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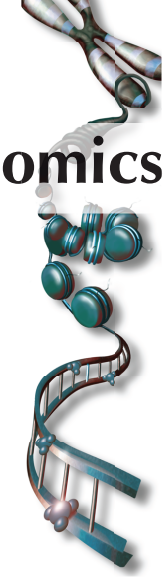
Epigenomics



Measured maternal prepregnancy anthropometry and newborn DNA methylation

Edwina H Yeung*[,1], Weihua Guan[2], Sunni L Mumford[1], Robert M Silver[3,4], Cuilin Zhang[1], Michael Y Tsai[5] & Enrique F Schisterman[1]

[1]Epidemiology Branch, Division of Intramural Population Health Research, Eunice Kennedy Shriver National Institute of Child Health & Human Development, 6710B Rockledge Drive 7004, Bethesda, MD 20817, USA

[2]Division of Biostatistics, School of Public Health, University of Minnesota, A460 Mayo Building, MMC 303, 420 Delaware St SE, Minneapolis, MN 55455, USA

[3]Department of Obstetrics & Gynecology, University of Utah, 50 North Medical Drive, Room 2B200, Salt Lake City, UT 84103, USA

[4]Intermountain Healthcare, Salt Lake City, 50 North Medical Drive, Salt Lake City, UT 84132, USA

[5]Department of Laboratory Medicine & Pathology, University of Minnesota Medical School, Minneapolis, MN 55455, USA

*Author for correspondence: Tel.: +1 301 435 6921; edwina.yeung@nih.gov

Aim: We examined maternal prepregnancy anthropometry and cord blood DNA methylation. **Methods:** Associations between maternal measures (i.e., weight, height, waist circumference, hip circumference, skinfolds, leptin) and methylation β-values at each CpG (measured by the Infinium MethylationEPIC BeadChip) were estimated among 391 singletons. **Results:** Total of 18% of mothers were obese (body mass index ≥ 30) and 27% centrally obese (waist-to-hip ratio ≥ 0.85). One Bonferroni significant CpG with respect to obesity (cg02975187) and two with central obesity (cg12053563, cg12549355) were identified ($p < 6 \times 10^{-8}$). A suggestive association ($p < 10^{-6}$) was observed at *SFRS8* with increasing body mass index. *SFRS8* was previously identified with propensity for weight gain in adults. **Conclusion:** While associations identified with multiple measures related to maternal adiposity suggest different pathways, methylation differences were small in magnitude.

First draft submitted: 29 June 2018; Accepted for publication: 23 October 2018; Published online: 8 January 2019

Keywords: DNA methylation • maternal obesity • newborn • pregnancy

Nationwide, the prevalence of obesity in the US remains high [1], and about 20% of pregnant women conceive with a body mass index (BMI) over 30 kg/m^2 [2]. Maternal obesity increases risk of macrosomia, large for gestational age and preterm birth. It has also been tied to increased childhood/adulthood adiposity and cognitive impairment [3–5]. It is now clear that a major mechanism through which prenatal exposures induce life-long effects on health is epigenetics [6]. Thus, examination of maternal obesity on newborn DNA methylation using an epigenome wide approach could identify fetal genes influenced by maternal obesity.

The Pregnancy and Childhood Epigenetics (PACE) consortium identified methylation sites associated with prepregnancy maternal BMI by meta-analysis of 19 cohorts (n = 9340) using the Infinium HumanMethylation450K chip on newborn DNA [7]. Over 9000 CpG sites were initially associated with maternal BMI examined linearly with percent DNA methylation. The group concluded that of these sites, eight may be causally related as associations were stronger with maternal rather than paternal BMI and direction of associations persisted to adolescence. However, the findings are considered preliminary since the magnitude of methylation differences were so small they may not be meaningful (<0.2%) [7]. Also, some of these studies used microarrays with relatively limited coverage [7]. Prior to this PACE investigation, other studies, including a few which also contributed data to the consortium, had identified yet other sites associated with maternal BMI [8–10].

Previous studies relied on BMI as the measure of adiposity from mostly self-reported prepregnancy weight and height. Thus, more direct measures of adiposity taken before pregnancy could be helpful in deciphering the impact of maternal adiposity by reducing measurement error. Also, central adiposity, as measured by waist circumference and

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in relation to subcutaneous fat by measured waist-to-hip ratio (WHR), is more strongly associated with metabolic dysregulation than high BMI. The genetic basis of these types of adiposity also differ, leading to individuals with propensity to deposit adiposity in different areas of their body [11], particularly in women [12]. Skinfold thicknesses may also better reflect total body fat, whereas leptin provides a measure of adipocyte function. Thus, we aimed to determine whether maternal prepregnancy adiposity measures (by BMI, waist/hip circumferences, skinfolds, serum leptin) are associated with the DNA methylation pattern of newborn cord blood in a preconception cohort using the Illumina EPIC microarray.

Materials & methods

Study population

The Effects of aspirin in gestation and reproduction (EAGeR) trial (2007–2011; NCT00467363) randomized women to LDA (i.e., 81 mg LDA and 400 μg folic acid) or placebo plus folic acid prior to pregnancy, to determine whether LDA could improve live birth rates in women who previously experienced one or two prior pregnancy losses [13]. Women between 18 and 40 years old, with a history of one or two prior pregnancy losses, no history of infertility, actively trying to conceive and with regular menstrual cycles of 21–42 days during the past year, were eligible for the trial. Among women who conceived during follow-up, 597 live births occurred. Beginning in 2009, the trial collected 10 ml cord blood from over 90% of the deliveries in the Utah site. IRB approval was attained prior to enrollment (UT, USA; IRB #1002521).

Anthropometric measures

Maternal BMI (kg/m^2) was calculated based on clinically measured weight and height at baseline prior to pregnancy. BMI categories followed standard WHO definitions with BMI < 25.0 as reference (due to only 9 women who are underweight BMI < 18.5), 25.0- < 30.0 as overweight and BMI ≥ 30 as obese. Waist and hip circumference (cm) as well as subscapular, suprailiac and triceps skinfolds were also measured at baseline prior to pregnancy. Skinfolds were summed together and examined as one measure. WHR above 0.85 was used to define central obesity by WHO criteria [14]. Baseline preconception anthropometrics were collected prior to randomization. Visits occurred during days 2–4 of each participant's menstrual cycle. Participants were followed for up to six cycles while attempting pregnancy and then throughout pregnancy for those that conceived. The median time between the baseline preconception visit and the 8-week gestation visit during pregnancy was 4 months (range from 3 to 8 months). Hence, anthropometric measures were taken approximately 2 months prior to women's estimated day of conception.

Laboratory methods

Leptin was measured from maternal blood samples collected at baseline prior to pregnancy using ELISA (3.3% interassay CV). Cord blood was centrifuged and separated into plasma and buffy coat. Samples were thereafter frozen at -80°C and in 2017 shipped to the University of Minnesota for DNA extraction and analysis. We processed 429 cord blood buffy coat samples; 30 samples did not have sufficient DNA for analyses and a total of 391 mother–child pairs were included in the analysis. DNA underwent bisulphite conversion with standardized kits (e.g., Zymo EZ DNA MethylationTM kit, Zymo, CA, USA; Supplementary Figure 1). The Infinium MethylationEPIC BeadChip microarray was used to measure genome-wide DNA methylation [15,16].To control for batch effects, samples were randomly ordered. Samples from two EAGeR newborns one male and one female yielding high amounts of DNA were used as internal controls. DNA from the female newborn was replicated 11 times and DNA from the male newborn was replicated ten times. A female and male were chosen to ensure sex differences in reproducibility may be examined. Plate and positions were tracked to account for technical variability in analyses.

Statistical analysis

Data cleaning

Methylation data were processed using the minfi package in R [17], including the identification of failed probes and scaling with Illumina control probes to determine methylation values. The β value was determined for each of the CpG sites by the fluorescent signals (β = Max (M, 0)/[Max(M,0) + Max(U, 0) + 100) [18]. β values approaching 1 are completely methylated and those close to 0 are unmethylated. Background and dye-bias corrections were applied.

Quantile normalization was used to normalize β values between two types of probes [19]. The purpose of this step is to eliminate potential probe type bias (type I vs II probes). Cell type mixture was then estimated on the full set of normalized data (FlowSorted.CordBlood.450K package) [20]. Principal component analysis was performed to further detect outliers and samples mismatched in sex compared against information from electronic medical records. 5 samples with sex mismatch were further excluded. We extracted the detection p-value for each methylation measure (per site per sample) and filtered data which failed detection p-value ($p > 0.01$). β values were replaced as missing if either detection p-value > 0.01 or bead counts < 3. We removed samples and CpG sites with low passing rate (<97%) based on detection p-value and bead counts. After probe removal, 831,807 CpG probes remained. Probes identified from the EPIC chip which may be affected by gene polymorphisms or can potentially cross-hybridize were further removed [21].

Modeling exposure

We evaluated the association between maternal adiposity and methylation differences. Maternal BMI was modelled both continuously and as 2-level BMI categories (reference nonobese vs obese). Waist circumference, WHR, SST and leptin were examined continuously. Central obesity was modeled dichotomously.

Modeling outcomes

Linear mixed effects models were used to test associations between methylation β values at each CpG site and exposures of interest with adjustment for covariates. Batch effects (as covariates of chip and row) were accounted for through random effects. To account for multiple testing, Bonferroni correction which divided the alpha level of 0.05 with the number of CpGs tested was applied ($p < 6.01 \times 10^{-8} = 0.05/831{,}807$). We did not further account for testing of each of the anthropometric measures as central adiposity may differ in impact from total adiposity (i.e., waist circumference vs BMI).

Covariates

Covariates evaluated include maternal age, race, smoking status, income, marital status, parity, infant sex and cell count distribution. To be parsimonious, the final models included maternal age, income, infant sex and cell count distribution as marital status, race, smoking and parity were not associated with maternal obesity (all $p > 0.7$). Cell counts were estimated based on a recent cord blood reference using *minfi* in R [20], including B-cell, CD-4+ T cells, CD-8+ T cells, granulocytes, monocytes, NK-cells and nucleated red blood cells (RBC). A major difference previously identified between adult versus cord blood cell count distribution in terms of DNA contribution from buffy coat samples is the proportion of nucleated RBCs [20]. In sensitivity analysis, models were also run without adjustment for infant sex and/or cell count.

Annotation

The Illumina database was primarily used for identifying gene annotations except where missing, in which case UCSC genome browser was searched to augment genes within 5 kb of the CpG site.

Results

Women averaged 28 years of age, were predominantly white (97%), married (95%) and had more than high school education (90%). (Table 1) BMI averaged 25.2 kg/m^2, with 18% obese and 27% centrally obese. Maternal obesity was not associated with smoking ($p = 0.79$), marital status ($p = 0.84$) or parity ($p = 0.99$). It was, however, associated with lower income ($p = 0.01$). Although this study was nested in a randomized trial of LDA, methylation did not differ by aspirin treatment groups (data not shown). Prepregnancy BMI was correlated with waist circumference ($r = 0.88$), WHR ($r = 0.44$), sum of skinfolds (SST; $r = 0.71$) and leptin ($r = 0.82$).

We identified several associations between methylation sites and maternal obesity, which were Bonferroni genome-wide significant ($p < 6.01 \times 10^{-8}$). (Table 2) One CpG was associated with maternal obesity defined by BMI (i.e., cg02975187 on chromosome 22). In examining the distribution of the methylation at this site by obesity status (Supplementary Figure 2), there was a cluster of newborns from obese women with high levels of hypomethylation in this region. The clustering might suggest a single nucleotide polymorphism cis- or trans-acting with the CpG identified. However, we lacked genotype data to directly confirm. One CpG was hypomethylated in newborns of mothers with central obesity as defined by WHR (i.e., cg12053563 on chromosome 14). No other

Table 1. Maternal characteristics of newborns with DNA methylation data in effects of aspirin in gestation and reproduction.

Characteristics	Mean (SD) or n (%)
N	391[†]
Age (years)	28.37 (4.48)
White	379 (97%)
High school or more	350 (90%)
Parous	232 (59%)
Married	373 (95%)
2 previous losses	133 (34%)
Randomized to LDA	200 (51%)
Never smoke	357 (91%)
Male infant sex	190 (49%)
Gestational diabetes	9 (2%)
BMI (kg/m^2)	25.16 (5.56)
Normal weight	227 (59%)
Overweight	92 (24%)
Obese	69 (18%)
Weight (kg)	69.31 (16.87)
Height (cm)	165.31 (15.63)
Waist circumference (cm)	85.38 (13.47)
Hip circumference (cm)	105.22 (11.79)
Waist-to-hip ratio	0.81 (0.07)
Central obesity (WHR $\geq$ 0.85)	107 (27%)
Subscapular skinfold (units)	19.95 (9.20)
Suprailiac skinfold (units)	22.35 (10.86)
Triceps skinfold (units)	25.41 (9.72)
Sum of skinfolds (units)	67.71 (27.16)

[†]Three women were missing weight at baseline, 1 was missing skinfold measures.
BMI: Body mass index; LDA: low dose aspirin; WHR: Waist-to-hip ratio; SD: Standard deviation.

associations were Bonferroni significant but suggestive associations ($p < 1 \times 10^{-6}$) included one CpG associated with obesity and eight additional CpGs with central obesity.

For the continuous measures of adiposity and leptin, no Bonferroni significant associations arose (Table 2). Of the six suggestive sites associated with continuous BMI, four of them were identified regardless of adjustment for infant sex and cell count (Supplementary Table 1), while two were only identified after adjustment (cg02918970 on chromosome 14, cg13460858 on chromosome 21). The CpGs identified using other measures of adiposity were compared against the sites for BMI to better understand the overlap in the identified sites. The two CpGs identified for waist circumference (cg05072085, cg14420357) were also associated with BMI, suggesting they may not be specific for central adiposity. Leptin also was associated with this latter site (cg14420357). However, the two CpG sites with WHR did not overlap with sites associated with BMI. Most associations were with decreased methylation, except for SST, which tended to be associated with increased methylation. No overlap was seen for these six suggestive CpGs and others. Nine additional CpG sites were identified in association with preconception leptin levels, not identified by any of the anthropometric measures. Although there was correlation between the associations for leptin and BMI, no sites were strongly associated with both phenotypes. These observations suggest that the maternal adiposity as measured by BMI differs from maternal adipocyte function as measured by leptin. Genome inflation in all models was minimal (Supplementary Table 2).

In sensitivity analysis, we evaluated the impact of model adjustment. Supplementary Table 1 shows associations minimally adjusted for maternal age and income. Twelve CpG sites were Bonferroni genome-wide significant with continuous BMI ($p < 6 \times 10^{-8}$) and 59 additional sites had suggestive levels of significance ($p < 6 \times 10^{-6}$). Four associations reached Bonferroni genome wide significance for other measures of adiposity (Supplementary Table 1; cg05072085 with WC, cg13536409 with SST, cg10578324 with leptin) but were attenuated ($p < 6 \times 10^{-6}$)

Table 2. Maternal adiposity related measures and newborn DNA methylation in effects of aspirin in gestation and reproduction (n = 391).

BMI (per kg/m^2)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg02918970	-0.00158	0.000294	7.73E-08	0.851	10	70621889	*STOX1*	
	cg03550075+	0.001377	0.0002632	1.66E-07	0.850	12	132278448	*SFRS8*	S Shore
	cg02236945+	-0.000756	0.0001479	3.24E-07	0.876	2	191269737	*MFSD6*	N Shelf
	cg05072085‡+	-0.000665	0.000133	5.77E-07	0.855	8	134198574	*WISP1*	
	cg13460858	-0.000878	0.0001774	7.38E-07	0.148	9	96720652	*BARX1*	Island
	cg02975187+	-0.002525	0.0005135	8.75E-07	0.855	22	18512423	*FLJ41941*	
Obesity (BMI ≥30 kg/m^2)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg02975187	**-0.04081**	**0.007332**	**2.62E-08**	0.855	**22**	**18512423**	***FLJ41941***	
	cg10739592	0.01975	0.003751	1.39E-07	0.815	8	129962331		
WC (per cm)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg05072085‡+	-0.000274	0.00005379	3.58E-07	0.855	8	134198574	*WISP1*	
	cg14420357‡	-0.000332	0.0000667	6.44E-07	0.830	6	158464617	*SYNJ2*	
WHR (per unit)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg05905044	-0.0879	0.01747	4.90E-07	0.745	17	10710010	*TMEM220-AS1*	
	cg12848697	-0.06905	0.01373	4.94E-07	0.837	18	13133368		N Shelf
Central obesity (WHR ≥ 0.85)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg12053563	**-0.01162**	**0.001983**	**4.58E-09**	0.835	**14**	**29637580**		
	cg12549355	-0.01113	0.00207	7.54E-08	0.732	21	11126993		
	cg09073263	-0.01584	0.003148	4.87E-07	0.754	20	61771440		
	cg09324752	-0.006796	0.001363	6.15E-07	0.812	22	39546207	*CBX7*	N_Shore
	cg06958525	-0.008351	0.001676	6.26E-07	0.853	2	108370346	*GACAT1*	
	cg08807496	-0.01196	0.002402	6.43E-07	0.794	2	241831618	*C2orf54*	S_Shelf
	cg07560064	0.006758	0.001366	7.46E-07	0.058	16	22018867	*C16orf52*	N_Shore
	cg19402503	0.01647	0.003343	8.33E-07	0.754	11	128211576		
	cg15392364	0.01398	0.002848	9.16E-07	0.713	1	206976689	*IL19*	
SST (per unit)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg11416447	8.83E-05	0.00001631	6.19E-08	0.070	7	86781551	*DMTF1*	N Shore
	cg13536409+	0.0003112	0.00005912	1.41E-07	0.269	7	158498471	*NCAPG2*	S Shore
	cg02588321+	0.0002254	0.00004607	9.94E-07	0.192	7	22485037	*MGC87042*	

Models adjusted for maternal age, income, infant sex and cell type distribution (estimated by cord blood reference).
Associations shown with $p < 10^{-6}$. (In bold, Bonferroni genome significance at $p < 6.01 \times 10^{-8}$).
+ indicates significant for model 1 (adjusted for age and income).
‡same CpG site identified as for another adiposity measure (regardless of model 1 or 2).
BMI: Body mass index; SE: Standard error; SST: Sum of skinfolds; WC: Waist circumference; WHR: Waist-to-hip ratio.

Table 2. Maternal adiposity related measures and newborn DNA methylation in effects of aspirin in gestation and reproduction (n = 391) (cont.).

BMI (per kg/m^2)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
Leptin (per ng/ml)	CpG site	β	SE	p-value	Mean	Chr	Position	Gene	Relation to CpG island
	cg19625347	-1.93E-07	3.69E-08	1.73E-07	0.888	3	133721889	*SLCO2A1*	
	cg01872122	-2.25E-07	4.33E-08	2.15E-07	0.869	5	16940481	*MYO10*	S_Shelf
	cg07637375	-2.73E-07	5.30E-08	2.73E-07	0.835	16	84210542	*DNAAF1*	
	cg10578324	-4.20E-07	8.19E-08	2.82E-07	0.855	5	133187068		
	cg03278488	2.22E-07	4.45E-08	5.83E-07	0.126	16	85044408	*ZDHHC7*	N_Shore
	cg14420357	-2.54E-07	5.16E-08	8.82E-07	0.830	6	158464617	*SYNJ2*	
	cg05364570	-2.08E-07	4.24E-08	8.92E-07	0.854	16	72984764	*ZFHX3*	
	cg01889485	3.94E-07	8.03E-08	9.15E-07	0.780	2	2233529	*MYT1L*	
	cg06542302	-2.62E-07	5.35E-08	9.41E-07	0.858	12	132924218		S_Shore
	cg16139161	-4.15E-07	8.48E-08	9.78E-07	0.851	7	150069806	*REPIN1*	Island

Models adjusted for maternal age, income, infant sex and cell type distribution (estimated by cord blood reference).
Associations shown with $p < 10^{-6}$. (In bold, Bonferroni genome significance at $p < 6.01 \times 10^{-8}$).
+ indicates significant for model 1 (adjusted for age and income).
‡same CpG site identified as for another adiposity measure (regardless of model 1 or 2).
BMI: Body mass index; SE: Standard error; SST: Sum of skinfolds; WC: Waist circumference; WHR: Waist-to-hip ratio.

after adjustment for cell count and infant sex. We also compared results additionally adjusting for sex but not cell count with those adjusting for cell count. The greatest impact of cell count adjustment was on the BMI results. Supplementary Table 3 shows the 14 Bonferroni corrected significant associations when models were adjusted for only age, sex and income in comparison to when models were additionally adjusted for cell count. 11 of these genes were no longer even suggestively significant ($p > 1 \times 10^{-6}$) after cell count adjustment. As expected, some of the CpGs removed were related to the immune pathway (e.g., *RAB44*, *ITGB2*), although for most, the gene functions are unclear with regard to methylation differences.

Discussion

In this epigenome wide analysis, we found that maternal adiposity as clinically measured prior to pregnancy does not have a strong impact on offspring DNA methylation patterns at birth. Prepregnancy obesity as defined by BMI was associated with a Bonferroni corrected significant association identified (cg02975187; *FLJ41941*) and central obesity was associated with one further CpG. However, the biological relevance of these three sites are uncertain. Further suggestive associations were found with BMI and other measures of adiposity prior to pregnancy.

CpGs identified with BMI at or near gene regions include cg02918970 (*STOX1*), cg03550075 (*SFRS8*), cg02975187 (*FLJ41941*), cg14420357 (*SYNJ2*), cg02236945 (*MFSD6*), cg05072085 (*WISP1*) and cg13460858 (*BARX1*). The first four CpGs were in the introns of the genes and the latter three are near (±5 kb) the listed gene. Of these, *SFRS8* methylation in subcutaneous adipose tissue was recently found to be associated with propensity to gain weight [22]. In LIPOGAIN, weight gain in response to high fat feeding for 7 weeks among 31 individuals, was predicted by baseline adipose tissue DNA methylation levels of multiple genes including *SFRS8* based on the Illumina 450K microarray [22]. The associated CpG identified (cg10437240) differed from the current analysis (cg03550075), but suggests a potential mechanism of inheritance from mother to offspring. Of the other CpGs, *cg05072085* is near (~4.5 kb) the *WISP1* gene found to be associated with adipose tissue inflammation and differentiation [23–25]. The other annotated genes have been previously identified primarily in the development of some cancers where metabolic disturbance and/or insulin resistance played a role. In particular, we observed that cg14420357 (i.e., *SYNJ2*) methylation was lower in association with multiple measures of adiposity including BMI, waist circumference and leptin levels. Although no studies have reported its effects specific to methylation differences, variants in the gene are associated with colorectal cancer [26,27]. Hence, they may serve to support epidemiologic findings suggesting that maternal obesity confers offspring risk of colorectal cancer incidence, particularly in men [28]. Shared common risk factors may also explain these associations. Nevertheless, the high number of genes identified that also are related to cancer may be an artifact of CpGs being originally investigated in tumor cells.

Heterogeneity remain for the associations between maternal obesity/BMI and offspring cord blood methylation among studies [7]. Similar to PACE, previous associations were not replicated in our current analysis, including the eight CpGs identified by the consortia for each unit increase in maternal BMI [7], the CpG identified among a subgroup of black children from the Boston Birth Cohort (cg01422136) using the Illumina 27K beadchip [9], and the CpGs from the Avon Longitudinal Study of Parents and Children (ALSPAC; 1991–1992) in their work prior to joining the consortium [29]. The heterogeneity could be due to limitation of sample size or difference in targeted population. However, when we compared our results to those publish in PACE, a general agreement is observed. Among the top 104 CpG sites in PACE (Sharp *et al.*, 2017; Supplementary Table 4), we have 96 CpG sites in common after QC in our study. There were 33 CpG sites with $p < 0.05$ in our model 1 results, and 14 in model 2 results. A one-sided binomial test for null hypothesis of proportion = 0.05 gave a p-value of 2.9×10^{-19} for model 1 results comparison, and 0.0003 for model 2 comparison. The effects sizes also agree with what PACE reported mostly (Supplementary Figure 3).

Since we were the only group to assess other measures of adiposity or leptin levels prior to pregnancy, our results could not be compared with previous studies. For maternal central adiposity, while waist circumference was similar to BMI, WHR was associated with methylation at two sites, one of which (cg05905044) is annotated to *TMEM220-AS1*, a noncoding RNA on chromosome 17 with high expression in stomach, liver and intestinal tissues but unknown in function [30]. A long noncoding RNA, LINC00675, near this region (~3 kb) is associated with tumor suppression in gastric cancer [31]. The other site (cg1284869) is not near a known gene region. The suggestive associations between maternal preconception leptin and newborn methylation implicated yet different genes. Three of the CpGs were located at genes tied to adiposity. Specifically, *REPIN1* (cg16139161) is associated with adipocyte cell size and glucose transport [32]. *ZFHX3* (cg05364570) was identified by GWAS of rare obesity variants [33].

MYT1L (cg01889485) is only expressed in neuronal tissues with protein functions tied to development of the fetal nervous system. There also is some evidence of mutations in the gene being associated with cognitive disabilities in children, along with development of obesity pointing to upstream dysregulation of the hypothalamus [34]. This marker, if replicated, could explain ties between maternal obesity through leptin and potential neurodevelopmental outcomes [35]. Two CpGs were located at or near genes related to cancer development. Cg01872122 was within ~4.5 kb of *MYO10*, in the myosin subfamily and important for intracellular movements, has been implicated in many cancers [36]. *DNAAF1* (cg07637375) codes for cilium-specific proteins has been associated with testicular cancer [37] and neural tube defects [38]. Neural tube defect risk linearly increases with maternal BMI [39] but the specific role of leptin in these associations may be pursued [40]. *SLCO2A1* (cg19625347) encodes for a prostaglandin transporter protein involved in clearance of prostaglandin from numerous tissues with loss of function mutations tied to chronic enteropathy [41]. Remaining sites had no known nearby genes (cg10578324, cg06542302). Similarly, the sites associated with SST had no known function or were related to cancer/development. Moreover, these CpGs were hyper- rather than hypomethylated as SST increased.

We show results by different covariate models (i.e., Model 1: age, income vs Model 2: age, income, sex, cell type) as it is unclear whether infant sex and cell type distribution should be adjusted for and included in the models. Newborn sex is not likely on the causal pathway since there is little evidence that maternal obesity alters the secondary sex ratio [42], and preserving it in statistical adjustment is unlikely to be biased. On the other hand, cell type is likely on the causal pathway between maternal adiposity and newborn methylation, with clear evidence that immune cell distribution differs by maternal obesity at delivery [43]. In this setting, adjustment for cell type may lead to 'overadjustment' [44]. Additionally, the cell count references, whether cord blood or otherwise, may have inaccuracies leading to measurement error. Levels of DNA methylation from a mixture of cells are intrinsically counting different cell types and not adjusting for cell count leaves results inconclusive for identifying nonimmunologic pathways. DNA from cord blood is composed not just of leukocytes but of nucleated RBC [20], further complicating matters. Importantly, genomic inflation was decreased with the addition of cell type in the model and half of the CpGs were identified regardless of the covariates adjusted. We show how cell count adjustment factors matter in the case of maternal BMI (Supplementary Table 3). As expected, it reduces associations with sites particularly related to immune function. Potentially in investigations of how maternal obesity may lead to later risk of offspring asthma or allergies [45] or other immunological disorders, adjustment of cell count should be carefully considered.

In addition to statistical modeling, the heterogeneous results compared with previous studies may also be due to the use of different microarrays and the assessment of obesity taken prior to pregnancy versus self-report. Although self-reported weight and height are highly concordant with measured values, clinical measures are less prone to measurement error. These and other differences make it difficult to conclude whether studies identifying different CpGs suggest that they are not reproducible. On the other hand, residual confounding may have impacted studies on different levels as maternal obesity has heterogeneous etiologies. DNA methylation is tissue specific, and neonatal adipose tissue may be the more appropriate target for evaluating future risks of obesity. However, some evidence in adulthood obesity suggests overlap in methylation of CpGs in white blood cells and adipose tissue [46]. In comparison to the 23 top sites commonly differentially methylated in both leukocytes and adipocytes [46], we did not find overlap. Placental methylation may also provide more clues to differences [47]. Of note, we used preconception levels of leptin which may differ from pregnancy levels of leptin as the placenta also is a source of leptin [48]. However, as leptin plays a role in blastocyst formation and other early developmental pathways [48], having preconception levels removes the issues of teasing apart sources of leptin if measures were taken only during pregnancy.

Our study was strengthened by the measurement of all maternal anthropometry and leptin prior to pregnancy by trained technicians in a clinical setting. The exposure assessment conducted is not subject to reporting errors. Our study also had several limitations. Primarily, our sample size limited our ability to detect smaller effects. However, similar to the large consortia findings [7], very small methylation differences were observed and it is unclear how biologically meaningful these small differences are (<1%). While paternal adiposity might play a role in conjunction with maternal adiposity [49], we lacked paternal information to evaluate further. We were also unable to pyrosequence the CpGs but previous report shows high correlation between methylation measured by microarray technologies and by pyrosequencing [50]. Lastly, folic acid is a known methyl donor and its provision to all women in the study prior to conception and through pregnancy, while reducing a source of confounding, might have reduced generalizability should folic acid modify the impact of maternal obesity on methylation differences. However,

conflicting findings from animal models have suggested that folic acid could either be adiposity promoting [51] or inhibiting [52,53]. More evidence on the impact of maternal folic acid and other dietary factors on newborn methylation is required to further understand its role.

Conclusion & future perspective

In conclusion, maternal prepregnancy adiposity does not lead to profound alterations on cord blood methylation in newborns. Further research is needed to understand the biological mechanisms through which maternal obesity leads to transgenerational effects. Such examination includes potentially isolating specific cell types. Moreover, the neonate's own anthropometry, as influenced by parental adiposity in conjunction with other pregnancy exposures, may be a more proximal determinant of cord blood DNA methylation. Future investigation of these connections could also provide insight into the associations observed between birth size and risk of cardiovascular disease [54]. Unlike genetic studies, epigenetic studies are subject to confounding bias and thus greater use of randomized trial designs could also help understand the causal influence of exposures on DNA methylation in the short and long term.

Summary points

- DNA methylation in newborn cord blood was examined for differences by preconception measures of maternal adiposity and leptin.
- Previous studies relied on maternal recall or used early pregnancy measures rather than preconception measures which are not influenced by fetal/placental weight.
- The Illumina EPIC microarray was used, with data from 391 cord blood samples.
- After adjusting for estimated cell type composition, few associations at Bonferroni level of significance were observed.
- A suggestive association between increased preconception body mass index and DNA methylation of the *SFRS8* gene body was observed.
- *SFRS8* was previously found to be associated with propensity for adult weight gain.
- Additional associations were observed depending on type of maternal measure (i.e., central adiposity vs total adiposity by sum of skinfolds identified different CpGs).
- Hypomethlyation was largely observed with increasing adiposity/leptin except for sum of skinfolds.
- Differences in methylation in association with adiposity phenotypes were generally weak.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/epi-2018-0099

Financial & competing interests disclosure

This work was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIH, Bethesda, MD, USA) under contract numbers HHSN267200603423, HHSN267200603424, HHSN267200603426 and HHSN275201300023I-HHSN2750008.

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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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