However, since the interactions between age and BMI or weight were not statistically significant in the second set of models, the results do not provide evidence for deflection. Given the estimates and *p* values of some interaction terms (*p* < .2) between early teen adiposity measures and age in models of both *H19* and *HSD11B2*, we recommend examination of this question in future studies with adequate statistical power.

Since the epigenome is reprogrammed shortly after fertilization, it is important to acknowledge that in utero environmental exposures can have long-lasting impacts on individual epigenetic profiles and contribute to the 'baseline' for each individual. While exposures during other developmental periods (i.e., infancy, childhood, adolescence) are not expected to have as great of an impact on epigenetic profiles compared with in utero exposures, cumulative and continued exposures and conditions such as obesity or inflammation may have subtle

impacts on maintenance of DNA methylation profiles in dividing cells, especially in tissues with high turnover, such as blood cells. DNA methylation profiles are important for health; associations between DNA methylation and risk for cardiometabolic complications, cancer, polycystic ovary syndrome, and more are widely published.[45] The reverse direction—the impact of disease states on DNA methylation—is also plausible but less well studied. One of the potential mechanisms by which adiposity could impact DNA methylation levels is through induction of oxidative stress (OS). It is widely accepted that accumulation of adipose tissue in the visceral compartment is considered an active endocrine organ, releasing a variety of biologically active adipocytokines or adipokines.[46] Due to the complex interplay between adipokines, overweight/obesity leads to chronic low-grade inflammation with permanently increased OS.[46] Meanwhile, other work has provided evidence that elevated OS can transiently alter the epigenome by modulating the activity of enzymes responsible for demethylation of DNA and deacetylation of histones.[47] Integrating the pieces of evidence above provides biological plausibility for the impact of early-life adiposity on maintenance of DNA methylation profiles with ageing. Future analyses on this hypothesis could incorporate biomarkers of OS along with longitudinal epigenetic profiling.

The results reported in this pilot study are largely null, yet several factors limited our ability to detect evidence for environmental deflection by adiposity. First, the sample size was restricted to participants with repeat archived DNA samples, and statistical power to detect interactions with small to medium effect sizes was extremely limited. None of the associations observed would be significant at a Bonferroni corrected p-value accounting for multiple testing ($p < .001$). Second, we quantified DNA methylation at only LINE-1 repetitive elements and two adiposity-related genes; as such, we are likely missing many key gene regions of interest including those that have been associated with BMI in children in other studies.[9] Third, cell type composition influences DNA methylation levels at many loci, and we did not have cell type differentials to adjust for at all time points of sample collection. Confounding bias from changing cell type composition is expected to be minimal for *H19*, an imprinted gene which is stable across tissues.[28] Fourth, unlike animal studies, cohort studies are complex and we may not be controlling for all important confounders or beneficial factors (e.g., micronutrients) that could protect against any detrimental effects of obesity on DNA methylation patterns. Finally, we included adiposity measures commonly collected in clinical settings, e.g., BMI, weight and waist circumference, expecting each to offer some insights in evaluating whether weight status and fat distribution may relate to changes in DNA methylation of growth-related genes across childhood and adolescence. Nevertheless, future research that relies on measures of adipose tissue via DEXA or MRI would provide more accurate information on total fat and fat distribution.

In summary, we conducted a pilot epidemiological study to examine the associations of early-life adiposity with repeated measurements of DNA methylation at three key developmental time periods (birth, early, and late-teen). We also estimated the interaction between childhood adiposity measures and age to investigate deflection of age-related DNA methylation levels through adolescence. While our results were largely null and the statistical power to detect interactions was low, we provide some preliminary evidence for the association between early-life adiposity and DNA methylation at *H19*, and potential interactions between age and early teen adiposity on *H19* and *HSD11B2* methylation. Future studies with larger sample sizes should incorporate multiple measures of DNA methylation across childhood, ideally using an epigenome-wide approach, in order to investigate deflection of age-related DNA methylation trajectories during childhood and adolescence by obesity. Remaining questions that could be assessed with such studies include whether: (1) the magnitude of effects vary according to obesity status (i.e. in obese versus normal weight children); (2) adiposity impacts DNA methylation at other growth-, visceral fat metabolism- or hormone related genes; (3) adiposity has a stronger effect at certain developmental periods, especially during childhood adiposity rebound timing[48]; and (4) additional endogenous and exogenous factors modulate the association.

It is known that a complex interplay between genetic and environmental factors influence child growth, development, and outcomes including childhood obesity. We often think of epigenetics as a molecular mechanism linking the child's broader environment to adverse health outcomes. It is now becoming clear that obesity itself can act like an adverse 'environmental exposure'—leading to inflammation, oxidative stress,[3] and a modified epigenome.[7] These subtle biological changes can propagate risk for further health complications. We believe it is important to understand the extent to which childhood obesity/adiposity modifies the child epigenome, as this is a molecular mechanism that can be targeted for further study to identify subtle alterations in biological pathways that could contribute to further metabolic complications as children age. Small effect sizes, as we observe here, are what is commonly observed in children's health studies involving epigenetics,[49] and the impact of such small effects across a multitude of genes could be important for health.

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CONFLICT OF INTEREST

No conflict of interest is declared.

ORCID

Alejandra Cantoral https://orcid.org/0000-0002-1397-8476

Karen E. Peterson https://orcid.org/0000-0003-0471-1427

Jaclyn M. Goodrich https://orcid.org/0000-0002-8289-9253

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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