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# Early pregnancy dyslipidemia is associated with placental DNA methylation at loci relevant for cardiometabolic diseases

Marion Ouidir<sup>1</sup> , Xuehuo Zeng<sup>2</sup>, Tsegaselassie Workalemahu<sup>1</sup>, Deepika Shrestha<sup>1</sup>, Katherine L. Grantz<sup>1</sup>, Pauline Mendola<sup>1</sup>, Cuilin Zhang<sup>1</sup> & Fasil Tekola-Ayele<sup>\*1</sup>

<sup>1</sup>Epidemiology Branch, Division of Intramural Population Health Research, Eunice Kennedy Shriver National Institute of Child Health & Human Development, National Institutes of Health, Bethesda, MD 20892-7004, USA

<sup>2</sup>Division of Intramural Population Health Research, Eunice Kennedy Shriver National Institute of Child Health & Human Development, National Institutes of Health, Bethesda, MD 20892-7004, USA

\*Author for correspondence: [ayeleft@mail.nih.gov](mailto:ayeleft@mail.nih.gov)

**Aim:** To identify placental DNA methylation changes that are associated with early pregnancy maternal dyslipidemia. **Materials & methods:** We analyzed placental genome-wide DNA methylation (n = 262). Genes annotating differentially methylated CpGs were evaluated for gene expression in placenta (n = 64). **Results:** We found 11 novel significant differentially methylated CpGs associated with high total cholesterol, low-density lipoprotein cholesterol and triglycerides, and low high-density lipoprotein cholesterol. High triglycerides were associated with decreased methylation of cg02785814 (*ALX4*) and decreased expression of *ALX4* in placenta. Genes annotating the differentially methylated CpGs play key roles in lipid metabolism and were enriched in dyslipidemia pathways. Functional annotation found *cis*-methylation quantitative trait loci for genetic loci in *ALX4* and *EXT2*. **Conclusion:** Our findings lend novel insights into potential placental epigenetic mechanisms linked with maternal dyslipidemia.

**Trial Registration:** [ClinicalTrials.gov](https://clinicaltrials.gov), NCT00912132.

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**Keywords:** cardiometabolic diseases • dyslipidemia • epigenome-wide association study • lipids • placental DNA methylation • placental gene expression

Dyslipidemia during pregnancy has been linked with increased risk of pregnancy loss, preeclampsia, gestational diabetes, preterm birth, large for gestational age fetuses and high birth weight babies [1–6]. Studies have shown that maternal dyslipidemia may program lipid metabolism in the offspring and contribute to the development of adult cardiometabolic diseases [7,8]. Offspring exposed to maternal dyslipidemia during fetal life may be at higher risk of cardiometabolic diseases in later life [9–12], such as dyslipidemia, atherosclerosis, hypertension, obesity and Type 2 diabetes. This concept of fetal programming of cardiometabolic diseases in later life is well documented [13], however the mechanisms are not clearly understood.

As a mediator of the maternal-fetal exchange, the placenta can play a role in the programming of health and disease in adulthood [14,15]. Maternal dyslipidemia may impact placental growth and function [16]. For instance, our group previously found that low maternal high-density lipoprotein cholesterol (HDLc) status was associated with accelerated epigenetic aging of the placenta [17], which demonstrate epigenetic modification in the placenta linked to maternal lipid levels. Recent studies have suggested that placental epigenetic alterations could be the mechanistic links between the intrauterine environment during fetal life and health outcome in later life [15,18,19]. Two candidate gene studies reported that lower first trimester maternal HDLc blood levels was associated with higher placental DNA methylation levels at the lipoprotein lipase (LPL) gene locus [20], and lower third trimester HDLc was associated with higher placental DNA methylation levels at the *ABCA1* gene locus [21]. Higher placental DNA methylation at *ABCA1* locus was associated with lower *ABCA1* mRNA levels [21], suggesting that placental epigenetic alterations may contribute to regulation of cholesterol transfer from the mother to the fetus [22]. However,

to our knowledge, there is no published epigenome-wide association study (EWAS) of maternal dyslipidemia in placenta.

In the present study, we performed an EWAS to identify placental DNA methylation of cytosine-(phosphate)-guanine (CpG) sites associated with maternal dyslipidemia status (i.e., high plasma total cholesterol, low high-density lipoprotein cholesterol [HDLc], high low-density lipoprotein cholesterol [LDLc] and high triglycerides) in early pregnancy. We also tested correlations between the DNA methylation levels of the top significant CpG sites and expression of the corresponding annotated genes in placenta. Finally, we evaluated whether CpG sites which were previously implicated in lipid traits in adult blood, were differentially methylated in placenta in relation to maternal dyslipidemia in our study.

## Methods

### Study population

The *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) Fetal Growth Studies – Singleton cohort included pregnant women aged 18 to 40 years old with viable singleton pregnancy, who planned to deliver at participating clinic sites. Specifically, the study included 2334 nonobese pregnant women (pre-pregnancy body mass index [BMI] between 19.0 to 29.9 kg/m<sup>2</sup>) and 468 obese pregnant women (pre-pregnancy BMI between 30.0 and 44.9 kg/m<sup>2</sup>) [23]. In both groups, pregnant women were excluded if they reported having major medical condition (such as cancer, autoimmune disease, diabetes, HIV or AIDS, chronic renal disease and psychiatric disorder). In addition, nonobese pregnant women were excluded if they reported chronic hypertension, past adverse pregnancy outcomes and self-reported behavioral risk factors such as use of cigarettes, illicit drugs or alcohol in the months prior to pregnancy. Women were enrolled between 8 weeks and 0 days (8w0d) and 13 weeks and 6 days (13w6d) between July 2009 and January 2013 from 12 clinic sites within the USA. The study was approved by institutional review boards at NICHD, each participating clinical sites and data coordinating centers. Placental samples were available from 312 women and the final analytical population was composed of 262 women who had plasma lipid levels at enrollment and placental DNA methylation data (see below for details). None of these women took lipids lowering medication.

### Maternal blood lipid levels

Plasma lipids were measured using maternal nonfasting blood samples collected at enrollment (mean  $\pm$  standard deviation [SD]: 12.7  $\pm$  0.9 gestational weeks, Table 1). Total cholesterol, HDLc and triglyceride concentrations were directly measured from plasma stored in -80°C freezers [24], using the Roche COBAS 6000 chemistry analyzer (Roche Diagnostics, IN, USA). The inter-assay laboratory coefficients of variation were 2.2, 3.2 and 2.3% for total cholesterol, HDLc and triglycerides, respectively. LDLc was calculated using the Friedewald formula [25]. Lipid levels were dichotomized using the third report of the National Cholesterol Education Program (NCEP III) criteria from nonpregnant population, as there is no clear reference for lipid levels during pregnancy [26]. Total cholesterol as normal total cholesterol (<200 mg/dl) versus high total cholesterol ( $\geq$ 200 mg/dl), HDLc as normal HDLc (>50 mg/dl) versus low HDLc ( $\leq$ 50 mg/dl), LDLc as normal LDLc (<100 mg/dl) versus high LDLc ( $\geq$ 100 mg/dl) and triglycerides as normal triglycerides (<150 mg/dl) versus high triglycerides ( $\geq$ 150 mg/dl).

### Placental DNA methylation measurement & quality control

Placental samples (n = 312) were obtained within one hour of delivery. Placental parenchymal biopsies measuring 0.5 cm  $\times$  0.5 cm  $\times$  0.5 cm were taken from the fetal side, collected directly below the fetal surface of the placenta. Samples were placed in RNALater and frozen for molecular analysis. Processing of the placental biopsies was performed at the Columbia University Irving Medical Center and details have been described previously [27]. DNA was extracted from the placental biopsies and was assayed using Illumina's Infinium Human Methylation450 Beadchip (Illumina Inc., CA, USA). Standard Illumina protocols were followed for background correction, normalization to internal control probes and quantile normalization. The resulting intensity files were processed with Illumina's GenomeStudio, which generated average  $\beta$ -scores (i.e., the fraction of methylated sites per sample by taking the ratio of methylated and unmethylated fluorescent signals at each queried CpG) and detection p-values (that characterize the chance that the target sequence signal was distinguishable from the negative controls). Stringent quality control procedures were followed as previously described [28]. A total of 301 placental samples and 409,101 CpGs that passed the quality control filters were included in the EWAS. Of the 301 pregnant women with placental DNA methylation data that passed quality filters, 262 women who had blood lipid levels at enrollment were included in

**Table 1. Characteristics of study final analytical population (n = 262).**

Characteristics	Mean ± SD or n	(%)
<b>Maternal age, years</b>	27.5 ± 2.5	
<b>Gestational age at enrollment, weeks</b>	12.7 ± 0.9	
<b>Maternal prepregnancy BMI</b>		
– <25 kg/m <sup>2</sup>	183	(69.8)
– ≥25 and <30 kg/m <sup>2</sup>	71	(27.1)
– ≥30 kg/m <sup>2</sup> (obese women)	2	(0.8)
– Missing	6	(0.4)
<b>Maternal race/ethnicity</b>		
– Non-Hispanic White	65	(24.8)
– Non-Hispanic Black	61	(23.3)
– Hispanic	88	(33.6)
– Asian	48	(18.3)
<b>Parity</b>		
– 0	127	(48.5)
– 1 or more	135	(51.5)
<b>Infant sex</b>		
– Male	131	(50)
– Female	131	(50)
<b>Maternal dyslipidemia</b>		
– High total cholesterol (≥200 mg/dl)	77	(29.4)
– Low HDLc (≤50 mg/dl)	44	(16.8)
– High LDLc (≥100 mg/dl)	122	(46.6)
– High triglycerides (≥150 mg/dl)	52	(19.8)

Lipid levels dichotomized using the third report of the National Cholesterol Education Program (NCEP III) criteria [26].  
HDLc: High-density lipoprotein cholesterol; LDLc: Low-density lipoprotein cholesterol; SD: Standard deviation.

these analyses. Raw DNA methylation  $\beta$ -values are prone to heteroscedasticity. For improved model fitting in our analyses,  $\beta$ -values were converted to M-value scale by using the formula:  $M\text{-value} = \log_2(\beta/[1-\beta])$ . All analyses were performed by fitting the methylation M-values as recommended [29]. Any resulting infinity or missing M-values (6.0%) were imputed by the  $k$ -nearest neighbors method, setting  $k = 10$  [30].

### Placenta RNA quantification for gene expression

RNA was available for 80 placentas (42 male and 38 female) and was extracted using TRIZOL reagent (Invitrogen, MA, USA) and sequenced using the Illumina HiSeq2000 system. The expression of the transcripts were quantified using Salmon [31], which accounts for experimental attributes and biases such as fragment GC-content bias that are commonly observed in RNA-seq data. Participants with RNA-seq, DNA methylation and blood lipids level at enrollment (n = 64) were used to test correlation between DNA methylation and mRNA gene expression levels.

### Statistical analysis

#### *Epigenome-wide analyses*

EWAS's were performed for each dyslipidemia trait (high total cholesterol (≥200 mg/dl), low HDLc (≤50 mg/dl), high LDLc (≥100 mg/dl) and high triglycerides (≥150 mg/dl)) as a predictor and placenta DNA methylation at each CpG site as the response variable using the R/Bioconductor package limma [32]. Placental genome-wide SNP genotype data were used to estimate ten genotype-based principal components (PCs) representing population structure. The R package 'prcomp' was used to calculate PCs on the samples' percent methylation profiles [33]. EWAS analyses were implemented using adjusted robust linear regression models that use iterated re-weighted least squares [34]. EWAS were adjusted for self-reported maternal race/ethnicity, maternal age in years, offspring sex, methylation sample plate (n = 5), the first three methylation PCs and the first ten genotype PCs to account for population structure [35] and putative cell-mixture estimated using Surrogate Variable Analysis (SVA) components

( $n = 20$ ) to account for latent source of noise such as batch effects and cell composition [36–38]. To account for the inflation of statistical test in EWAS, we implemented the Bayesian method to obtain BACON-corrected inflation estimates ( $B\lambda$ ) and BACON-corrected p-values using the R/Bioconductor package BACON [39]. Corrected p-values from BACON were then controlled for false discovery rate (FDR), giving BACON-corrected FDR p-values. Quantile-quantile plots of p-values and the corresponding inflation estimates before ( $\lambda$ ) and after ( $B\lambda$ ) BACON-correction are reported in Supplementary Figure 1. The inflation statistic was close to one after BACON correction (Supplementary Figure 1). Genome-wide Manhattan plots were used to report results from EWAS (Figure 1).

#### *Gene expression analyses*

We estimated the correlation between placenta DNA methylation and mRNA gene expression levels of the corresponding annotated genes. Significant statistical correlations were considered with FDR p-values  $< 0.05$ . Association between mRNA levels of the genes mapping to dyslipidemia-associated CpGs in our study (BACON-corrected FDR p-values  $< 0.05$ ) and the corresponding dyslipidemia trait (high total cholesterol, low HDLc, high LDLc and high triglycerides) was tested using the R/Bioconductor package DESeq2 [40]. DESeq2 implemented negative binomial generalized linear models and estimated dispersion and logarithmic fold changes to quantify differential expression. Adjustment factors in the model included maternal race/ethnicity, maternal age in years, fetal sex and the first ten genotype PCs. The Benjamini–Hochberg adjusted p-value of the Wald test was used to correct for multiple testing.

#### *Canonical pathway analysis*

Genes annotating the top epigenome-wide significant CpGs (BACON-corrected FDR p-values  $< 0.05$ ) were further explored. We identified canonical pathways, networks, and disease and biological functions involved using the ‘Core Analysis’ function in Ingenuity Pathway Analysis (IPA, Qiagen, CA, USA, [www.qiagen.com/ingenuity](http://www.qiagen.com/ingenuity)). Statistically significant over-represented canonical pathways were determined by Fisher’s exact test followed by adjustment for multiple testing using the Benjamini–Hochberg method.

#### *Functional annotation & analysis*

To identify SNPs that may influence DNA methylation at the CpG sites in *cis* (i.e., within 1 megabase on either side of a gene’s transcript start site [TSS] [41]), the top significant CpG sites (BACON-corrected FDR p-values  $< 0.05$ ) were queried in the mQTL database ([www.mqtl.org/](http://www.mqtl.org/)) that documents methylation quantitative trait loci (meQTL) in human blood at serial time points across the life-course: at birth, childhood, adolescence, middle age and pregnancy [42]. Then, the *cis*-meQTL SNPs identified through the query were annotated using tools and databases including Functional Mapping and Annotation (FUMA) [43], HaploReg v4.1 [44] and Genotype-Tissue Expression (GTEx) [45].

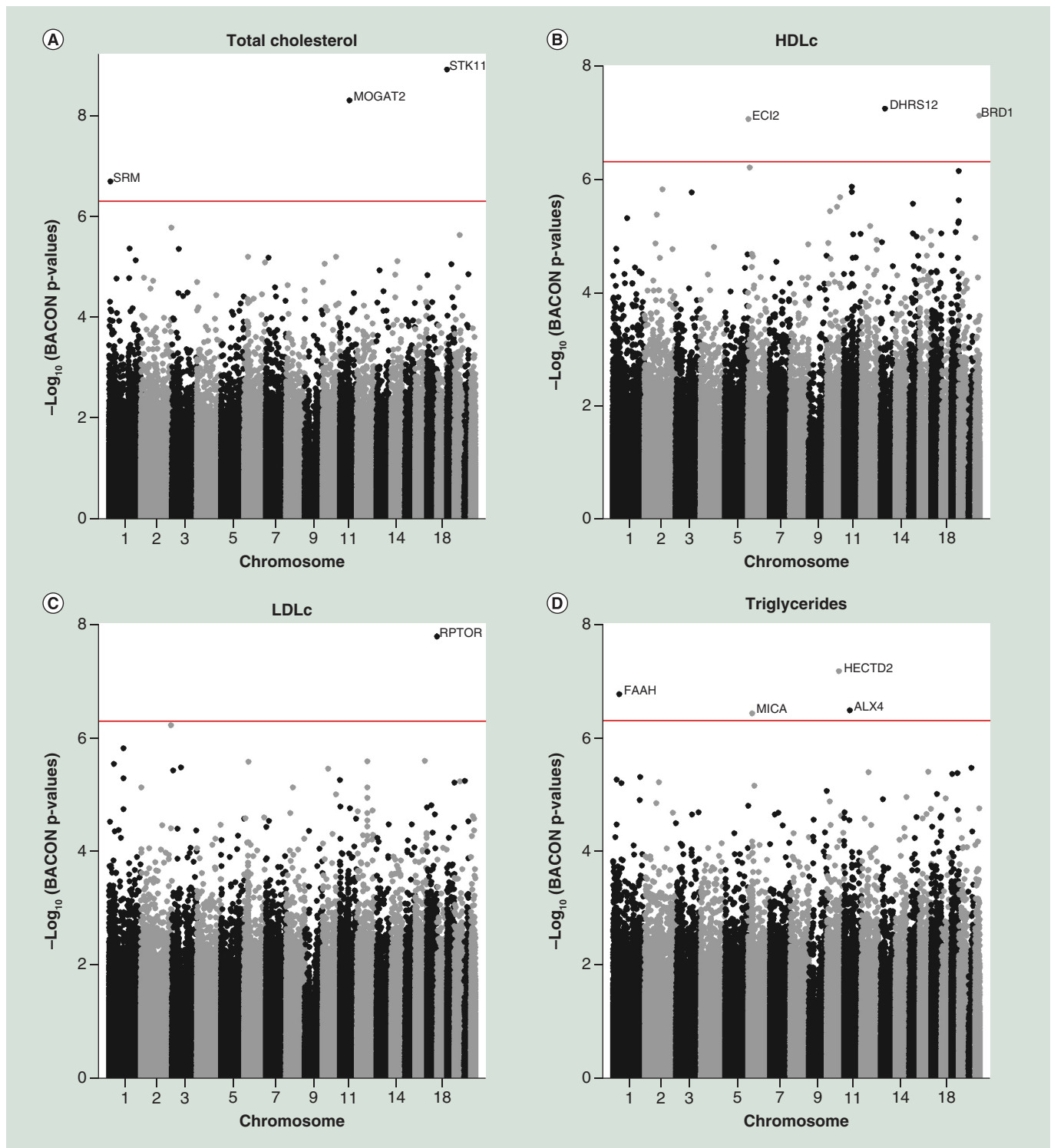
To test whether the *cis*-meQTL SNPs modify the association between dyslipidemia and DNA methylation in placenta, we repeated the EWAS for the lipid trait as a predictor and placenta DNA methylation at each CpG site as the response variable that included an interaction term between the lipid level and the SNP genotypes in placenta as well as the covariates included in the primary analyses.

#### *Evaluation of previously reported CpGs associated with lipid-related traits*

A PubMed literature search was conducted to identify CpG sites previously associated with lipid traits by EWAS (Supplementary methods). Ten studies met search eligibility criteria and were confirmed independently by two co-authors. We identified 434 unique CpG sites associated with lipid traits in adult blood [46–53] or CD4<sup>+</sup> T cells [54,55], out of them 112, 240, 56 and 74 CpGs were reported to be associated with total cholesterol, HDLc, LDLc and triglycerides, respectively. We examined whether the 434 CpGs were associated with the four dyslipidemia traits in placenta in our data. CpGs with nominal p-values  $< 0.05$  were considered to be statistically significant associations providing evidence for overlap in our study.

## Results

Among the 262 women included in the analysis, 29% had high total cholesterol, 17% had low HDLc, 47% had high LDLc and 20% had high triglycerides at enrollment (Table 1). See Supplementary Table 1 for description of the pregnancy outcomes.



**Figure 1. Manhattan plots of associations between maternal dyslipidemia and DNA methylation in placenta.** HDLc: High-density lipoprotein cholesterol; LDLc: Low-density lipoprotein cholesterol.

**Table 2. Top-significant (BACON-corrected false discovery rate p-values < 0.05) placental DNA methylation loci associated with maternal dyslipidemia status (n = 262).**

Dyslipidemia trait	CpG	Chr	Position	Relation to Island	Gene	Gene Group	$\beta$	(95% CI)	BACON-corrected p-value	BACON-corrected FDR p-value
High vs normal total cholesterol	cg20637199	19	1226534	Island	<i>STK11</i>	Body	0.004	(0.002–0.005)	1.21E-09	0.0005
	cg10695180	11	75427562	OpenSea	<i>MOGAT2</i>	TSS1500	0.013	(0.008–0.019)	4.97E-09	0.0010
	cg10490147	1	11120546	Island	<i>SRM</i>	TSS1500	-0.004	(-0.006–-0.002)	2.00E-07	0.0273
Low vs normal HDLc	cg26851608	13	52378480	Island	<i>DHRS12</i>	TSS1500 TSS200	-0.003	(-0.005–-0.002)	5.89E-08	0.0124
	cg00156001	22	50195025	N.Shore	<i>BRD1</i>	Body	-0.014	(-0.020–-0.009)	7.81E-08	0.0124
	cg17182202	6	4153258	OpenSea	<i>ECI2</i>		-0.015	(-0.019–-0.010)	9.09E-08	0.0124
High vs normal LDLc	cg02047211	17	78932697	N.Shelf	<i>RPTOR</i>	Body	-0.012	(-0.015–-0.008)	1.61E-08	0.0066
High vs normal triglycerides	cg07255925	10	93073139	OpenSea	<i>HECTD2</i>	Body	-0.011	(-0.015–-0.007)	6.70E-08	0.0274
	cg09326404	1	46956839	Island	<i>FAAH</i>		0.049	(0.029–0.069)	1.71E-07	0.0350
	cg02785814	11	44338707	Island	<i>ALX4</i>		-0.019	(-0.030–-0.009)	3.33E-07	0.0386
	cg04985482	6	31382065	OpenSea	<i>MICA</i>	Body	-0.035	(-0.049–-0.020)	3.77E-07	0.0386

Adjusted for maternal self-reported race/ethnicity, maternal age in years, fetal sex, methylation sample plate, first three methylation principal component, the first ten genotype principal components and Surrogate Variable Analysis.

Dyslipidemia trait: High total cholesterol  $\geq 200$  mg/dl, low HDLc  $\leq 50$  mg/dl, high LDLc  $\geq 100$  mg/dl and high triglycerides  $\geq 150$  mg/dl.

Position: Based on hg19 human reference genome.

Relation to island: Shore = 0–2 kb from island, Shelf = 2–4 kb from island, N = upstream (5') of CpG island, S = downstream (3') of CpG island.

Gene Group: TSS200 = 0–200 bases upstream of the TSS; TSS1500 = 200–1500 bases upstream of the TSS; Body = Between the ATG and stop codon; irrespective of the presence of introns, exons, TSS, or promoters; 5'UTR = Within the 5' untranslated region, between the TSS and the ATG start site; 3'UTR = Between the stop codon and poly A signal.

ATG: Start codon coding for methionine; Chr: Chromosome; CpG: Cytosine-(phosphate)-guanine; FDR: False discovery rate; HDLc: High-density lipoprotein cholesterol; LDLc: Low-density lipoprotein cholesterol; TSS: transcriptional start site.

### Epigenome-wide analyses

In total, maternal dyslipidemia in early pregnancy was significantly associated with placental DNA methylation at 11 CpG sites (BACON-corrected FDR p-values < 0.05, nominal p-values ranging from  $1.21 \times 10^{-9}$  to  $3.77 \times 10^{-7}$ , Table 2 & Figure 1). High total cholesterol was associated with increased methylation of cg20637199 (*STK11*) and cg10695180 (*MOGAT2*) and decreased methylation of cg10490147 (*SRM*). Low HDLc was associated with decreased methylation of cg26851608 (*DHRS12*), cg00156001 (*BRD1*) and cg17182202 (*ECI2*). High LDLc was associated with decreased methylation of cg02047211 (*RPTOR*). High triglycerides were associated with decreased methylation of cg07255925 (*HECTD2*), cg02785814 (*ALX4*) and cg04985482 (*MICA*) and increased methylation of cg09326404 (*FAAH*). The highest change in methylation was between high triglycerides and cg09326404 (*FAAH*) in which there was a 4.9% higher placental DNA methylation (95% CI: 2.9–6.9%) among women with high triglycerides compared with women with normal triglycerides.

### Gene expression analyses

DNA methylation at cg26851608 (which was found to be hypomethylated with low HDLc) was inversely correlated with the placental gene expression of *DHRS12* ( $r = -0.25$ , p-value = 0.044, n = 64; Table 3). High triglycerides were significantly associated with decreased placental gene expression of *ALX4* (FDR adjusted p-value =  $1.93 \times 10^{-2}$ , Table 4).

### Canonical pathways

The genes mapping to the differentially methylated CpG sites were enriched in canonical IPA disease and function pathways including dyslipidemia (p-value =  $1.37 \times 10^{-4}$ ), energy homeostasis (p-value =  $2.44 \times 10^{-4}$ ) and abnormal morphology of embryonic tissue (p-value =  $4.84 \times 10^{-4}$ ) (Table 5). The top canonical IPA network was related to Cell Cycle, Endocrine System Development and Function and Carbohydrate Metabolism (Supplementary Figure 2; Supplementary Tables 2 & 3).

### Functional annotation analyses

In the mQTL database, 137 SNPs in *EXT2* and *ALX4* genes were cis-meQTL with cg02785814 (*ALX4*) across different stages of life (Supplementary Table 4). Visualization of cg02785814 is presented in Supplementary Figure

**Table 3. Correlations between DNA methylation at the top differentially methylated cytosine-(phosphate)-guanine sites and gene expression in placenta (n = 64).**

CpG site	Gene	Spearman correlation	p-Value
cg20637199	<i>STK11</i>	0.223	0.077
cg10695180	<i>MOGAT2</i>	0.042	0.743
cg10490147	<i>SRM</i>	-0.112	0.379
cg26851608	<i>DHRS12</i>	-0.253	<b>0.044</b>
cg00156001	<i>BRD1</i>	-0.014	0.912
cg17182202	<i>ECI2</i>	-0.112	0.377
cg02047211	<i>RPTOR</i>	-0.190	0.133
cg07255925	<i>HECTD2</i>	-0.081	0.527
cg09326404	<i>FAAH</i>	0.016	0.899
cg02785814	<i>ALX4</i>	0.093	0.464
cg04985482	<i>MICA</i>	0.092	0.471

Note: Bold value denotes p-value <0.05.  
CpG: Cytosine-(phosphate)-guanine.

**Table 4. Association between maternal dyslipidemia status and expression of genes near the top significant DNA methylation cytosine-(phosphate)-guanine sites (n = 64).**

Dyslipidemia trait	Gene	Log2FoldChange	SE	P-value	Adjusted p-value
High vs normal total cholesterol	<i>STK11</i>	-0.021	0.107	0.846	0.99996
	<i>MOGAT2</i>	-0.407	4.013	0.919	0.99996
	<i>SRM</i>	-0.346	0.121	<b>0.004</b>	0.29049
Low vs normal HDLc	<i>DHRS12</i>	-0.088	0.131	0.501	0.99996
	<i>BRD1</i>	0.121	0.114	0.288	0.99996
	<i>ECI2</i>	-0.294	0.134	<b>0.029</b>	0.77191
High vs normal LDLc	<i>RPTOR</i>	0.094	0.114	0.410	0.99999
High vs normal triglycerides	<i>HECTD2</i>	0.024	0.190	0.898	0.99998
	<i>FAAH</i>	-0.077	0.168	0.646	0.99998
	<i>ALX4</i>	-16.265	4.310	<b>1.61E-04</b>	<b>1.93E-02</b>
	<i>MICA</i>	0.062	0.962	0.948	0.99998

Note: Bold value denotes p-value <0.05.  
Adjusted for maternal self-reported race/ethnicity, maternal age in years, fetal sex and the first ten genotype principal components.  
Dyslipidemia trait: High total cholesterol ≥200 mg/dl, low HDLc ≤50 mg/dl, high LDLc ≥100 mg/dl and high triglycerides ≥150 mg/dl.  
CpG: Cytosine-(phosphate)-guanine; FDR: False discovery rate; HDLc: High-density lipoprotein cholesterol; LDLc: Low-density lipoprotein cholesterol.

**Table 5. Top 10 pathways of diseases and biological function from Ingenuity Pathway Analysis.**

Categories	Functions	Diseases or functions annotation	p-Value	Molecules
Lipid Metabolism, Small Molecule Biochemistry	Catabolism	Catabolism of fatty acid	7.30E-05	<i>ECI2, FAAH</i>
Cell Death and Survival, Embryonic Development	Cell death	Cell death of mesenchymal cells	8.50E-05	<i>STK11, ALX4</i>
Hematological Disease, <b>Metabolic Disease</b>	<b>Dyslipidemia</b>	<b>Dyslipidemia</b>	1.37E-04	<i>MOGAT2, RPTOR, FAAH</i>
Organismal Functions	Energy homeostasis	Energy homeostasis	2.44E-04	<i>MOGAT2, STK11, FAAH</i>
Cell Cycle	senescence	Senescence of connective tissue cells	3.82E-04	<i>STK11, FAAH</i>
Developmental Disorder, Embryonic Development, Organismal Development, Tissue Morphology	Abnormal morphology	Abnormal morphology of embryonic tissue	4.84E-04	<i>RPTOR, STK11, BRD1, ALX4</i>
Developmental Disorder, Embryonic Development, Organismal Development, Tissue Morphology	Abnormal morphology	Abnormal morphology of zone of polarizing activity	4.99E-04	<i>ALX4</i>
Lipid Metabolism, Small Molecule Biochemistry	Acylation	Acylation of 1-oleoylglycerol	4.99E-04	<i>MOGAT2</i>
Lipid Metabolism, Small Molecule Biochemistry	Acylation	Acylation of 2-oleoylglycerol	4.99E-04	<i>MOGAT2</i>
Cancer, Organismal Injury and Abnormalities, Reproductive System Disease	Adenoma	Adenoma malignum	4.99E-04	<i>STK11</i>

Note: Bold text highlights lipid-related pathways.



3. FUMA identified six lead SNPs (rs80053551, rs73541707, rs56338244, rs3845266, rs72892167, rs1349831) out of the 137 *cis*-meQTL SNPs. When we included the six lead SNPs in the EWAS, we found a significant interaction between rs73541707, rs56338244 and rs72892167 and maternal triglycerides status (Supplementary Table 5). According to the GTEx and HPA database, gene *EXT2* displayed the highest expression in the placenta and *ALX4* displayed the highest expression in breast and endometrium tissues (Supplementary Figure 5).

### Evaluation of previously reported CpGs associated with dyslipidemia

Out of 434 unique CpG sites that have been previously reported to be associated with dyslipidemia, 130 CpGs (30%) were significantly associated with dyslipidemia in our data (raw p-values < 0.05, Supplementary Table 6). From the 434 CpG sites, 112, 240, 56 and 74 were reported to be associated with total cholesterol, HDLc, LDLc and triglycerides, respectively. Among the 112 CpGs previously related to total cholesterol, 59 (58%) showed directionally consistent associations with total cholesterol in our data (Supplementary Table 7). Similarly, 108 (46%) CpGs, 29 (56%) and 41 (58%) showed directionally consistent associations with HDLc, LDLc and triglycerides in our data, respectively (Supplementary Table 7). There were no published maternal lipid EWAS in placenta or cord blood.

### Discussion

We conducted the first placental EWAS of early pregnancy maternal dyslipidemia and found 11 differentially methylated CpG sites. The top differentially methylated CpG sites map to genes enriched in pathways including dyslipidemia, energy homeostasis and morphology of embryonic tissue. We found corroborating evidence from DNA methylation, gene expression and meQTL analyses for the role of the *ALX4* gene in dyslipidemia due to high triglycerides. Specifically, high maternal triglycerides were associated with both decreased methylation of cg02785814 annotated to the *ALX4* gene and decreased expression of the same gene in placenta. Lastly, 130 out of 434 previously reported CpG sites in adults blood known to be associated with lipid traits, displayed significant differential methylation in placenta associated with maternal dyslipidemia in our study.

Our study identified differentially methylated CpG sites mapping to genes such as *MOGAT2*, *ECI2*, *DHRS12*, *FAAH*, *SRM*, *BRD1*, *ALX4* and *RPTOR*, which have biological functions relevant for lipid metabolism or other metabolic traits. Specifically, *MOGAT2* is a member of the acylglycerol o-acyltransferase family of genes and encodes a crucial protein in the absorption of dietary fat by the small intestine. It is highly expressed in human small intestine and white adipose tissue [56]. A study has found decreased expression of *MOGAT2* after fish oil supplementation among dyslipidemic men [57], and the gene has been associated with HDLc in a meta-analysis of genome-wide association studies (GWASs) [58]. *ECI2* encodes a key mitochondrial enzyme involved in  $\beta$ -oxidation of unsaturated fatty acids and its antisense gene *AL136309.4* has been associated with blood metabolite levels [59]. Furthermore, mouse deficient in *eci2* accumulate unsaturated fatty acyl groups in ester lipids and deposit large amounts of triglycerides in hepatocytes (steatosis) [60]. *DHRS12* encodes a short-chain dehydrogenases/reductases family enzyme that metabolizes compounds including lipids and is highly expressed in fat [61]. *FAAH* encodes a protein responsible for the hydrolysis of several fatty acid amides [62]. Some of these loci have also been associated with cardiometabolic diseases by GWAS. For example, *SRM* has been associated with fat-free mass [63]; *BRD1* and *ALX4* have been associated with blood pressure [64,65]; and *RPTOR* has been associated with BMI [66–68], obesity [69] and hypertension [65,70]. Previous studies have reported higher risk of cardiometabolic disease among children of mothers with dyslipidemia during pregnancy [9–12], and suggested that the placental response to dyslipidemia may be a mediator. One study has reported that placental DNA methylation at *ABCA1* locus was associated with maternal blood HDLc level and lower *ABCA1* mRNA levels, suggesting that placental epigenetic changes may play a role in cholesterol transfer from the mother to the fetus [22], as epigenetic changes in blood cells have been linked to end-product feedback control of lipid metabolism [47]. In line with these previous studies, we reported epigenetic alterations linked with maternal dyslipidemia in genes known to be associated with cardiometabolic diseases. Together with these previous evidences, our findings suggest that placental epigenetic markers of maternal dyslipidemia may give insights into early mechanisms that underlie the etiology of cardiometabolic diseases in later life. The relationship between DNA methylation and dyslipidemia traits is complicated by DNA sequence variation, which may contribute to both traits [71]. We found that genetic variants near the *EXT2* and *ALX4* genes which are highly expressed in placenta and female reproductive tissues had regulatory effects on cg02785814. *ALX4* encodes a paired-like homeodomain transcription factor expressed in the mesenchyme of developing bones, limbs, hair, teeth and mammary tissue. *ALX4* is essential for embryonic morphogenesis [72]. *EXT2* encodes one of

two glycosyltransferases involved in the chain elongation step of heparan sulfate biosynthesis [73]. Several GWASs have reported *ALX4* and/or *EXT2* loci associated with gout disease [74], response to antidepressants [75], Type 2 diabetes [76], blood pressure [65] and LDLc blood levels [77]. These findings provide evidence that specific placental epigenetic modifications may result from complex interactions between the fetal genome and the intrauterine environmental exposure such as maternal dyslipidemia. Future multi-omic studies hold the potential to unravel the early life mechanisms in fetal development and disease risk in later life.

To our knowledge, there have been no prior placenta-specific EWAS of dyslipidemia-related traits. Houde *et al.* have investigated the association between DNA methylation at *LPL* and *ABCA1* genes quantified by sodium bisulfite pyrosequencing in cord blood and placenta and dyslipidemia among Canadian women [20,21]. Placental DNA methylation at *LPL*-CpG3 was negatively associated with maternal third trimester HDLc blood level, and placental DNA methylation at *LPL*-CpG2 was negatively associated with cord blood HDLc levels. Placental DNA methylation levels at both CpG sites were negatively correlated with placental *LPL* gene expression [20]. Maternal first trimester HDLc blood level was negatively correlated with *ABCA1* DNA methylation on the maternal side of the placenta but not on the fetal side, but no association was observed with cord blood HDLc level [21]. In our study, no CpG sites annotated to either *LPL* or *ABCA1* exhibited significant differential DNA methylation associated with maternal dyslipidemia.

We found that several CpG sites in adult blood and CD4<sup>+</sup> T cells reported to be associated with lipid levels in previous EWASs [46–55] exhibited significant differential DNA methylation in placenta associated with maternal dyslipidemia. Some of these CpGs map to genes implicated in lipid metabolism such as *CPT1A*, *ABCG1*, *SREBF1* and *APOB*. For example, cg00574958, cg09737197 and cg17058475 (*CPT1A*) that have been negatively associated with triglycerides and total cholesterol in adults [46–51,54,55] showed significant differential methylation in placenta associated with HDLc and LDLc in our data; cg02370100 and cg07397296 (*ABCG1*) that have been positively associated with triglycerides levels in adults [48,50,78] were positively associated with higher total cholesterol and triglycerides and lower HDLc; cg08129017 and cg20544516 (both annotated to *SREBF1*) were positively associated with triglycerides levels in adults [48] and in our data; cg03350299 (*APOB*) was positively associated with LDLc levels in adults [48] and in our data. The *CPT1A* gene plays an important role in triglyceride metabolism [79]; *ABCG1* is involved in the transport of cholesterol and phospholipids in macrophages [80]; *SREBF1* encodes a transcription factor that binds to the sterol regulatory element-1, which is localized at the promoter of the LDL-receptor gene and other genes involved in sterol biosynthesis [81]; and *APOB* is involved in the production of LDLc [82].

We recognize that our study has potential limitations. We were not able to identify another independent cohort to replicate our findings, because similarly designed cohorts are not common. However, we compared our results with previous published EWASs. Maternal lipid levels, placental DNA methylation and gene expression profiles vary during pregnancy [83], and epigenome-wide analysis cannot conclude a causal effect because DNA methylation may be a cause or consequence of dyslipidemia, a recurrent challenge in epigenomic studies [84]. However, a Mendelian randomization study demonstrated that differential DNA methylation observed in adult blood is more likely to be a consequence but not a cause of variations in lipids levels [47]. No such studies have been done using placenta samples. In addition, our criteria for dyslipidemia are from a nonpregnant population while the lipid levels in our study were measured during early pregnancy (between 8w0d and 13w6d). Studies including data from a subcohort of our own NICHD Fetal Growth Studies have shown that pregnant women's blood lipid levels decrease during the first 6 weeks and then increase throughout pregnancy [6,24,85,86], and hence our measuring windows are likely to reflect women's pre-pregnancy dyslipidemia status. However, after the first trimester of pregnancy, maternal blood lipid levels can change dramatically [6]. Blood samples have been stored in  $-80^{\circ}\text{C}$  freezers before analyses were done. Variability between fresh and frozen samples stored in  $-80^{\circ}\text{C}$  is small, with metabolomic compounds having both stable levels and good concordance [87]. Future studies are needed to understand mechanisms other than gene expression such as regulation of splicing, gene activation and transcription factors that may mediate the associations between dyslipidemia and DNA methylation changes found in this study [88].

Several unique strengths of our study deserve mention. Our study involved diverse race/ethnic participants that provided placenta samples at delivery. We were able to adjust for genotype-based PCs in addition to methylation-based PCs (which are widely used in most EWASs, despite being a poor proxy for ancestry) [35], effectively minimizing spurious associations due to population stratification [89]. To date, there is no reference for cell type composition for placenta. Therefore, we implemented a validated reference-free adjustment for cell type proportion variation implemented in SVA [90]. Further correction for genomic inflation was performed using BACON, a method demonstrated to maximize study power while controlling for FDR [39].

## Conclusion

In conclusion, this first EWAS revealed that maternal dyslipidemia in early pregnancy was associated with placental DNA methylation and gene expression. Notably, our study found corroborating evidence for associations of maternal high triglycerides with hypomethylation of a CpG site annotated to *ALX4* and decreased placental expression of *ALX4*. Some of these loci including *MOGAT2*, *ECI2*, *DHRS12*, *FAAH*, *SRM*, *BRD1*, *ALX4* and *RPTOR* are known for their relevance in vascular and structural development of the placenta. These data provide insights into the potential early mechanisms underlying the etiology of cardiometabolic diseases in later life.

## Future perspective

Findings from the present study shed further light on the importance of optimizing women's cardiometabolic health in early pregnancy or even before pregnancy to improve maternal and child health. Future multi-omic studies will have the potential to unravel pathways that may link mechanisms during fetal development and disease risk in later life.

### Summary points

- We conducted the first placental epigenome-wide association study of maternal dyslipidemia.
- Maternal high total cholesterol ( $\geq 200$  mg/dl) was associated with placental DNA methylation at three cytosine-(phosphate)-guanine (CpG) sites (*STK11*, *MOGAT2* and *SRM*).
- Maternal low high-density lipoprotein cholesterol ( $\leq 50$  mg/dl) was associated with placental DNA methylation at three CpG sites (*DHRS12*, *BRD1* and *ECI2*).
- Maternal high low-density lipoprotein cholesterol ( $\geq 100$  mg/dl) was associated with placental DNA methylation at a CpG site near *RPTOR*.
- Maternal high triglycerides ( $\geq 150$  mg/dl) were associated with placental DNA methylation in four CpG sites (*HECTD2*, *FAAH*, *ALX4* and *MICA*) and decreased placental gene expression of *ALX4*.
- Many genes found to be associated with dyslipidemia are known for their relevance in vascular and structural development of the placenta and cardiometabolic diseases, suggesting potential early mechanisms underlying the etiology of cardiometabolic diseases in later life.

### Supplementary data

To view the supplementary data that accompany this article please visit the journal website at: [www.futuremedicine.com/doi/suppl/10.2217/epi-2019-0293](http://www.futuremedicine.com/doi/suppl/10.2217/epi-2019-0293)

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### Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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