Maternal pre-pregnancy obesity, offspring cord blood DNA methylation, and offspring cardiometabolic health in early childhood: an epigenome-wide association study

Chantel L. Martin [a], Dereje Jima[b,c], Gemma C. Sharp [d], Lauren E. McCullough[e], Sarah S. Park[f], Kymberly M. Gowdy[g], David Skaar[f], Michael Cowley[f], Rachel L. Maguire[f], Bernard Fuemmeler[h], David Collier [i], Caroline L. Relton[d], Susan K. Murphy [j], and Cathrine Hoyo[b,f]

[a]Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, NC, USA; [b]Center of Human Health and the Environment, North Carolina State University, Raleigh, USA; [c]Bioinformatics Research Center, North Carolina State University, Raleigh, NC, USA; [d]Medical Research Integrative Epidemiology Unit, Bristol Medical School, Population Health Sciences, University of Bristol, Bristol, UK; [e]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, GA, USA; [f]Department of Biological Sciences, North Carolina State University, Raleigh, NC, USA; [g]Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University, Greenville, NC, USA; [h]Department of Health Behavior and Policy, Virginia Commonwealth University, Richmond, VA, USA; [i]Department of Pediatrics, Brody School of Medicine, East Carolina University, Greenville, NC, USA; [j]Division of Reproductive Sciences, Department of Obstetrics and Gynecology, Duke University School of Medicine, Durham, NC, USA

ABSTRACT

Pre-pregnancy obesity is an established risk factor for adverse sex-specific cardiometabolic health in offspring. Epigenetic alterations, such as in DNA methylation (DNAm), are a hypothesized link; however, sex-specific epigenomic targets remain unclear. Leveraging data from the Newborn Epigenetics Study (NEST) cohort, linear regression models were used to identify CpG sites in cord blood leukocytes associated with pre-pregnancy obesity in 187 mother-female and 173 mother-male offsprings. DNAm in cord blood was measured using the Illumina HumanMethylation450k BeadChip. Replication analysis was conducted among the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort. Associations between pre-pregnancy obesity-associated CpG sites and offspring BMI z-score (BMIz) and blood pressure (BP) percentiles at 4–5-years of age were also examined. Maternal pre-pregnancy obesity was associated with 876 CpGs in female and 293 CpGs in male offspring (false discovery rate <5%). Among female offspring, 57 CpG sites, including the top 18, mapped to the *TAPBP* gene (range of effect estimates: −0.83% decrease to 4.02% increase in methylation). CpG methylation differences in the *TAPBP* gene were also observed among males (range of effect estimates: −0.30% decrease to 2.59% increase in methylation). While technically validated, none of the *TAPBP* CpG sites were replicated in ALSPAC. In NEST, methylation differences at CpG sites of the *TAPBP* gene were associated with BMI z-score (cg23922433 and cg17621507) and systolic BP percentile (cg06230948) in female and systolic (cg06230948) and diastolic (cg03780271) BP percentile in male offspring. Together, these findings suggest sex-specific effects, which, if causal, may explain observed sex-specific effects of maternal obesity.

Background

More than 9% of preschool-aged children 2–5 years of age are classified as obese, as are 18% of children ages 6–11 years and 21% of 12–19 years of age in the U.S [1]. Although the prevalence of childhood obesity may be plateauing, the prevalence in males and ethnic minorities continues to increase [2]. Childhood obesity is associated with higher blood pressure (BP), recurrent wheezing, and metabolic disease, which are consistent risk factors for common chronic diseases and conditions in later life, including type 2 diabetes, coronary heart disease, some cancers, and life-long obesity [3–10].

Data from the last 15 years have demonstrated that the first 1,000 days, the period from conception to two years of age, represents a critical developmental window in which exposures can play a major role in the programming of physiological systems involved in growth, energy metabolism, adipogenesis, appetite and glucose-insulin homeostasis of the

developing offspring [11]. Both pre-pregnancy obesity and excessive gestational weight gain (GWG) independently contribute to cardiometabolic function, including obesity, in offspring [3–5]. With 25% of conceptions occurring among obese women in the U.S [12]., maternal obesity before and during pregnancy is perhaps one of the most common intrauterine risk factors for childhood obesity for which we have limited mechanistic insights.

While mechanisms by which maternal obesity is linked to childhood obesity are still unclear, mounting evidence from animal and human studies supports that a wide range of *in utero* exposures [10,13–17], including pre-pregnancy obesity and gestational weight gain [18–22], are associated with epigenetic modifications, including DNA methylation of cytosine-phosphate-guanine (CpG) sites, which can in turn alter the transcriptional capacity of genes important in metabolism. A recent meta-analysis comprising 19 studies with >9,000 participants with DNA methylation measured in umbilical cord blood leukocytes using CpG methylation arrays identified 86 maternal obesity-associated CpG sites; however, there was no evidence of enrichment for chromosomal regions or disease pathways [19]. In addition, sex-specific data relating maternal obesity to CpG methylation is lacking and limited research exists with follow-up into childhood to estimate the impact of pre-pregnancy related CpG methylation on cardiometabolic health [18]. To fill this gap, we conducted an epigenome-wide association analysis to determine whether associations between maternal pre-pregnancy obesity and CpG methylation, measured in umbilical cord blood leukocytes, varied by offspring sex, and whether maternal pre-pregnancy obesity-associated CpG methylation was associated with offspring cardiometabolic phenotypes, body mass index (BMI) and BP at 4–5 years of age.

Results

The distribution of maternal and offspring characteristics for 360 mother-offspring pairs are summarized in Table 1. African-American women comprised almost half (48%) of the NEST study sample. Participating women were approximately 25 years of age and older (72%), educated beyond high school (70%), and reported not smoking during pregnancy (68%). Approximately 48% had a pre-pregnancy BMI less than 25kg/m^2, 21% were overweight (BMI 25–29.99 kg/m^2), and 31% were obese (BMI ≥30 kg/m^2). Among the obese, 12% were Class III obese (≥40kg/m^2).

Pre-pregnancy obesity and CpG methylation

Among female offspring, pre-pregnancy obesity was associated with differential methylation at 6,148 CpG sites (FDR <0.05; Supplemental Table 1), corresponding to 1,034 genes. Adjustment for cell proportion reduced the number of significant CpG sites to 876 corresponding to 101 genes (Supplemental Table 2; Figure 1). Among male offspring at birth, 781 CpG sites were associated with pre-pregnancy obesity (FDR <0.05); Supplemental Table 3. Adjustment for estimated cell proportions reduced the number of significant CpG sites to 293 corresponding to 31 genes (Supplemental Table 4; Figure 2). After adjusting for cell type estimates, lambdas (λ) were close to one for the mother-female offspring model (λ = 0.95) and mother-male offspring models (0.78). In a sensitivity analysis, we examined the association between maternal pre-pregnancy obesity and cell type proportions, where we observed significant positive associations among female offspring (Supplemental Table 5). Specifically, maternal obesity was associated with increased CD4+T-cells, B-cells, and granulocytes. We did not observe any associations in male offspring.

Table 2 provides the top 20 genes with the most robust methylation differences for female offspring of obese and non-obese pregnant women before and after adjusting for cell proportions. After adjustment for cell proportion, the most robust methylation differences that were associated with maternal obesity corresponded to *TAPBP* (57 CpGs), *RP11* (13 CpGs), *NFE2L3* (6 CpGs), *GLIPR1L2* (11 CpGs), and *MFSD1* (10 CpGs). Of the 57 CpG sites annotated to the *TAPBP* gene, 48 were associated with higher offspring methylation (range of effect estimates: 0.83% to 4.02% increase in methylation; range of associated p-values: 1.31×10^{-10} to 1.61×10^{-12}).

Table 3 summarizes the 20 genes with the most robust methylation differences among male offspring of obese versus non-obese pregnant women. Prior to adjustment for cell proportion, the most

Table 1. Selected baseline characteristics of study population (n = 360) mother-offspring pairs, Newborn Epigenetics STudy (NEST).

	N	% or Mean	SD
Maternal age			
<25 years	101	28.1	
25–29 years	88	24.4	
≥30 years	171	47.5	
Maternal ethnicity			
African American	172	47.8	
European	159	44.2	
Other	29	8.1	
Maternal educational attainment			
Less than high school	32	8.9	
High school graduate	77	21.4	
More than high school	251	69.7	
Maternal cigarette smoking			
Smoked during pregnancy	115	32.2	
Did not smoke during pregnancy	242	67.8	
Maternal BMI before pregnancy			
<25kg/m2	173	48.1	
25–29.9 kg/m2	75	20.8	
≥30kg/m2	112	31.1	
Child sex			
Female	187	51.9	
Male	173	48.1	
Birth weight			
<2500g	62	17.2	
≥2500g	298	82.8	
Breastfeeding status in first 3 months			
Never breastfed	69	19.2	
Exclusively breastfed	56	15.6	
Bottle and breastfed	109	30.3	
Child BMI ≥85th percentile (age 4–5)			
Not overweight/obese	160	67.2	
Overweight/obese	78	32.8	
Child BMI ≥95th percentile (age 4–5)			
Not obese	194	81.5	
Obese	44	18.5	
Child Elevated BP (age 4–5)			
No	137	85.6	
Yes	23	14.4	
Gestational age at delivery, months	360	38.3	2.3
Birth weight, grams	359	3108.2	667.5
BMI for age z score (age 4–5)	238	0.34	1.66
SBP percentile	160	57.98	27.65
DBP percentile	160	67.83	22.91

robust differences were at CpG sites in or near *GFI1* (9 CpGs), *HOXA3* (15 CpGs), and *WDFY4* (17 CpGs). After adjustment for cell proportion, robust differences remained at CpG sites in or near *GFI1* (10 CpGs). Notably, CpG methylation differences in the *TAPBP* gene (17 CpGs) were also observed among male offspring. Of the 17 CpG sites that mapped to the *TAPBP* gene, 15 were associated with higher methylation; however, the magnitude of male-specific associations was smaller than those for female offspring (range of effect estimates: 0.79%

to 2.59%; range of associated p-values: 5.57×10^{-3} to 6.07×10^{-3}; Supplemental Table 2).

Validation study

To determine whether findings identified using the Illumina HumanMethylation450k BeadChip (HM450k) array could be replicated in an independent dataset within the same cohort, we used more accurate pyrosequencing to quantify methylation at the *TAPBP* gene among male and female offspring born to obese (n = 20) and non-obese women (n = 24). We found that the CpG sites that flanked and included cg14419102 in *TAPBP* gene had ~2% higher methylation in offspring of obese women when compared to non-obese women (vs. 3% from the array). The relationship between pre-pregnancy obesity and higher methylation persisted at 311 bp chr6:28,943,509–28,943,820 region in female offspring at sequence regions flanking cg18353226 and cg06375761 near *TAPBP*, where significant methylation differences were the least robust (<2%), chr6:33,312,579–33,312,730.

Replication study – ALSPAC

HM450K methylation array data from 751 mother-offspring pairs in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort from Bristol, UK was used as our replication dataset. Prior to multiple testing correction, maternal pre-pregnancy obesity was associated with differential methylation at 10 CpGs of *TAPBP* gene among female offspring and at 12 CpGs of the same gene among males after adjusting for cell proportion. Among the 10 CpGs of *TAPBP* gene identified in female offspring, only two overlapped with those identified in the NEST cohort, of which one (cg07500019) showed a positive association in both ALSPAC and NEST. Of the 12 CpGs of the *TAPBP* gene identified in males, none overlapped with those identified in NEST. Also, none of the CpGs of the *TAPBP* gene identified in ALSPAC survived correction for multiple testing in female or male offspring (Bonferroni correction for 227 tests – total CpGs that mapped to *TAPBP* gene – P-value<0.00022).

Table 2. Top 20 genes with differentially methylated CpGs in offspring cord blood associated with maternal obesity before and after adjustment for cell proportions, among females (n = 187).

	Unadjusted for cell proportion					Adjusted for cell proportion				
Annotated Gene[a]	# of CpG sites	Chr.	Median Methylation Difference[b]	P-value[c] (min, max)		Annotated Gene[a]	# of CpG sites	Chr.	Median Methylation Difference[b]	P-value[c] (min, max)
TAPBP	30	chr6	0.76%	$3.39e^{-26}$, $2.65e^{-02}$		TAPBP	57	chr6	0.91%	$1.09e^{-12}$, $4.24e^{-02}$
HMHB1	7	chr5	2.83%	$7.40e^{-10}$, $2.26e^{-03}$		RP11-109A6.3	13	chr10	-0.90%	$1.04e^{-17}$, $1.18e^{-02}$
WT1	25	chr11	-1.93%	$1.57e^{-07}$, $2.64e^{-02}$		NFE2L3	6	chr7	4.40%	$2.00e^{-07}$, $3.69e^{-05}$
PSMB9	11	chr6	-1.41%	$1.73e^{-06}$, $1.80e^{-02}$		GLIPR1L2	11	chr12	2.96%	$3.30e^{-07}$, $3.48e^{-06}$
DDR1	30	chr6	-1.48%	$2.31e^{-06}$, $4.33e^{-02}$		MFSD1	10	chr3	-1.12%	$2.26e^{-06}$, $3.00e^{-02}$
PCDHGA4	38	chr5	-1.52%	$4.01e^{-06}$, $4.97e^{-02}$		HOXC4	22	chr12	-1.78%	$5.71e^{-06}$, $4.30e^{-02}$
PPT2	42	chr6	-0.97%	$4.02e^{-06}$, $4.41e^{-02}$		LSP1	14	chr11	0.56%	$8.30e^{-06}$, $1.89e^{-02}$
GLIPR1L2	11	chr12	3.20%	$4.10e^{-06}$, $7.31e^{-06}$		WT1	16	chr11	-1.89%	$2.01e^{-05}$, $3.60e^{-02}$
BRCA1	26	chr17	0.94%	$4.72e^{-06}$, $3.80e^{-02}$		DDR1-006	14	chr6	-1.70%	$4.49e^{-05}$, $4.52e^{-02}$
FLJ12825	8	chr12	-1.80%	$5.37e^{-06}$, $2.01e^{-02}$		CKMT2	9	chr5	-1.54%	$5.23e^{-05}$, $3.70e^{-02}$
FBXO27	9	chr19	-2.3%	$6.22e^{-06}$, $1.44e^{-02}$		MEST	15	chr7	1.33%	$7.55e^{-05}$, $4.51e^{-02}$
LYPD3	8	chr19	-1.57%	$6.68e^{-06}$, $1.13e^{-02}$		APOB	12	chr2	-2.09%	$7.97e^{-05}$, $2.08e^{-03}$
TMEM187	12	chrX:	-1.65%	$9.09e^{-06}$, $4.66e^{-03}$		LINC00336	9	chr6	2.25%	$9.39e^{-05}$, $1.12e^{-03}$
CSNK1E	11	chr22	-1.82%	$9.17e^{-06}$, $3.17e^{-02}$		C17orf64	9	chr17	-2.06%	$1.50e^{-04}$, $1.25e^{-03}$
GRB7	13	chr17	-1.10%	$1.04e^{-05}$, $2.06e^{-02}$		PCDHB15	4	chr5	-2.41%	$3.47e^{-04}$, $3.11e^{-03}$
BAT1	29	chr6	1.13%	$1.06e^{-05}$, $1.70e^{-02}$		PRR5L	5	chr11	3.64%	$4.97e^{-04}$, $6.38e^{-04}$
EIF2C2	5	chr8	1.78%	$1.07e^{-05}$, $1.33e^{-05}$		HOXB5	3	chr17	1.51	$6.07e^{-04}$, $1.18e^{-02}$
MFSD1	10	chr3	-1.05%	$1.13e^{-05}$, $3.64e^{-02}$		SEPT8-003	10	chr5	-1.63%	$6.64e^{-04}$, $2.36e^{-02}$
GPSM3	10	chr6	1.68%	$1.39e^{-05}$, $2.60e^{-02}$		STK10	4	chr5	-1.45%	$6.75e^{-04}$, $1.17e^{-03}$
ATF7	1	chr12	7.16%	$1.69e^{-05}$		PCDHGB8P-002	7	chr5	-2.88%	$4.20e^{-04}$, $2.48e^{-02}$

[a]Annotated genes shown according to FDR-corrected p-value in ascending order
[b]Median Methylation difference across all significant CpG sites annotated to denoted gene
[c]Minimum and maximum p-value for significant CpG sites annotated to denoted gene
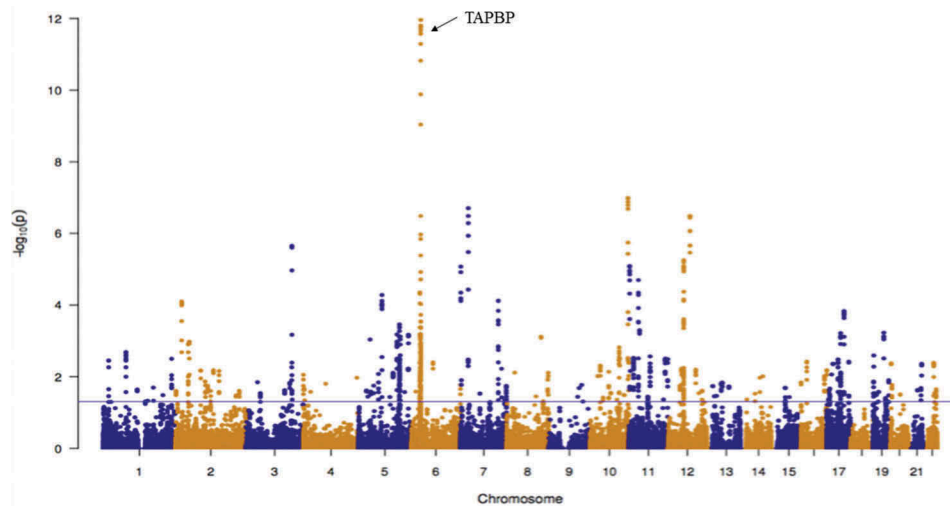
Figure 1. Manhattan plot for the associations between maternal pre-pregnancy obesity and offspring cord blood DNA methylation among females (n = 187) after adjustment for estimated cell proportions. A total of 876 CpGs had an FDR-adjusted P-value <0.05 (solid horizontal blue line).

Associations between differential methylation of TAPBP and offspring cardiometabolic outcomes.

We evaluated whether methylation at CpGs identified in NEST near *TAPBP* were also associated with offspring BMI z-score and systolic and diastolic BP percentiles at 4–5 years of age. In female offspring, we found evidence for association between two pre-pregnancy obesity-related *TAPBP* CpG sites and BMI z-score [cg23922433: β = 1.58, 95% CI: 0.73, 0.14; cg17621507: β = −1.18, 95% CI: −2.22, −0.16], but not BP (Table 4). Another two *TAPBP* CpG sites were associated with systolic BP percentile [cg06230948: β = 9.54, 95% CI: 1.16, 17.93; cg07955457: β = 23.49, 95% CI: 0.03, 46.96]. Among male offspring, one CpG site was associated with higher systolic BP percentile [cg06230948: β = 8.11, 95% CI: 0.29, 15.95] and one CpG site was associated with lower diastolic BP percentile [cg0378027: β = −23.70, 95% CI: −41.17, −6.23].

Assessment of functional importance

To further examine the functional significance of the chromosome 6 genomic region containing pre-pregnancy obesity associated CpGs mapping to TAPBP (28 CpG sites in female and 18 CpG sites in male offspring), we used the UCSC Genome Browser to visualize the ~700bp region of statistically significant differential methylation identified by Illumina HM450K Methylation arrays (Chr6: 28,911,468– 28,912,166, GRCh37/hg37; Supplemental Figure 1). This region has 12 consecutive CpG sites with p-values <5x10-5 and contains another 13 CpG sites not included in the HM450K methylation array. This region contains the noncoding RNA LINC01556, and ENCODE data shows H3K27 methylation and partial overlap with two DNase I hypersensitive sites that also contain binding sites for more than 30 transcription factors, man of them related to cardiometabolic risk. The total extent of the DNase hypersensitive sites, transcription factor binding regions, and histone H3K27 methylation is 1600bp, centered on the sequence region of differential methylation we found.

In order to determine the potential functional significance of genes corresponding to differentially methylated CpGs in males (31 significant genes) and females (101 significant genes), we utilized the Database of Genotypes and Phenotypes (dbGaP) within the Disease/Drugs category of Enrichr [23,24]. The three most significantly associated categories in males were narcolepsy, body weight, and atherosclerosis, and in females, lymphocytes, ulcerative colitis, and hypertension. However, after p-value adjustment using the Benjamini-Hochberg method, these results were not significant. As a comparative measure, we also analyzed the 77 genes in the PACE

Table 3. Top 20 genes with differentially methylated CpGs in offspring cord blood associated with maternal obesity before and after adjustment for cell proportions, among males (n = 173).

	Unadjusted for cell proportion				Adjusted for cell proportion			
Annotated Gene[a]	# of CpG sites	Chr.	Median Methylation Difference[b]	P-value[c] (min, max)	Annotated Gene	# of CpG sites	Chr.	Median Methylation Difference	P-value (min, max)
GFI1	9	chr1	3.74%	$3.48e^{-10}$, $1.04e^{-03}$	CYP26C1	10	chr10	1.73%	$1.15e^{-08}$, $9.35e^{-03}$
HOXA3	15	chr7	1.37%	$2.15e^{-9}$, $3.84e^{-02}$	GFI1	10	chr1	2.42%	$1.15e^{-08}$, $2.92e^{-02}$
WDFY4	17	chr10	−1.94%	$2.43e^{-08}$, $3.55e^{-02}$	STMN1	16	chr1	−1.49%	$1.15e^{-08}$, $2.95e^{-02}$
AGPAT1	13	chr6	−1.09%	$2.67e^{-08}$, $4.38e^{-06}$	CPT1B	14	chr22	1.76%	$3.29e^{-05}$, $4.40e^{-03}$
RNF5	2	chr6	−0.70%	$2.67e^{-08}$, $2.67e^{-08}$	HOXA5	33	chr7	1.14%	$3.29e^{-05}$, $4.26e^{-02}$
RNF5P1	22	chr6	−1.02%	$6.54e^{-08}$, $1.90e^{-04}$	ZNF135	10	chr19	1.47%	$2.07e^{-04}$, $5.94e^{-03}$
STMN1	15	chr1	−1.50%	$1.41e^{-07}$, $3.90e^{-02}$	MOV10L1	8	chr22	1.33%	$7.80e^{-04}$, $9.00e^{-04}$
GABBR1	18	chr6	1.59%	$1.61e^{-07}$, $3.18e^{-03}$	PIGZ	3	chr3	2.84%	$1.15e^{-03}$, $3.12e^{-03}$
CYP26C1	9	chr10	1.64%	$3.15e^{-06}$, $3.84e^{-02}$	HOXA	8	chr7	1.21%	$2.53e^{-03}$, $7.84e^{-03}$
FOXK2	6	chr17	3.10%	$7.97e^{-06}$, $1.56e^{-05}$	C17orf98	8	chr17	−2.28%	$3.12e^{-03}$, $4.41e^{-02}$
TLR5	5	chr1	2.18%	$9.32e^{-06}$, $4.97e^{-03}$	TAPBP	17	chr6	1.22%	$4.01e^{-03}$, $3.90e^{-02}$
CUX2	8	chr12	−2.28%	$1.57e^{-05}$, $4.07e^{-05}$	TLR5	5	chr1	1.35%	$4.20e^{-03}$, $3.73e^{-02}$
CMYA5	12	chr5	3.2%	$2.15e^{-05}$, $4.79e^{-04}$	CMYA5	12	chr5	2.78%	$5.23e^{-03}$, $2.97e^{-02}$
C1orf210	8	chr1	1.52%	$2.44e^{-05}$, $2.09e^{-02}$	POU2AF	3	chr11	1.50%	$5.23e^{-03}$, $3.72e^{-02}$
PIGZ	3	chr3	3.05%	$7.96e^{-05}$, $2.55e^{-04}$	KIAA0101	9	chr15	−0.87%	$5.41e^{-03}$, $7.07e^{-03}$
CSNK1E	10	chr22	−1.52%	$9.77e^{-05}$, $1.52e^{-03}$	PTPN3	2	chr9	0.65%	$5.41e^{-03}$, $5.76e^{-03}$
PCDHB3	1	chr5	−6.60%	$1.15e^{-04}$	BRSK2	2	chr11	5.4%	$6.25e^{-03}$, $8.78e^{-03}$
CPT1B	14	chr22	1.65%	$1.62e^{-4}$, $1.41e^{-02}$	RNU6	5	chr1	2.35%	$7.26e^{-03}$, $2.32e^{-02}$
KIAA0101	9	chr15	−1.25%	$1.75e^{-04}$, $2.70e^{-04}$	TMEM72	4	chr10	3.47%	$7.31e^{-03}$, $1.10e^{-02}$
BRD2	12	chr6	1.23%	$1.78e^{-04}$, $3.39e^{-02}$	AGPAT1	21	chr6	−0.78	$7.82e^{-03}$, $4.78e^{-02}$

[a] Annotated genes shown according to FDR-corrected p-value in ascending order
[b] Median Methylation difference across all significant CpG sites annotated to denoted gene
[c] Minimum and maximum p-value for significant CpG sites annotated to denoted gene

Table 4. Associations[a] of TAPBP DNA methylation sites[b] with child BMI z-score and elevated blood pressure at 4–5 years of age, among 238 female and male offspring in NEST cohort.

| TAPBP gene[c, d]	Females			Males		
	BMI z-score β (95% CI)	Systolic Blood Pressure Percentile β (95% CI)	Diastolic Blood Pressure Percentile β (95% CI)	BMI z-score β (95% CI)	Systolic Blood Pressure Percentile β (95% CI)	Diastolic Blood Pressure Percentile β (95% CI)
cg23922433	**1.58 (0.73, 0.14)**	4.23 (−22.46, 30.92)	10.51 (13.64, 34.66)	–	–	–
cg16632096	0.28 (−1.08, 1.63)	−10.24 (−35.84, 15.37)	−11.44 (−34.65, 11.77)	–	–	–
cg17621507	**−1.18 (−2.22, −0.16)**	−2.77 (−25.12, 19.59)	2.67 (−17.64, 22.97)	–	–	**−23.70 (−41.17, −6.23)**
cg03780271	0.21 (−1.22, 1.63)	2.64 (−22.29, 27.56)	4.29 (−18.34, 26.91)	−0.32 (−1.19, 0.55)	−5.78 (−30.15, 15.59)	−8.59 (−26.19, 9.00)
cg12589538	−0.09 (−1.21, 1.03)	0.70 (−20.09, 21.50)	5.63 (−13.22, 24.48)	0.04 (−0.81, 0.89)	3.55 (−20.11, 27.22)	−14.60 (−38.11, 8.92)
cg11796996	−0.08 (−2.51, 0.87)	−2.10 (−33.46, 29.25)	3.65 (−24.82, 32.12)	−0.59 (−1.69, 0.51)	11.01 (−20.65, 42.68)	−5.05 (−28.18, 18.08)
cg02863594	0.59 (−0.95, 2.12)	−23.56 (−50.58, 3.46)	6.15 (−18.82, 31.11)	−0.58 (−1.71, 0.56)	22.31 (−8.27, 52.90)	0.41 (−20.70, 21.53)
cg01253676	−0.03 (−1.38, 1.31)	−9.21 (−33.86, 15.43)	0.51 (−23.95, 22.97)	−0.21 (−1.22, 0.80)	9.22 (−18.95, 37.40)	−4.68 (−22.25, 12.99)
cg20098791	0.25 (−0.84, 1.35)	−10.25 (−29.39, 8.89)	−1.06 (−18.56, 16.45)	−0.13 (−1.02, 0.77)	9.41 (−14.18, 33.00)	−3.01 (−19.52, 13.50)
cg18353226	0.28 (−0.80, 1.35)	−6.19 (−25.22, 12.84)	−0.92 (−18.25, 16.41)	0.12 (−0.68, 0.92)	7.84 (−14.20, 29.88)	−3.38 (−17.16, 10.39)
cg14419102	0.32 (−0.57, 1.22)	−12.56 (−28.44, 3.31)	−0.07 (−14.58, 14.71)	0.28 (−0.39, 0.95)	5.60 (−12.82, 24.01)	−2.47 (−13.11, 8.17)
cg06375761	0.26 (−0.46, 0.97)	−9.30 (−22.28, 3.68)	−2.58 (−14.51, 9.34)	0.12 (−0.41, 0.64)	7.60 (−6.55, 21.75)	−3.56 (−16.92, 9.80)
cg26083458	0.05 (−0.85, 0.95)	−7.73 (−24.27, 8.81)	2.66 (−12.44, 17.75)	0.26 (−0.41, 0.93)	6.29 (−11.56, 24.13)	0.59 (−16.32, 17.51)
cg13638257	−0.30 (−1.49, 0.89)	−0.84 (−21.85, 20.16)	5.82 (−13.21, 24.86)	−0.08 (−0.92, 0.77)	15.05 (−7.33, 37.44)	−0.77 (−10.54, 9.00)
cg05700142	0.21 (−0.47, 0.89)	−5.47 (−18.17, 7.23)	3.65 (−7.91, 15.21)	0.05 (−0.44, 0.53)	9.61 (−3.29, 22.51)	4.15 (−5.56, 13.86)
cg01654446	0.06 (−0.68, 0.81)	−2.96 (−17.24, 11.32)	3.64 (−9.31, 16.60)	−0.28 (−0.74, 0.17)	4.56 (−8.46, 17.57)	7.93 (−11.37, 27.22)
cg18930100	−0.24 (−1.44, 0.97)	−3.94 (−26.46, 18.58)	3.09 (−17.37, 23.55)	−0.58 (−1.47, 0.31)	24.21 (−1.16, 49.57)	3.87 (−2.07, 9.81)
cg06230948	−0.13 (−0.60, 0.34)	**9.54 (1.16, 17.93)**	0.38 (−7.47, 8.23)	0.17 (−0.13, 0.47)	**8.11 (0.29, 15.95)**	0.15 (−20.65, 20.95)
cg08814531	−0.43 (−2.07, 1.21)	3.80 (−26.09, 33.69)	20.99 (−5.77, 47.75)	−0.01 (−1.11, 1.09)	4.76 (−23.05, 32.57)	7.18 (−11.61, 25.98)
cg03591496	−1.26 (−2.61, 0.28)	8.55 (−18.60, 35.70)	14.88 (−9.63, 39.38)	−0.28 (−1.28, 0.73)	18.04 (−6.87, 42.96)	–
cg20973878	0.92 (−0.72, 2.56)	−13.86 (−43.11, 15.40)	−20.93 (−47.25, 5.38)	–	–	–
cg27020289	−0.78 (−2.26, 0.70)	16.46 (−12.31, 45.23)	1.23 (−25.11, 27.57)	–	–	–
cg00274587	−0.29 (−1.60, 1.03)	−3.05 (−24.47, 18.37)	−5.59 (−25.02, 13.84)	–	–	–
cg00558147	0.09 (−1.49, 1.68)	6.69 (−19.27, 32.65)	9.95 (−13.56, 33.47)	–	–	–
cg00792719	0.92 (−0.59, 2.43)	11.61 (−16.31, 39.53)	−16.99 (−42.17, 8.20)	–	–	–
cg07500019	0.00 (−1.76, 1.76)	1.67 (−30.75, 34.09)	−8.62 (−38.01, 20.77)	–	–	–
cg07955457	−0.12 (−1.48, 1.25)	**23.49 (0.03, 46.96)**	18.71 (−2.72, 40.15)	–	–	–
cg27385940	−0.69 (−3.58, 2.20)	−1.88 (−13.38, 9.62)	−3.68 (−14.10, 6.74)	–	–	–

a Adjusted for maternal age at delivery, race/ethnicity, maternal pre-pregnancy obesity, and smoking status during pregnancy.
b All CpG sites listed are located in Chromosome 6.
c CpG sites listed were all associated with maternal pre-pregnancy BMI after accounting for multiple testing (q < 0.05). Associations between TAPBP gene CpGs and offspring BMI z-score and elevated blood pressure were not statistically significant after applying the Bonferroni test for multiple testing (female offspring p < 0.002 for 28 tests; male offspring p < 0.003 for 17 tests).
d CpGs were all located in the gene body of the TAPBP gene
Bold represents associations with p < 0.05.
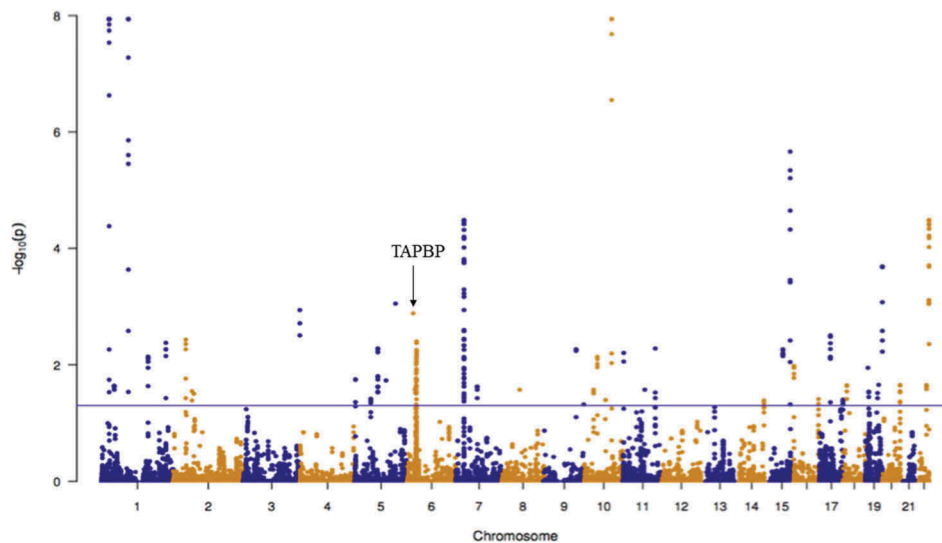
Figure 2. Manhattan plot for the associations between maternal pre-pregnancy obesity and offspring cord blood DNA methylation among males (n = 173) after adjustment for estimated cell proportions. A total of 293 CpGs had an FDR-adjusted P-value <0.05 (solid horizontal blue line).

study [19] using dbGaP. The three most significantly associated categories were insulin resistance, insulin, and lipoproteins, however, after p-value adjustment, these results were not significant. Given that our analysis was sex-specific whereas the PACE study was not, we note the limitation in our functional comparisons between these studies. We do highlight that indicators of cardiometabolic function (atherosclerosis, body weight, hypertension, and insulin resistance) were identified as potential categories of importance in both studies.

Discussion

To our knowledge, this is the first epigenome-wide analysis to examine sex-specific associations between maternal pre-pregnancy obesity, offspring DNA methylation in cord blood, and offspring BMI and BP. We identified multiple differentially methylated CpG sites in umbilical cord blood among male and female offspring of obese mothers compared with non-obese mothers. A key finding of our study was CpGs in or near the *TAPBP* gene were highly associated with maternal obesity in both male and female offspring. Specifically, we identified 74 maternal obesity-related CpG sites (57 CpGs in female and 17 CpGs in male offspring) corresponding to the *TAPBP* gene on chr6, located in the group of genes involving the class 1 major histocompatibility complex. Associations were most

striking among female offspring, where methylation differences of approximately 5% were detected in 21 consecutive CpG sites within a genomic region spanning 700bp. The number and density of the significant CpG sites of the *TAPBP* gene identified were suggestive of a regulatory region warranting further investigation. Therefore, the findings were validated by pyrosequencing supporting the hypothesis that the vulnerability of fetal homeostasis differs by offspring sex. We further evaluated the association between pre-pregnancy maternal obesity-related CpG sites of *TAPBP* gene with offspring cardiometabolic outcomes and found links to BMIz and BP percentile in female offspring and systolic and diastolic BP percentile in male offspring.

While many differentially methylated CpGs of *TAPBP* gene were identified in relation to maternal pre-pregnancy obesity in the NEST cohort, none were replicated in the ALSPAC cohort. Reasons for lack of replication are unclear, however, we note that the prevalence and severity of pre-pregnancy maternal obesity were very different between the two cohorts – this might account for the discrepant findings. Among women participating in NEST, the mean BMI was 29 kg/m^2, with an upper limit of 65 kg/m^2, and the prevalence of pre-pregnancy maternal obesity was 31%. ALSPAC cohort had a lower mean BMI (22 kg/m^2) and prevalence of maternal obesity (5%). It also is possible that differences in confounding structures across the two

populations could account for our results not replicating among the ALSPAC cohort. Differences in ethnic distributions across the NEST and ALSPAC cohorts may also contribute to the lack of replication – ALSPAC cohort comprised ~96% European ethnicity, while NEST comprised 48% African American and 44% European Americans. CpG sites of *TAPBP* were also not identified in the larger Pregnancy and Childhood Epigenetics (PACE) consortium, a study of 23 independent cohorts [19]. The PACE consortium conducted an epigenome-wide DNA methylation study to identify CpG sites associated with maternal BMI at the start of pregnancy, where maternal overweight/obesity was defined as BMI $\geq$ 25 kg/m^2. Differential methylation at CpG sites of *TAPBP* gene may only be associated with pre-pregnancy obesity among individuals with more severe levels of obesity and in a sex-specific manner, yet associations by sex were not examined. Lastly, we did not identify any CpG sites that overlapped with the 86 significant CpGs in the PACE consortium, which is also likely due to the heterogeneity across studies. Therefore, we suggest future studies replicate our findings in cohorts with similar population characteristics.

The significance of pre-pregnancy obesity associated methylation levels observed in *TAPBP* gene is unclear and requires gene- and network-specific functional experiments to elucidate mechanisms underlying these associations. However, hyper-methylation of multiple CpG sites in *TAPBP* at birth was recently shown to be associated with increased risk of cardiometabolic endpoints (e.g., insulin sensitivity) among five-year old Australian children [25]. Our data contributes to the novel findings that hypermethylation of regulatory sequences of *TAPBP* may be an important link between maternal obesity and offspring cardiometabolic dysfunction.

Sex-specific physiological and behavioral responses to periconceptional obesity have been repeatedly demonstrated in animal models (e.g., fed high-fat diets before conception) and observational studies of paternal obesity [26–28]. However, sex-specific human data linking maternal obesity to periconceptional or prenatal obesity epigenetic response are limited. Obesity in the male or female parent at conception has been associated with methylation differences at birth

of regulatory sequences of genes known to increase obesity risk in human offspring, including those targeting *BDNF* and *IGF2*, or identified from methylation arrays [10,13,14,29,30].

In our sex-specific replication analysis of CpG sites of the TAPBP gene and maternal pre-pregnancy obesity, initial statistically significant associations for many CpG sites did not survive multiple comparisons correction. *TAPBP* encodes the transporter associated with tapasin antigen processing (TAP) binding protein, a transmembrane glycoprotein that mediates the interaction between newly formed MHC class 1 molecules and TAP, an interaction essential for optimal peptide loading onto the MHC and antigen display. [31]. While mechanisms linking maternal obesity to sex-specific epigenetic response are still unknown, higher methylation levels of *TAPBP* such as those found in our study might decrease tapasin via decreased transcriptional activity, leading to impaired immune responses in offspring. Decreased tapasin has been shown to lower CD8 + T-cell responses in vitro [32–35]. Indeed, tapasin has also been associated with the autoimmune disease rheumatoid arthritis [36–38], and like obesity, this condition is also associated with chronic inflammation. Both excess adipose tissue and endothelial dysfunction are associated with elevated levels of pro-inflammatory cytokines, such as Interleukin-6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α). In mice, tapasin is activated by the cytokines IFN-γ and IFN-β, and to a lesser extent, TNF-α [39]. Taken together, our results are consistent with regulatory function for the identified TAPBP CpG sites with further experimental studies needed to confirm these findings. In addition to further interrogating the 700bp chr6 region contained within the HLA complex, evaluating whether these differentially methylated genes serve as biomarkers of immune dysfunction or as early indicators of an already altered state of inflammation might contribute to early obesity risk assessment [40,41].

In addition to the *TAPBP* gene, multiple CpG sites (ranging from 2–33 per gene) were also robustly associated with maternal pre-pregnancy obesity in female (e.g., *GLIPR1L2, DDR1,* and genomically-imprinted *WT1*) and male (e.g., STMN1, CYP26C1, TLR5, CMYA5, PIGZ, and genomically-imprinted *GFI1*) offspring. These genes are posited to have

multiple annotated functions including cellular processes such as cell proliferation, apoptosis, differentiation and oncogenesis. Several also play major roles in activation of immune response and inflammation. *DDR1* has been shown to contribute to the development of Th17-dependent inflammatory diseases [42,43]. Other genes, such as *TLR5* and *STMN1*, are involved in pathways linked to obesity and obesity-related cancer [44]. *TLR5* is a member of the toll-like receptor family which has been linked to inflammation, obesity and metabolic dysfunction in both humans and animal models [42,43,45–47]. Further, the gut microbiome has been shown to be impacted by *TLR5* transcriptional activity in mice, which resulted in metabolic syndrome – hyperlipidemia hypertension, insulin resistance, and increased adiposity. *STMN1*, also known as stathmin 1, may have a role in cell cycle progression and cell migration [48,49]. Expression of *STMN1* has been shown to interact with BMI to influence colorectal cancer outcomes [44]. Further work is warranted to examine the potential mechanisms by which perturbation of these genes might mediate the observed associations between maternal obesity and offspring cardiometabolic dysfunction outcomes such as blood pressure and obesity.

This prospective study, conducted in an ethnically and socioeconomically diverse population, has several strengths that include: the use of an established, highly reproducible methylation array; a comprehensive comparison of both maternal and childhood obesity by infant sex; and prospective follow-up of offspring into early childhood at 4–5 years of age. Despite these strengths, study limitations exist. First, the functional assessment included a small number of genes as inputs in the functional analysis tool, which limited the interpretation of the findings from this analysis. Second, pre-pregnancy obesity-associated CpG methylation was measured in cord blood leukocytes, yet the cell fractions driving these associations remain unknown, and should be investigated in the future. Third, maternal pre-pregnancy weight was based on self-reported data, which may have resulted in exposure misclassification. Fourth, with CpG methylation data available only at birth, we cannot exclude the possibility that postnatal modifications alter cardiometabolic status in children. Finally, although paternal obesity is correlated with maternal obesity, and paternal obesity is associated with altered CpG methylation in offspring [29,49], paternal anthropometric data were available on only 92 of the 360 children with DNA methylation measurements in NEST [50], precluding paternal sex-specific analyses.

In summary, we found multiple CpG sites that were differentially methylated at birth in relation to maternal obesity, the most striking of which was the *TAPBP* that was also associated with either weight status or elevated blood pressure, depending on the sex of the offspring. While multiple other CpG sites were also significantly associated with maternal obesity, the *TAPBP* gene is associated with immune-response and supports one mechanism by which maternal obesity increases the risk of offspring obesity and other cardiometabolic phenotypes may be via epigenetic modification of immune response before birth. Our data highlight the importance of this region for future interrogation to gain mechanistic insights linking pre-pregnancy obesity with the development of obesity in children.

Methods

Study population

Study participants for the current analysis were enrolled as part of the Newborn Epigenetics STudy (NEST), a pre-birth cohort study designed to improve our understanding of the role of environmental influences on epigenetic responses and phenotypes in children. Between 2005 and 2009, pregnant women receiving prenatal care at Duke Maternal Fetal Medicine Clinic who intended to use Duke Hospital for their obstetrics care were enrolled using a recruitment protocol whose details have been described previously [51,52]. Inclusion criteria were age 18 years or older, English speaking, and intent to use Duke University Medical Center for obstetric care for the index pregnancy. From these, women intending to move before the first birthday of the offspring, relinquish custody of the index child, or who had confirmed human

immunodeficiency virus (HIV) infection, were excluded. A total of 1,101 eligible women were approached, 895 (81%) consented and were enrolled, and umbilical cord blood was successfully collected from 741 infants. The current study is limited to the 360 mother-child pairs with pre-pregnancy BMI, DNAm, and sex data; these pairs were comparable to the remainder of the cohort, with respect to maternal age at delivery, race/ethnicity, BMI, offspring weight, although these women had a higher education than the remainder of the cohort (p < 0.05). The study protocol was approved by the Duke University and Durham Regional Hospital Institutional Review Boards.

Data collection

Upon enrollment, participants completed a questionnaire, which queried women on socio-demographic, lifestyle and health characteristics. At delivery, parturition data were obtained from medical records, and infant cord blood specimens were collected in EDTA-treated tubes, and subsequently centrifuged using standard protocols to allow for collection of plasma and buffy coat for DNA extraction (Qiagen, Valencia, CA, USA) and stored at −80°C until required. Offspring were followed annually and at each follow-up, caretakers completed a caretaker-administered questionnaire regarding the feeding, sleep, and social and physical activity habits of the child. Medical records were also used to augment follow-up data.

Assessment of maternal body size
The enrollment questionnaire solicited information on maternal pre-pregnancy anthropometric measurements, including current height in meters (m) and usual pre-pregnancy weight in kilograms (kg) from which BMI was calculated and expressed as kg/m^2. In these analyses, participants were defined as non-obese if their BMI was less than $30 \ kg/m^2$, and obese if their BMI was equal to or greater than $30 \ kg/m^2$, according to the World Health Organization classifications [53].

Assessment of childhood cardiometabolic outcomes
Data to compute offspring BMI z-score and BP percentiles were obtained from medical records

and directly measured height and weight. Sex-specific body mass index (BMI) percentiles and BMI z-score were computed based on the Centers for Disease Control and Prevention protocols [54]. BP percentiles were calculated using the norms recommended by the National Education Program Working Group on High Blood Pressure in Children and Adolescents [55].

Assessment of covariates
Women self-reported race/ethnicity, level of education, and cigarette smoking status at enrollment. At delivery, medical records were abstracted to obtain maternal age at delivery and parturition data including mode of delivery, infection in labor, gestational age at birth, and infant birth weight and sex. In addition to offspring weight and height, follow-up questionnaires also queried mothers on breastfeeding for each of the 12 months following delivery, and breastfeeding duration was dichotomized at three months. *A priori*, we considered infant sex as a potential modifier of the association between maternal obesity and offspring methylation as emerging data suggest epigenetic perturbations may be likely sex-specific [26,56]. Therefore, all CpG analyses were stratified by infant sex.

DNA methylation

Methods for DNAm analyses have been described in detail elsewhere [57,58]. Briefly, cord blood was collected at delivery and genomic DNA was extracted from buffy coat specimen based on standard precipitation procedure. Bisulfite conversion was performed using the EZ-96 DNAm kit (Zymo Research Corporation, Irvine, CA, USA) according to manufacturer instructions. Illumina's GenomeStudio Methylation module version 1.0 (Illumina, Inc.) was used to calculate the methylation fractions as the beta-value [$\beta = M/(M + U + 100)$], where M is the intensity of the methylated allele and U is the intensity of the unmethylated [59]. These beta values were log transformed to obtain the log ratio ($\log[\beta/(1 - \beta)]$) and the detection p-value for each value, which represents a statistical test for the difference between the signal for each CpG and background noise.

Data pre-preprocessing and normalization were preformed using the *minfi* package in R [60]. Data verification plots (q-q plots) were created for all children and for each sex. A MethylSet object containing only methylated and unmethylated signals was created using the *preprocessRaw* function and beta-value densities were inspected to assess sample quality. We performed quantile normalization using *preprocessQuantile* function from the minfi package. The normalization procedure was applied to the methylated and unmethylated intensities separately. Since probe types and probe regions were confounded and DNA methylation varies across regions we set the stratify options to true. This normalization step also addressed outlier samples and removed poorly performing samples of both methylated and unmethylated channels when small intensities were close to zero. Batch normalization was preformed using ComBat [61] function from sva R package using plate as batch variable. A detection p-value is returned for every genomic position in every sample. A small p-value indicates a good position and significant above the background level. We excluded probes with a detection p-value > 0.01 in more than 20% of the samples. Potential outliers were removed based on the values beyond the lower and upper outer fences (values < 25th percentile minus 3 x interquartile range and values > 75th percentile + interquartile range) based on PACE guidelines [19]. To pursue CpG methylation marks responsive to pre-pregnancy obesity, we also excluded probes that overlap with SNPs using *dropLociWithSnps* function in *minfi* package in R, as SNPs inside probe bodies or at nucleotide extensions can alter data interpretation from downstream analysis. The missing data were imputed using *champ.impute* function from the ChAMP [62] R package using combine method. A total of 486,427 probes on the Illumina Methylation 450k array comprising type I probes (N = 135,476), type II probes (N = 350,036), control probes (N = 850) and SNP probes (N = 65) were assessed. After removal of control probes, probes with a detection p-value > 0.01 in more than 20% of the samples, and those mapping to SNPs, 422,916 CpGs remained from the robust linear regression in 360 umbilical cord blood samples comprised of 173 males and 187 females.

DNA methylation validation

Targeted pyrosequencing among an independent sample of NEST offspring, who were randomly selected from obese and non-obese mothers, was employed to validate two sequence regions. Assays were designed to flank both CpG sites of the most robust CpG methylation differences (p-values = $1e^{-12}$) in *TAPBP* and those of weaker association (p-values = $1e^{-5}$) from the HM450K methylation array in chromosome 6 (Chr6), referred to here as region 1 and region 2. Primers targeting Chr6 maternal obesity region 1, (Hg38) chr6:28,943,503–28,943,827, are Chr6Ob1F – 5′ AGG AAA ATA GAA GAG GAA TTT GT-3′; Chr6Ob1R – 5′ [Btn]CAA AAA ATA CTA AAA AAA AAA CC-3′; Chr6Ob1S – 5′ AAT ATT TTA TAT TGG TAG-3′, primers to Chr6 maternal obesity region 2–2, (Hg38) chr6:33,312,586–-33,312,726, are Chr6Ob2-2F – 5′ cac CAG TGT AAT AGG AGT TAT TTA ATT T-3′; Chr6Ob2-2R – 3′ [Btn]ctg aAA AAC AAA AAC TCT TAC ACT C-3′. Bases in lowercase for ChrOb2-2 primers are non-genomic sequences added in order to optimize primer annealing temperatures. Amplification conditions were as follows: 95⁰C – 5′; 94⁰C – 20s, 55⁰C – 20s, 68⁰C – 40s, repeated 45X; 94⁰C – 3′. For pyrosequencing, primer Chr6Ob1S was used for Chr6Ob1 amplicons, primer Chr6Ob2-2F was used for Chr6Ob2-2 amplicons. These assays were used to analyze 25ng bisulfite converted leukocyte DNA of n = 44 offspring, 20 of them born to obese women. Validation of DNAm quantitation used genomic DNA controls with 0% and 100% methylation (Qiagen, Inc.) mixed to generate methylation at 0%, 20%, 40%, 60%, 80%, and 100%.

Statistical analysis

Multivariable linear regression analyses were utilized to estimate the association between pre-pregnancy obesity (BMI≥30 vs. BMI<30 kg/m²) and CpG site methylation. These analyses were adjusted for maternal age, maternal cigarette smoking during pregnancy, ethnicity/race, parity. Cell type proportion were also adjusted for using the *estimateCellCounts* function in the *minfi* R package and the Reinius et al. reference dataset [63,64]. The Houseman blood reference dataset

was utilized to estimate cell type proportion to compare our findings to existing studies that also used the Houseman reference (i.e., PACE consortium). We also adjusted for multiple comparisons using a false discovery rate (FDR) [65]. As a sensitivity analysis, we examined the sex-specific associations between maternal pre-pregnancy obesity and cell type estimates. The most robustly associated CpG sites, based on number of CpG sites with differential methylation and location within the gene, were then examined for associations with offspring BMI z-score and BP percentiles at age 4–5 years using multivariable linear regression analyses adjusted for maternal age at delivery, race/ethnicity, maternal pre-pregnancy obesity, and smoking status during pregnancy.

DNA methylation replication

Replication was accomplished by analyzing HM450K methylation array data using an identical analysis plan and covariate adjustment set in 751 mother-infant pairs (n = 367 mother-male pairs and n = 384 mother-female pairs) in the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort, despite the lower prevalence of maternal obesity than NEST participants (ALSPAC = 5% vs. NEST = 31%). ALSPAC is a general population pregnancy cohort that initially recruited 14,541 pregnancies with a due date between April 1991 and December 1992 in Avon, UK [66,67]. The study website contains details of all the data that is available through a fully searchable data dictionary (http://www.bris.ac.uk/alspac/researchers/data-access/data-dictionary). Written informed consent was obtained for all ALSPAC participants and ethical approval was granted from the ALSPAC Law and Ethics Committee and the local Research Ethics Committees. Methylation data were generated as part of the Accessible Resource for Integrated Epigenomics Studies (ARIES) project as described previously [68]. Data were normalized using the functional normalization method within the R package meffil [69]. CpG methylation was also measured in mixed cord blood leukocytes.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Chantel L. Martin http://orcid.org/0000-0003-1907-0810
Gemma C. Sharp http://orcid.org/0000-0003-2906-4035
David Collier http://orcid.org/0000-0002-3323-1900
Susan K. Murphy http://orcid.org/0000-0001-8298-7272

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