Integrative Analysis of Gene-Specific DNA Methylation and Untargeted Metabolomics Data from the ELEMENT Cohort

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ABSTRACT: Epigenetic modifications, such as DNA methylation, influence gene expression and cardiometabolic phenotypes that are manifest in developmental periods in later life, including adolescence. Untargeted metabolomics analysis provide a comprehensive snapshot of physiological processes and metabolism and have been related to DNA methylation in adults, offering insights into the regulatory networks that influence cellular processes. We analyzed the cross-sectional correlation of blood leukocyte DNA methylation with 3758 serum metabolite features (574 of which are identifiable) in 238 children (ages 8-14 years) from the Early Life Exposures in Mexico to Environmental Toxicants (ELEMENT) study. Associations between these features and percent DNA methylation in adolescent blood leukocytes at LINE-1 repetitive elements and genes that regulate early life growth (*IGF2, H19, HSD11B2*) were assessed by mixed effects models, adjusting for sex, age, and puberty status. After false discovery rate correction (FDR $q < 0.05$), 76 metabolites were significantly associated with LINE-1 DNA methylation, 27 with *HSD11B2*, 103 with *H19*, and 4 with *IGF2*. The ten identifiable metabolites included dicarboxylic fatty acids (five associated with LINE-1 or *H19* methylation at $q < 0.05$) and 1-octadecanoyl-rac-glycerol ($q < 0.0001$ for association with *H19* and $q = 0.04$ for association with LINE-1). We then assessed the association between these ten known metabolites and adiposity 3 years later. Two metabolites, dicarboxylic fatty acid 17:3 and 5-oxo-7-octenoic acid, were inversely associated with measures of adiposity ($P < .05$) assessed approximately 3 years later in adolescence. In stratified analyses, sex-specific and puberty-stage specific (Tanner stage = 2 to 5 vs Tanner stage = 1) associations were observed. Most notably, hundreds of statistically significant associations were observed between *H19* and LINE-1 DNA methylation and metabolites among children who had initiated puberty. Understanding relationships between subclinical molecular biomarkers (DNA methylation and metabolites) may increase our understanding of genes and biological pathways contributing to metabolic changes that underlie the development of adiposity during adolescence.

KEYWORDS: Metabolic programming, epigenetics, DNA methylation, *IGF2, H19, HSD11B2*, LINE-1, adolescence, biomarkers, adiposity, children's health

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Introduction

Prevalent conditions such as obesity and cardiovascular and metabolic diseases arise from the complex interplay between genetics and environmental factors including diet, physical activity, and environmental toxicants. The developmental origins of health and disease (DOHaD) theory postulates that the early life environment influences metabolic programming and increases the risk for developing these conditions later in life.[1,2] Subclinical metabolic and epigenetic changes that occur in mid-childhood and adolescence could provide insight into the physiological processes that are altered early in life that ultimately lead to disease development.

The metabolome can serve as a surrogate composite measure of metabolism and biological function. Metabolomics have the potential to uncover mechanisms of disease development and may serve as predictive biomarkers of risk, including in children and adolescents.[3-9] For example, in the Early Life Exposure in Mexico to Environmental Toxicants (ELEMENT) cohort, 7 identifiable metabolites from untargeted serum metabolomics analysis among girls and 3 among boys (ages 8-14 years) were associated with age-specific risk for metabolic syndrome.[5] In Project Viva, branched chain

* Denotes equal contribution.

amino acids measured in children's serum at ages 6 to 10 years were associated with decreased glucose in boys and increased triglycerides in girls 5 years later.[7] The mechanisms by which the early life environment influences these metabolic patterns are relatively unknown and may involve epigenetic regulation.

Epigenetic modifications regulate gene expression without altering the genetic code, are heritable (mitotically and in some cases meiotically), and include DNA methylation and histone modifications. The epigenome is particularly sensitive to environmental perturbation during the early stages of gestation when disrupted patterns can be propagated across subsequent cell divisions. As such, associations between DNA methylation and gestational exposures to maternal dietary intake and various toxicants[10-18] suggest that epigenetic programming is 1 mechanism underlying the DOHaD. There is evidence that early life epigenetic changes program individuals to be susceptible to developing metabolic syndrome[19,20] and obesity.[21,22]

Epidemiological and animal-model studies provide evidence for relationships between the epigenome (specifically, DNA methylation) and the metabolome.[23-26] For example, in a cross-sectional study of 172 female twins aged 32 to 80 years with untargeted metabolomics and epigenome-wide DNA methylation data from the Infinium 27K BeadChip, circulating levels of C-glycosyltryptophan were associated with blood leukocyte DNA methylation at CpG sites in 3 genes. Interestingly, birth weight was associated with C-glycosyltryptophan in adulthood, and the association between this metabolite and DNA methylation in 1 of the 3 genes (*WDR85*) replicated in an additional sample of 350 adults from the same cohort.[24] Thus, epigenetic-metabolite relationships may play a role in complex phenotypes throughout the lifespan and may serve as a link between early life conditions and health and disease. Yet to our knowledge, no studies have examined relationships between DNA methylation and metabolites in children or adolescents.

In this study, we hypothesize that epigenetic regulation of environmentally-labile growth and adiposity-related genes is associated with metabolite profiles in childhood, and these metabolites are in turn associated with adolescent adiposity. To address these hypotheses, we examine relationships between DNA methylation and serum metabolites in children (ages 8-14 years) from the ELEMENT study (n = 238). We specifically assess associations between 3758 metabolites and venous blood leukocyte DNA methylation at 3 genes (*HSD11B2*, *IGF2*, and *H19*) and long-interspersed elements-1 (LINE-1), a global indicator of repetitive element DNA methylation status among all children and in stratified analyses by sex or pubertal stage. We then assess relationships between epigenetic-associated metabolites and measures of adiposity in ELEMENT children returning for follow-up on average 3 years later (n = 212).

Methods

Study population

This study used data from participants of Cohorts 2 and 3 of the ELEMENT project, a group of sequentially enrolled mother-child cohorts from 3 maternity hospitals in Mexico City.[27] Initially, 1459 women were recruited from 1997 to 2004 during pregnancy or at the time of delivery. Full details regarding all of the ELEMENT cohorts have been previously described.[27-30] Children were subsequently followed up at multiple visits from birth to adolescence. This paper includes data from 238 ELEMENT children who attended 1 follow-up visit between 2011 and 2012 at peripuberty (ages 8-14 years) and provided fasting blood leukocyte and serum samples for DNA isolation and metabolomics, respectively. At the peripubertal study visit, child age was reported, and fasting blood samples were obtained to isolate DNA and to analyze metabolite concentrations in serum. Most of the children (n = 212) attended an additional follow-up visit approximately 3 years later when anthropometry measures were repeated (BMI, waist circumference, skinfold thicknesses).

Prior to participation, study procedures were explained to mothers and children. Mothers provided written consent upon enrollment in the study, and children also provided assent. 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 the University of Michigan.

Assessment of DNA methylation

DNA was isolated from blood leukocytes using the PaxGene Blood DNA kit (PreAnalytiX, Switzerland) and bisulfite converted via the Epitect kit (Qiagen, Valencia, CA) or the EZ DNA Methylation kit (Zymo Research, Irvine, CA) as previously described.[31] Percent of methylated cells was quantified at LINE-1 and growth- and metabolism-related genes (H19 imprinted maternally expressed transcript (non-coding), *H19*; hydroxysteroid (11-beta) dehydrogenase 2 *HSD11B2*; and insulin-like growth factor 2, *IGF2*, a paternally expressed imprinted gene). These genes and the LINE-1 biomarker were selected due to evidence for environmental-lability by various exposures in early development[15,31-33] and for evidence of associations with adiposity-related outcomes in childhood or beyond.[34-36]

Percent DNA methylation was quantified via the pyrosequencing platform[37] using assays previously developed by us for LINE-1[38] and *HSD11B2*[15] and by others for *H19*.[33] Details on each region are included in Supplemental Table S1. Methylation at *IGF2* was quantified via the Sequenom EpiTYPER platform[39] using a previously developed assay.[40] Briefly, sequences were amplified from approximately 50 ng bisulfite-converted DNA using HotStartTaq Master Mix (Qiagen). Each PCR batch (experimental plate) contained at

least 2 controls of known methylation status (0 and 100%). For pyrosequencing, Pyro Q-CpG software was used to compute percent methylation reads at 4 to 5 CpG sites per gene from the PyroMark MD Pyrosequencer (Qiagen), and this software incorporates internal quality control checks (eg, bisulfite conversion control). Samples were run in duplicate with reads averaged. For *IGF2*, methylation was quantified at 5 cleaved units representing 7 total CpG sites via the Sequenom EpiTYPER platform.

Metabolomics

Metabolomics analyses were conducted at the Michigan Regional Comprehensive Metabolomics Resource Core (MRC^2). Fasting serum samples were collected in Mexico according to standard protocol, shipped to the US overnight on dry ice, and stored at −80°C until analysis. An untargeted assay was run in 8 batches of approximately 30 samples each using liquid chromatography-mass spectrometry (LC-MS) methodologies. Full details on the procedure have been previously described.[41] Samples were run in positive and negative electrospray ionization modes. Within- and between-batch normalization was achieved using spiked internal standards within each sample as well as sample pools run in each batch. Of the 9303 features detected, 3758 remained after removal of redundant compounds and those with >70% of values missing.

Anthropometry

Waist circumference was measured in duplicate to the nearest 0.1 cm with a non-stretchable tape (QM2000; QuickMedical).[42] Tricep and suprailiac skinfold thicknesses were measured in duplicate to the nearest 0.1 mm with a Lange skinfold caliper (Lange; Beta Technology). Child height was measured in duplicate to the nearest 0.1 cm with a stadiometer (Perspective Enterprises, Portage, WI, USA). Weight was measured with a Bamescale (model 420, Puebla, Mexico) and rounded to the nearest 0.1 kg. Age- and sex-specific BMI z-scores were calculated according to the World Health Organization criteria.[43] For all outcomes measured in duplicate, if intra-personal variability exceeded the measurement tolerance of ± 0.5 cm for waist circumference and height or 2.0 mm for skinfold thickness, an additional measurement was taken.

Covariates

Potential confounders in the relationship between metabolites and DNA methylation included maternal characteristics (age at birth of child, household socioeconomic status) and child characteristics (sex, age, pubertal status). A trained pediatrician assessed each child to determine Tanner stage on a scale of 1 (no development) to 5 (full development) for testicles, breasts, and pubic hair using standardized methods.[44] A dichotomous variable for pubertal onset was set to 1 if breast and/or pubic hair Tanner stages were >1 for girls, and if testicles or pubic hair Tanner stages were >1 for boys.

Metabolomics data processing

Metabolites were annotated by matching MS/MS fragmentation patterns, retention times, and ionization masses to metabolites within the MRC^2 compound library, allowing for the annotation of 574 known compounds. The remaining unannotated metabolites, considered unknown, were labeled by their neutral mass and retention times. Prior to formal analyses, systematic biases across batches were adjusted first by aligning the median of quality control samples in each batch with a global median,[45] and additional variations were accounted for by batch-specific random intercepts in the linear mixed-effects models. Metabolites were log-transformed, standardized using a z-score transformation (mean = 0, SD = 1), then imputed using the K-nearest-neighbour algorithm (K = 5) with the IMPUTE package in R. All 3758 metabolites were considered as outcomes in the statistical analysis.

DNA methylation data processing

DNA methylation at each gene region was examined for batch effects (ie, significant differences between experimental plates using ANOVA tests). All except the Sequenom-analyzed gene, *IGF2*, were found to exhibit batch effects in the bisulfite sequencing process. Therefore, methylation measurements for LINE-1, *HSD11B2*, and *H19* were standardized to controls of known methylation status included in each batch according to justified methods[15] and as we previously described.[31]

The quantification of DNA methylation involved several CpG sites for each region (4 for LINE-1, 5 for *HSD11B2*, and 4 for *H19*) or 5 units containing 7 total CpG sites for *IGF2* (due to the inability of the Sequenom EpiTYPER platform to resolve CpG sites on the same cleaved fragment). First, we considered a simple average of all sites or units at 1 region or a weighted average via principal component analysis (PCA). For LINE-1, *HSD11B2*, and *IGF2*, methylation differences in means and variances across CpG sites within the same region were apparent with low correlation between some of the sites. Then, to account for site-specific variability within each region we used a weighted average obtained by non-negative PCA transformation (NNPCA) available via the R package NSPRCOMP.[46-48] NNPCA constrains all factor loadings to be positive, which avoids cancellation of site-specific methylation in the resulting principal components (PCs). We chose 2 PCs (or weighted averages) for LINE-1, *HSD11B2*, and *H19* respectively, and 3 PCs for *IGF2* in the subsequent analyses.

Statistical analysis

All statistical analyses were conducted in the R software (version 3.3). Prior to modeling associations among metabolites and

DNA methylation outcomes, we examined the pairwise association of gene PCs with sociodemographic variables to identify potential confounders. Child's age, sex, and pubertal status were included in the LMM as these have been shown to be associated with metabolites in our previous work,[5] as well as DNA methylation at some loci in this study. Other available covariates were not associated with DNA methylation and/or metabolites. We fit a LMM to examine the association between each metabolite and DNA methylation PC pair —adjusting for child's age, sex, and pubertal status - where random intercepts were used to account for residual batch effects in the metabolite measurements. The R package NLME was utilized in the analysis involving 9 DNA methylation PCs and 3758 metabolites. To address the large number of hypotheses tested, we calculated q-values via the Benjamini-Hochberg method to account for false discovery rate.[49] A q-value less than 0.05 was considered a statistically significant metabolite-DNA methylation relationship.

Many studies using candidate gene DNA methylation as a predictor of outcomes model the data as averaged across CpG sites. Considering multiple PCs (also known as eigen-markers, but referred to as PCs in this study) allows us to not only preserve the maximum information of the original methylation data but also find signals that may not have been detected by a mean CpG site approach due to the averaging out of potentially opposite effects. The non-negative weights of NNPCA facilitate biological interpretation of the PCs as weighted sums of individual CpG sites. While we believe the NNPCA method better captures the variability in each genic region with no loss of interpretability, we ran the same association analysis replacing PCs (ie, weighted averages) with the mean DNA methylation (ie, equally weighted averages) across all CpG sites for each of the 4 regions in an effort to compare results with the more commonly used approach.

We conducted stratified analyses to explore whether there were sex- or pubertal status-specific associations between DNA methylation and metabolites. We re-ran the LMM stratified by (1) sex or by (2) pubertal status (no pubertal onset versus pubertal onset) to assess associations between DNA methylation PCs and metabolites. We considered associations with FDR $q < 0.05$ to be statistically significant.

Lastly among all children, we tested associations between known (identifiable) metabolites associated with DNA methylation and adiposity measures, for example, BMI z-score, waist circumference, triceps and suprailiac skinfold thicknesses measured at the follow-up visit. We applied multiple linear regression models, with the metabolites significantly associated with DNA methylation as predictors, adjusted for child age, sex and pubertal status at the first visit. We discuss estimated associations with P-value $<.05$.

Results

Descriptive statistics

Characteristics of the study population are summarized in Table 1. Of the 238 participants, 125 were female and 113 were male. Age at the peripubertal study visit ranged from 8 to 14 years. 41.9% of children had initiated puberty (Tanner stage >1) by the time of sample collection. At the follow-up visit, BMI z-score averaged 0.50 (SD = 1.28). Percent DNA methylation, averaged across the CpG sites included for each gene or region, is displayed in Table 1. Loadings for each PC from NNPCA are in Supplemental Table S2.

Table 1. Characteristics, DNA methylation at specific regions, and metabolites among peripubertal children from the early life exposures in Mexico to environmental toxicants (ELEMENT) cohort.

	N (%)	MEAN (SD)
Age (yrs)	238	10.43 (1.5)
Gender—Boys	113 (47.5)	
Gender—Girls	125 (52.5)	
Tanner Stage = 1	136 (58.1)	
Tanner Stage ⩾ 2	98 (41.9)	
DNA methylation (%)*		
Mean LINE-1 Methylation	233	78.5 (2.3)
Mean *HSD11B2* Methylation	236	9.2 (1.4)
Mean *IGF2* Methylation	219	45.2 (4.2)
Mean *H19* Methylation	235	58.3 (4.9)
Anthropometry at follow-up visit		
Age (yrs)—second visit	212	13.6 (1.7)
BMI for age z-score	211	0.5 (1.28)
Waist circumference (cm)	213	78.0 (10.6)
Triceps skinfold thickness (mm)	212	18.0 (6.8)
Suprailiac skinfold thickness (mm)	213	25.0 (13.8)

*DNA methylation levels standardized to batch for LINE-1, *HSD11B2*, and *H19*.

Associations between DNA methylation PCs and metabolites

We observed significant associations ($q < 0.05$) between known and unknown metabolites with PCs of each region (see Table 2 for known and Supplemental Table S3 for unknown metabolites). DNA methylation of LINE-1 repetitive elements was associated with 76 metabolites (20 with both PCs, 16 with PC1 only, and 40 with PC2 only). These included inverse associations with 2 dicarboxylic fatty acids and a positive association with 1-octadecanolyl-rac-glycerol. *HSD11B2* methylation was associated with 27 metabolites (3 with PC1, 24 with PC2), which included an inverse association with 16-bromo-9E-hexadecenoic acid. *H19* methylation was associated with 103 metabolites (one with both PCs, 21 with PC1, 81 with PC2). Inverse associations

Table 2. Significantly associated known metabolites with PCs of DNA methylation of LINE-1, *HSD11B2*, and *H19* ($q < 0.05$) among all children.

METABOLITE	SUPER PATHWAY	SUB PATHWAY	PC1 OF DNA METHYLATION		PC2 OF DNA METHYLATION	
			β (SE)	*Q*-VALUE	β (SE)	*Q*-VALUE
Associated with LINE-1						
FA 17:3 (DiC, diOH)	Lipid	Fatty Acid, Dicarboxylate, dihydroxy	−0.0737 (0.0152)	0.0000	−0.0709 (0.0186)	0.0349
1-octadecanoyl-rac-glycerol	Lipid	Monoacylglycerol	0.0499 (0.0136)	0.0439	0.0579 (0.0164)	0.0573
FA 10:1 (DiC)	Lipid	Fatty Acid, Dicarboxylate, hydroxy	−0.0613 (0.015)	0.0221	−0.0528 (0.0183)	0.1789
Associated with HSD11B2						
16-bromo-9E-hexadecenoic acid	Lipid	Halogenated fatty acids	−0.0764 (0.0207)	0.0439	−0.028 (0.0161)	0.5911
Associated with H19						
1-octadecanoyl-rac-glycerol	Lipid	Monoacylglycerol	−0.0311 (0.0066)	0.0000	0.011 (0.0102)	0.8020
FA 8:0 (DiC)	Lipid	Fatty Acid, Dicarboxylate	−0.03 (0.0072)	0.0000	0.0021 (0.0115)	0.9741
cycloheptane carboxylic acid	Organic acids and derivatives	Carboxylic Acid	−0.0281 (0.0073)	0.0221	0.0025 (0.0117)	0.9710
4-hydroxyphenylethanol	Amino acid	tyrosine metabolism pathway	−0.0242 (0.0078)	0.1310	−0.0483 (0.0118)	0.0221
FA 11:1 (DiC,OH)	Lipid	Fatty Acid, Dicarboxylate, hydroxy	−0.019 (0.0081)	0.3473	−0.0514 (0.012)	0.0000
FA 14:1 (DiC, OH)	Lipid	Fatty Acid, Dicarboxylate, hydroxy	−0.0073 (0.0071)	0.8201	−0.0462 (0.0114)	0.0221
5-oxo-7-octenoic acid	Lipid	Fatty acid, Keto	−0.0159 (0.0078)	0.4754	−0.0422 (0.0116)	0.0439

Models adjusted for child's age, sex, and pubertal status. There were no known metabolites associated with PCs of *IGF2* DNA methylation at $q < 0.05$. Only metabolites with at least one significant association at $q < 0.05$ are shown.

were observed with 3 dicarboxylic acids, 1-octadecanoyl-rac-glycerol, cycloheptanecarboxylic acid, 4-hydroxyphenylethanol, and 5-oxo-7-octenoic acid. Four unknown metabolites were associated with PC1 or PC2 of *IGF2* (Table S3).

DNA methylation means and metabolites

When gene-specific DNA methylation is the predictor in an analysis, the mean methylation across all included CpG sites in a region is often used. Thus, we ran a separate analysis using the DNA methylation means to compare overlap between results obtained with the NNPCA method (Supplemental Table S4). In general, our NNPCA analysis identified more statistically significant metabolites than the average DNA methylation analysis which is more commonly used. Of the significant metabolites in the mean analysis, 50 out of 77 for LINE-1, 2 out of 2 for *HSD11B2*, 22 out of 34 for *H19*, and 1 out of 1 for *IGF2* were also identified in the NNPCA analysis.

Sex-stratified analysis

In the sex-stratified analysis, DNA methylation at all 4 regions was associated with metabolites among both boys and girls (see Table 3 for number of associations with FDR $q < 0.05$ and Supplemental Table S5 for the list of metabolites with $q < 0.05$). Among boys, most of the statistically significant associations were with LINE-1 (Supplemental Table S5), including 2 saturated dicarboxylic fatty acids (11:0 and 9:0) with positive associations and an unsaturated dicarboxylic fatty acid (17:3) with an inverse association; the latter relationship also observed in all children (Table 2). Among girls in contrast to boys, most of the statistically significant associations were with *H19* or *HSD11B2* (Supplemental Table S5). Known metabolites inversely associated with *H19* methylation in girls include 5-oxo-7-ocetenoic acid and FA 8:0 DiC, which were also identified in the analysis of all children (Table 2).

Table 3. Number of metabolites significantly associated with DNA methylation at each region among all children and in stratified analyses ($q < 0.05$).

GENE	ALL CHILDREN	BOYS	GIRLS	PRE-PUBERTY	PUBERTAL ONSET
	N = 238	N = 113	N = 125	N = 136	N = 98
LINE-1					
PC1	36	30	5	16	99
PC2	60	28	2	4	111
HSD11B2					
PC1	3	2	2	0	7
PC2	24	0	28	3	13
H19					
PC1	22	2	13	9	242
PC2	82	1	15	0	175
IGF2					
PC1	1	2	1	0	0
PC2	3	5	2	1	1
PC3	0	0	0	1	0

Models of all children are adjusted for child's age, sex, and pubertal status. Sex-stratified models adjust for age and pubertal status. Puberty stage-stratified models adjust for sex and age. The number of metabolites from among 3758 associated with DNA methylation at the region at a false discovery rate of 5% ($q < 0.05$) are listed for each set of children. Pre-puberty was defined as Tanner stage = 1, and pubertal onset refers to Tanner stage = 2, 3, 4, or 5.

Pubertal stage-stratified analysis

When stratified by pubertal onset, a small number of statistically significant associations were observed among pre-pubertal children, primarily with PCs of LINE-1 and *H19* DNA methylation (Supplemental Table S6). Among pre-pubertal children, a primary bile acid metabolite (3,7-dihydroxy-5-cholestan-26-oic acid) was positively associated with LINE-1 methylation and a dicarboxylic fatty acid (11:1) was inversely associated with LINE-1, with additional associations between LINE-1 and fourteen unknown metabolites. The same dicarboxylic fatty acid was inversely associated with *H19* methylation among children with pubertal onset.

More significant associations were observed between DNA methylation and metabolites in the pubertal onset group (Table 3), including 137 and 305 metabolites associated with PC1 and/or PC2 of LINE-1 and *H19* methylation, respectively (Supplemental Table S6). Overlaps were observed in the all-children analysis including the known metabolites 5-oxo-7-octenoic acid, 4-hydroxyphenylethanol, and several dicarboxylic fatty acids. Additional metabolites associated with LINE-1 among pubertal children include a phosphatidylcholine, a phosphatidylserine, pinitol, methyl beta-d-galactoside, and taurolithocholate. In the pubertal onset group, 38 named metabolites and 267 unknown metabolites were associated with *H19* methylation. Inverse associations were observed with 22 saturated and monounsaturated dicarboxylic fatty acids with chain lengths from 8 to 20; this is 37% of dicarboxylic fatty acids captured in the entire dataset. The fatty acid oxidation metabolite, N-undecanoylglycine, was inversely associated with *H19*. Other metabolites associated with *H19* included 5-methoxytryptophol, 4-acetamidobutanoate, diethyl 2-methyl-3-oxosuccinate, methyl 8-2-2-formyl-vinyl-3-hydroxy-5-oxo-cyclopentyl-octanoate, porphobilinogen, and metabolites that likely stem from diet, supplement, or pharmaceutical use (benzoin, chloropheniramine, ibuprofen, and salsolinol).

Metabolites and child's adiposity measures

We examined relationships between the ten known metabolites associated with DNA methylation of at least 1 gene among all children ($q < 0.05$) and adiposity measured approximately 3 years later. Fatty acid intermediates, FA 17:3 (DiC, diOH) and 5-oxo-7-octenoic acid, were both inversely associated with adiposity measures ($P < .05$; Table 4). Both were associated with decreased skinfold thickness, and FA 17:3 (DiC, diOH) was also associated with decreased waist circumference.

Discussion

We observed statistically significant associations between serum metabolite levels and blood leukocyte DNA methylation of environmentally-labile genes related to physical growth and

Table 4. Associations between metabolites and child adiposity measures (model estimates and their SE are shown).

KNOWN METABOLITE	BMI FOR AGE *Z*-SCORE	WAIST CIRCUMFERENCE	TRICEPS SKINFOLD	SUPRAILIAC SKINFOLD
FA 10:1 (DiC)	−0.067 (0.088)	−1.117 (0.739)	−0.023 (0.442)	−1.017 (0.918)
FA 17:3 (DiC, diOH)	−0.095 (0.087)	−1.478 (0.732)*	−0.346 (0.443)	−1.936 (0.906)*
1-octadecanoyl-rac-glycerol	−0.137 (0.087)	−0.834 (0.739)	−0.843 (0.438)	−1.331 (0.914)
16-bromo-9E-hexadecenoic acid	−0.072 (0.086)	−0.337 (0.736)	0.018 (0.439)	−1.113 (0.91)
4-hydroxyphenylethanol	0.067 (0.087)	0.383 (0.743)	0.254 (0.442)	0.276 (0.921)
FA 8:0 (DiC)	0.077 (0.089)	0.801 (0.752)	0.31 (0.452)	0.885 (0.933)
FA 11:1 (DiC,OH)	0.054 (0.086)	0.432 (0.73)	0.313 (0.435)	0.421 (0.905)
FA 14:1 (DiC, OH)	−0.004 (0.087)	−0.622 (0.736)	0.059 (0.439)	−1 (0.911)
5-oxo-7-octenoic acid	−0.095 (0.086)	−1.412 (0.724)	−1.081 (0.429)*	−2.403 (0.891)**
cycloheptane carboxylic acid	0.068 (0.089)	0.783 (0.751)	0.296 (0.451)	1.022 (0.931)

Adiposity (BMI for age z-score, waist circumference [cm], and triceps or suprailiac skinfold thickness [mm] at the second adolescent study visit were modeled as outcomes adjusting for age, pubertal status, and sex among all children.
*$P < .05$, **$P < .01$.

metabolism in a sample of Mexican adolescents. Out of 3758 metabolites including 574 knowns, 76 metabolites were associated with DNA methylation of LINE-1, 27 metabolites with *HSD11B2*, 103 with *H19*, and 4 with *IGF2* at $q < 0.05$. The known identities among these were 7 fatty acids, a monoacylglycerol, a carboxylic acid, and a metabolite of the tyrosine metabolism pathway. Dicarboxylic fatty acids were inversely associated with LINE-1 and *H19* DNA methylation, which may reflect an increase in extra-mitochondrial fatty acid oxidation. Two metabolites - 5-oxo-7-octenoic acid and FA 17:3 DiC—were also inversely associated with adiposity measured in adolescence, suggesting a role for these metabolites as biomarkers or predictors of adiposity in children. Analyses stratified by sex revealed some statistically significant associations among girls and boys, though most were with unknown metabolites or with named metabolites that had already been identified in the analysis with all children. The analysis stratified by pubertal stage revealed hundreds of statistically significant associations between known and unknown metabolites, primarily with LINE-1 or *H19* DNA methylation, among children in the puberty onset group (Tanner stage>1). In particular, the relationship between *H19* DNA methylation and metabolites relevant to fatty acid oxidation and metabolism was evident in this group.

DNA methylation of LINE-1 repetitive elements serves as a partially representative biomarker of genome-wide DNA methylation.[50] LINE-1 is a family of repetitive elements that comprises nearly 17% of the human genome; these elements are typically hypermethylated to prevent retrotransposition and genomic instability.[51,52] LINE-1 methylation levels are associated with health outcomes as diverse as pubertal timing and cancer.[53,54] Dicarboxylic fatty acids, inversely associated with LINE-1 methylation, are formed as intermediates in extra-mitochondrial fatty acid oxidation within the endoplasmic reticulum and the peroxisomes,[55] potentially suggesting a link between a broad decrease in DNA methylation across the genome and increases in extra-mitochondrial fatty acid oxidation. The mechanism underlying this association is currently unknown. It is possible that LINE-1 methylation is serving as a proxy of DNA methylation of other environmentally-labile genes, some of which may play functional roles in fatty acid oxidation pathways. Previous studies have suggested a relationship between mitochondrial β-oxidation and extra-mitochondrial ω-oxidation with metabolic health in adolescents. Butte et al. observed decreases in dicarboxylic fatty acids in obese versus non-obese Hispanic children with an average age of 11 years (n = 803).[9] In a previous study of ELEMENT children, fat intake was associated with increased insulin resistance, and this seemed to be related to increased omega oxidation.[56] In the present study, the dicarboxylic FA 17:3 with 2 hydroxyl groups was inversely associated with waist circumference and skinfold thickness in adolescence, supporting that some dicarboxylic fatty acids may be a biomarker of adiposity. Whether measurement of dicarboxylic fatty acids in childhood could serve as an earlier or better biomarker of adiposity and its complications compared to typical clinical biomarkers remains to be tested.

In this study, we observed methylation at *H19* was associated with the largest number of metabolites (Supplemental Table S3), including a pattern of inverse associations with dicarboxylic fatty acids, a monoacylglycerol, carboxylic acid, and a tyrosine metabolite (4-hydroxyphenylethanol). When stratifying by pubertal status, we observed hundreds of statistically significant associations (Supplemental Table S6) between *H19* and metabolites among children who had started puberty (Tanner stage >1). The significant metabolites included 22 medium to long chain dicarboxylic fatty acids, comprising 37% of all dicarboxylic fatty acids captured in the dataset. DNA methylation at

IGF2 and *H19* was assessed at well-characterized differentially methylated regions (DMRs) that are critical for their unique regulation as imprinted genes.[33,57] There is evidence that epigenetic alteration at these DMRs from the early life environment is persistent—even into late adulthood[58]—and contributes to adverse health outcomes including low birth weight, adolescent adiposity, and adiposity in post-menopausal women.[34-36,57] While epigenetic regulation of *IGF2* and *H19* seem to play a role in obesity and metabolic conditions later in life, how or whether this starts to develop in childhood or adolescence is unknown. Examined separately, lower levels of dicarboxylic fatty acids have been previously associated with obesity in children[5,9] and *H19* methylation is positively associated with adiposity including among 17-year old children. [34,35] Our findings suggest DNA methylation and subsequently expression of *H19* may influence the formation of dicarboxylic fatty acids and development of adiposity and metabolic risk, especially among children who have initiated puberty. The biological pathway by which *H19* may affect fatty acid oxidation is currently unknown and an area of interest for future research. In this study, *H19* DNA methylation was also associated with decreased 5-oxo-7-ocetenoic acid; a medium chain unsaturated fatty acid that was inversely correlated with adolescent skinfold thickness in the same children. While the function of 5-oxo-7-ocetenoic acid is currently unknown, another straight-chain fatty acid of the same length (octanoic acid) inhibits adipogenesis.[59]

The application of metabolomics to children's health research has led to the identification of sets of metabolites associated with obesity,[8,60] insulin resistance,[56] and overall risk for metabolic syndrome[5] in children and adolescents. These metabolites hold promise for future development into screening tools to identify children at risk and for increasing understanding of disrupted biological pathways that lead to excessive adiposity and metabolic disorders. Recent rodent and epidemiological studies of adults have unveiled associations between the epigenome and metabolome that provide insight into early developmental epigenetic programming of metabolic pathways.[23-25,61] Combining epigenetic and metabolomics analyses in studies of children will accelerate our understanding of how early life epigenetic programming leads to the development of complex metabolic conditions later in life and will aid in the identification of key pathways to target to disrupt disease development. In these types of analyses, development stage of the children, especially pertaining to pubertal onset, is important to stratify by whenever possible.

There are several strengths to this study. Batch effects in both metabolite and DNA methylation data were addressed statistically to minimize error as batch effects are known to be problematic in both metabolomics and pyrosequencing analysis methods.[15,62] LMMs are a well-established tool for modeling covariance between measurements and lend themselves to adjustment for batch for the dependent variable via random intercept. For the primary analysis, NNPCA was used on all CpG sites within each gene instead of averaging methylation at CpG sites within the same region as the lability and the inter-individual variability of DNA methylation at each CpG site can differ.[63] We were able to annotate 574 of the 3758 metabolites included in the analysis.

Limitations of this study include the limited number of loci selected for DNA methylation analysis. With this candidate gene approach, we did not capture data on all genes and non-coding regions that are involved in metabolism. Moreover, DNA methylation at only 1 time period was quantified in 1 tissue type (blood leukocytes). Given the timing of the epigenetic and metabolomics analyses, reverse causation is possible for associations between DNA methylation and metabolites (ie, for associations between pharmaceutical metabolites and DNA methylation observed in the children with pubertal onset). The process of puberty and lifestyle changes when entering adolescence may induce epigenetic changes at many genes.[64] While we performed sex-stratified and pubertal onset-stratified analyses, our sample size precluded stratification by pubertal status within boys and girls separately. Such stratification will be important in future studies given the vast biological differences between boys and girls that emerge during and after puberty that influence metabolism and adiposity.

Future studies should emphasize longitudinal measurements of DNA methylation and utilize an epigenome-wide platform to identify novel genes or gene networks that are environmentally labile and influence growth and adiposity. Identification of these genes or non-coding regions would provide insight into the biological mechanisms influencing the developmental origins of adiposity and metabolic outcomes in childhood and adolescence. Ultimately, this knowledge will improve our understanding of subclinical indicators of metabolic health and biological pathways that can be screened and targeted earlier in life to break the chain of cardiovascular and metabolic disease risk as children age into adulthood.

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Authors' Contributions

EH, JG, and KP contributed to conception and design of the study. KP and MT designed and maintained the cohort and AM and AC collected data from the study participants. JG, DC, and JL performed epigenetic and metabolomics analyses. EH performed the statistical analysis with input from LT, JL, and PS. JG and EH wrote the first draft of the manuscript. All authors contributed to the final version of the manuscript and approved the submission.

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Supplemental material

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