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Differentially methylated regions interrogated for metastable epialleles associate with offspring adiposity

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ABSTRACT

Aim: Assess if cord blood differentially methylated regions (DMRs) representing human metastable epialleles (MEs) associate with offspring adiposity in 588 maternal-infant dyads from the Colorado Health Start Study.

Materials & methods: DNA methylation was assessed via the Illumina 450K array (~439,500 CpG sites). Offspring adiposity was obtained via air displacement plethysmography. Linear regression modeled the association of DMRs potentially representing MEs with adiposity.

Results & conclusion: We identified two potential MEs, *ZFP57*, which associated with infant adiposity change and *B4GALNT4*, which associated with infancy and childhood adiposity change. Nine DMRs annotating to genes that annotated to MEs associated with change in offspring adiposity (false discovery rate <0.05). Methylation of approximately 80% of DMRs identified associated with decreased change in adiposity.

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adiposity; biomarkers; childhood obesity; DNA methylation; metastable epialleles

1. Introduction

Approximately one in five children and adolescents aged 2–19 years in the United States has obesity [1], highlighting a major public health problem. Equally concerning is the sharp increase in the prevalence of Class I obesity among 2- to 5-year-olds that has been noted since the 2013–2014 National Health and Nutrition Examination Survey (NHANES) and has continued through at least NHANES 2015–2016 [1]. Research suggests that obesity in infancy predicts obesity in later childhood and adolescence [2,3] and portends increased risk of psychosocial problems, high blood pressure, dyslipidemia and abnormal glycemic status, including type 2 diabetes mellitus [4]. Differences in obesity risk may appear as early as infancy [2], which could suggest that the developmental factors driving obesity operate very early in life [5–7]. Genome-wide association studies have not been able to fully explain the total variance in risk conferred in childhood adiposity [5,8], and there is emerging evidence from human studies of the potential for epigenetic signatures, namely DNA methylation (DNAm), to serve as predictive biomarkers of obesity risk [9–12]. Although such studies are limited in their assessment of the downstream transcriptomic and proteomic functional outcomes, they do indicate early life developmental factors may be significant in contributing to phenotypic variation and identifying disease risk.

Human metastable epialleles (MEs) are unique genomic regions established during early embryogenesis that show systemic interindividual variation and stability across different tissues and may be influenced by preconceptional exposures [13,14]. Previous studies suggest that DNAm of these regions may partially explain interindividual phenotypic variability in disease risk that begins *in utero* [15–17]. In addition, DNAm of MEs has been suggested to play a role in energy balance and has previously been associated with obesity in adult and pediatric populations [18–21]. These key characteristics make MEs relevant as potential early life biomarkers for assessing risk of obesity and its related comorbidities from birth onward. Differential DNAm of ME regions in genes related to energy

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balance, nutrient signaling and metabolism and/or adipogenesis may thus serve as a novel biomarker at birth that predicts adiposity later in childhood and could trigger additional clinical monitoring and/or alternative nutritional recommendations or interventions to prevent excessive adiposity gain.

In this study, we aimed to determine if DNAm of MEs detectable in neonatal cord blood are associated with adiposity at birth and the change in adiposity from birth through 5 years of age. To the best of our knowledge, this is the first investigation to analyze cord blood DNAm of MEs and its specific association with adiposity at birth and the change in adiposity from birth to 5 months and 5 months to 5 years conducted amongst a large longitudinal prospective birth cohort. Our investigation is original given that we aimed to evaluate the association of DNAm of MEs with direct measures of fat mass instead of proxies such as BMI and we assessed the persistence of such methylation marks measured in cord blood at birth with adiposity change over known sensitive periods of auxological development in infancy and childhood. We hypothesized that cord blood DNAm patterns of differentially methylated positions within MEs and differentially methylated regions (DMRs) representative of MEs documented from the literature, particularly those associated with genes involved in energy balance, nutrient metabolism, growth and obesity, would be associated with adiposity at birth and change in adiposity from birth through 5 years of age, respectively. We further hypothesized that the association between DNAm of DMRs representing these MEs in cord blood and change in adiposity would persist from birth to 5 years, thus lending credence to their use as a novel biomarker for adiposity.

2. Materials & methods

2.1. Participants & study design

The Healthy Start Study is a longitudinal prospective pre-birth cohort (clinicaltrials.gov NCT02273297) that recruited 1410 pregnant women from outpatient obstetric clinics at the University of Colorado Hospital from 2009 to 2014 as previously described [22]. Briefly, women were eligible for participation if they were 16 years of age or older, pregnant with a single fetus, less than 24 weeks gestation at enrollment, had no history of extreme preterm birth nor stillbirth, and no self-reported history of diabetes, asthma, cancer or psychiatric illness. At the initial research visit, information on maternal age, education, gravidity, annual household income, race and ethnicity were obtained via self-report. Participants completed questionnaires, provided blood samples at a median of 17 weeks and 27 weeks' gestation, and authorized review of their medical records. Umbilical cord blood was collected at delivery on 867 mother–infant dyads, 600 of which were selected for an ancillary DNAm analysis based on availability of both maternal mid-pregnancy blood and urine samples and neonatal cord blood samples (R01ES022934). After five dyads withdrew consent and seven were removed from analysis due to inadequate quality of samples, 588 dyads remained for the present analysis. Our study subset of mother–infant pairs is representative of the full Healthy Start Study cohort in terms of demographic and clinical characteristics (Supplementary Table S1) [23]. Written informed consent was provided by all participants and study procedures were approved by the Colorado Multiple Institutional Review Board.

2.2. Umbilical cord blood collection, genomic DNA isolation & DNAm

Umbilical cord blood was collected at delivery and processed by the University of Colorado Clinical and Translational Science Institute Core Laboratory. Buffy coat fractions were separated and immediately stored at -80°C for later analyses. Genomic DNA was isolated from buffy coat samples using the QIAamp kit following manufacturer's instructions (Qiagen, Germantown, MD, USA) [24]. DNA purity was assessed via the NanoDrop 2000 Spectrometer (ThermoFisher Scientific, Waltham, MA, USA). DNA quantity and quality were determined using the Qubit Fluorometer (ThermoFisher Scientific, Waltham, MA, USA) and the Bioanalyzer 2100 (Agilent), respectively. Samples with a 260–280 nm ratio greater than 1:8 and a DNA Integrity Score (DNA) greater than 7 were used for DNAm analyses.

Genome wide DNAm was assessed by the University of Colorado Genomics and Microarray Core using the Illumina Infinium 450K Human Methylation array as previously described [24]. Briefly, 500 ng of genomic DNA underwent bisulfite conversion using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA). Bisulfite converted samples as well as commercially available positive and negative controls served as the input for the Illumina Infinium HumanMethylation450 BeadChip assay. Data were visualized with the GenomeStudio software and examined using both sample-dependent and sample-independent quality control criteria, and signal intensities and detection *p*-values of methylated and unmethylated probes were exported.

2.3. Infant & child body composition measures

Body composition (fat mass and fat free mass) was measured via air displacement plethysmography (ADP) within 3 days of birth, at approximately 5 months of age, and again at about 5 years of age using the PEAPOD

and BODPOD devices (Cosmed, Rome, Italy) as previously described [25]. These devices utilize a two-compartment model to estimate whole body fat mass and fat-free mass [26–29]. The validity and reliability of this body composition method has been assessed previously [27]. Adiposity was calculated as the percent of total mass that is fat mass. Two measures were taken, with a third obtained when adiposity differed by >2%, and the closest two measurements were averaged for analysis. Adiposity at birth, change in percent fat mass (%FM) (fat mass/total mass × 100) from birth to 5 months of age and from 5 months to 5 years of age were the main outcome measures for this analysis and were normally distributed.

2.4. DNAm quality control

We evaluated DNAm data from 588 individuals using Illumina's 450K array, with an original count of 484,261 probes. For quality control, one sample was removed for low median intensity using the Tukey far-out outliers method [30] with a cutoff of less than the 25th quantile or greater than the 75th quantile and six samples were removed due to mismatched sex between the clinical data and predicted sex from their methylation using the minfi getSex function (R version 4.0.2 (22 June 2020)) [31]. Probes were excluded if they had detection *p*-values (>0.05) in more than 10% of samples, a bead count <3 in at least 5% of samples, or a high detection *p*-value (>0.01) in more than 10% of samples. Cross-reactive probes (n = 27,349) were removed based on the investigations of Chen et al. [32] and probes with single nucleotide polymorphisms at the CpG interrogation and/or at the single nucleotide extension for any minor allele frequency (n = 17,272 probes) were excluded from the analysis of DMPs based upon Illumina's manifest and annotation information provided in the R package IlluminaHumanMethylation450kanno.ilmn12.hg19 (v 0.6.0). Probes on the X and Y chromosomes were included. Sex was adjusted for by including sex as a variable in the models. The final sample size was 439,281 probes and 588 participant samples (Figure 1).

2.5. Statistical analysis

Beta values for CpGs (i.e., the ratio of intensity at the methylated probe to the sum of the intensities of the methylated and unmethylated probes) were converted to M-values to better approximate a normal distribution, where M = log (beta/[1-beta]). The offset for the beta values was 100 (the default for the R packages minfi and lumi). Stratified quantile normalization was performed using the preprocessQuantile function in minfi [33]. Batch effects by sample plate were removed using ComBat [34]. For surrogate variable analysis, we used an intercept only model matrix with sample plate defined as the batch variable. Batch was adjusted during the preprocessing steps using ComBat as it could not be included in the model later. In addition, the sample size was large and samples were randomly allocated on the batches. We assessed the validity of ComBat, using multidimensional scaling (MDS) plots of normalized M values before/after ComBat batch correction and confirmed that clustering by plate on MDS plots was not observed. The MDS plots and batch correction steps are provided through GitHub.

Linear regression models were fit to estimate the association between adiposity at birth (outcome) and methylation M value (predictor) at each of the 927 CpG sites associated with MEs from the literature [16,17,35] using the Illumina Infinium 450K microarray after quality control measures were implemented (Figure 2). Linear regression models were also fit to estimate the association between the change in adiposity at each of the defined study time points, birth to approximately 5 months of age and approximately 5 months to approximately 5 years of age for each CpG on the array (a total of 439,281 probes) and then DMRs were identified via Comb-p [36]. Significant DMRs were then mapped to the nearest of the 927 CpGs annotated as MEs by genomic location [16,17,35]. We assessed the two-time periods separately as they may represent distinct sensitive periods for future adiposity risk. We also performed another analysis for DMR identification by implementing an alternative method, bumphunter [37,38].

Multiple comparisons were adjusted for by controlling the false discovery rate rate using the Benjamini–Hochberg procedure [39], across 439,281 *p*-values for the epigenome wide analysis (EWAS) and 927 *p*-values for the targeted DMP/ME analyses, with a level of significance set at 0.05.

Given the exploratory study aims, our main model evaluated infant race, ethnicity, sex, and gestational age as covariates (i.e., infant characteristics). We did not adjust for estimated cell proportions given that MEs are thought to be neither cell nor tissue specific, furthermore, DNAm was the predictor variable and not the outcome variable in our analyses [16,17]. In a secondary analysis, we accounted for potential effects of maternal pre-pregnancy BMI, gravidity, and prenatal smoking as these covariates have been associated with either offspring cord blood DNAm or the outcome of offspring growth or both in other studies [24,40,41]. Supplementary Figure S1 illustrates the forward model building approach used for the primary and secondary analyses and the covariates assessed. All analyses were conducted using R version 4.0.2 (2020-06-22) [31].

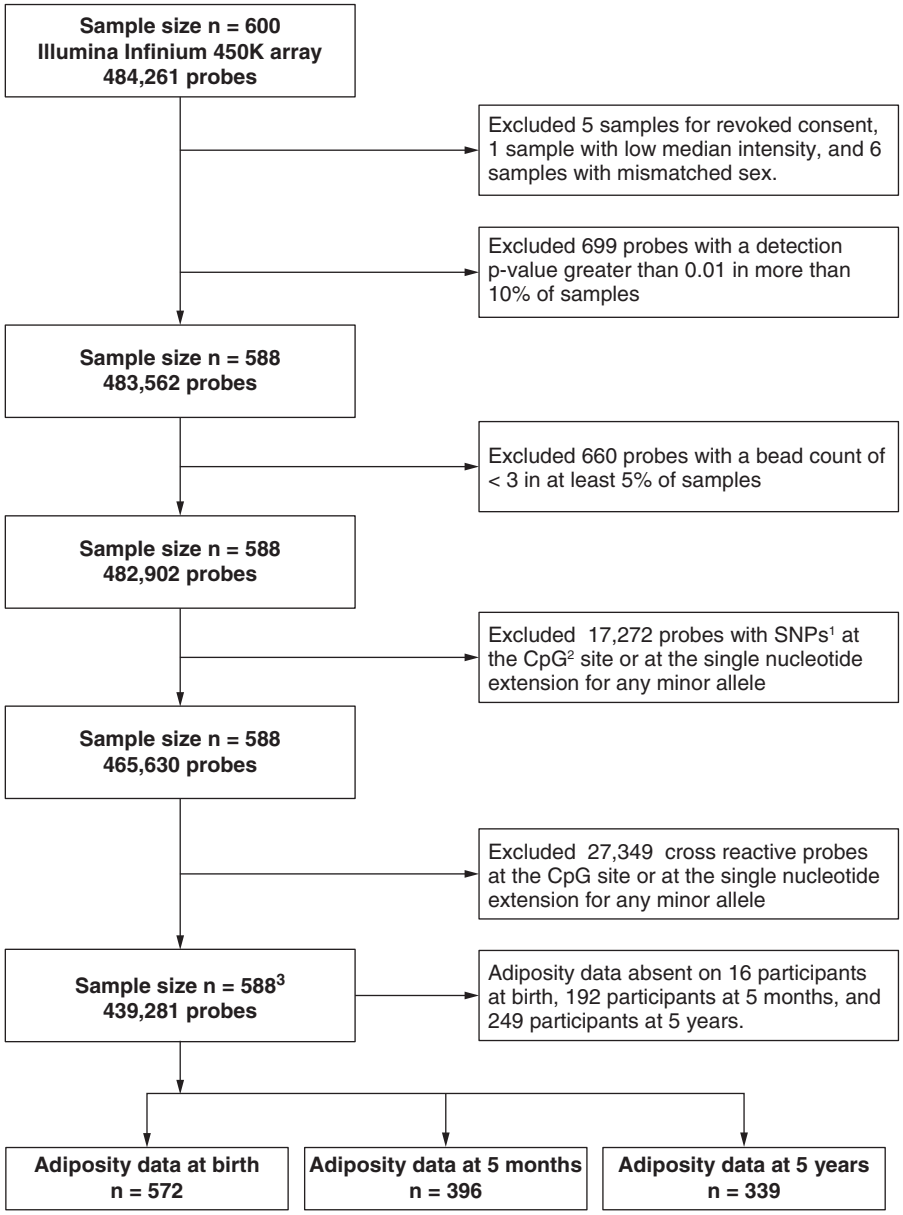

Figure 1. DNA Methylation Study quality control flow diagram and offspring participant flow diagram for adiposity data.
[1]SNP: Single nucleotide polymorphism; [2]CpG: Cytosine-guanine dinucleotide. [3]Further reduction in sample size for the three outcomes measures assessed (i.e., percent adiposity at birth, 5 months, and 5 years of age) is attributable to lack of adiposity measurements due to unavailability of the PEAPOD device as well as subject attrition.

2.6. Targeted analysis of DMPs near MEs & the association with adiposity at birth

We used the Illumina Infinium 450K microarray which measured DNAm across 439,281 CpG sites after quality control procedures (Figures 1 & 2). We performed a targeted analysis to determine if DMPs near MEs (i.e., 927 CpG sites referenced from the literature [16,17,35]) in infant cord blood were associated with adiposity at birth, change in adiposity from birth to 5 months of age and change in adiposity from 5 months to 5 years of age. Offspring cord blood DNAm at each DMP/ME served as the predictor and adiposity at birth, change in adiposity from birth to 5 months, and change in adiposity from 5 months to 5 years served as the outcomes with adjustment for selected covariates (Supplementary Figure S1).

2.7. Targeted analysis of DMRs near MEs & the association with change in adiposity

DMRs were identified using the Python package comb-p,[36] using the *p*-values from the untargeted epigenome-wide analysis in order to evaluate their

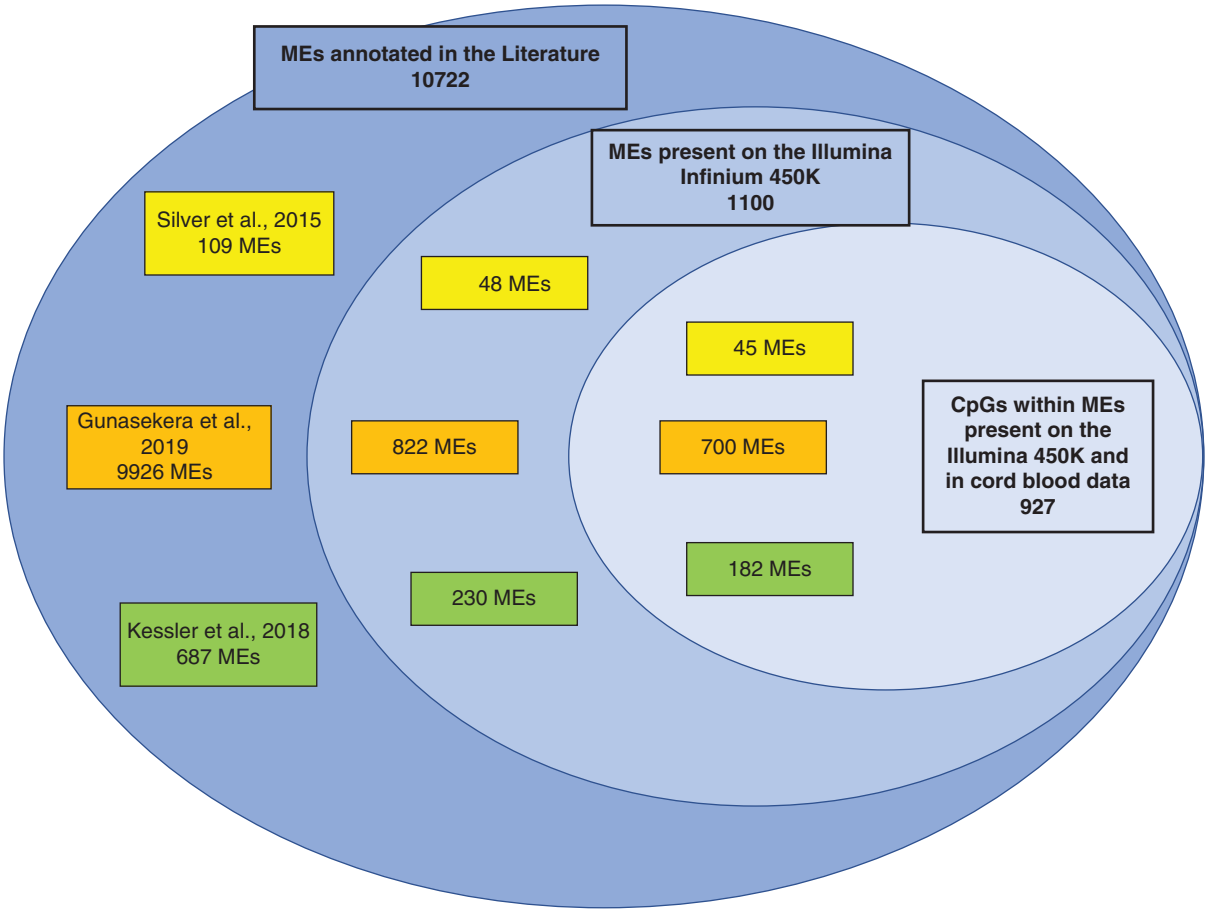

Figure 2. Metastable epiallele interrogation on the Illumina 450K and in Cord Blood by Literature Source.
Selection of MEs was based upon the following literature sources [16,17,35].
ME: Metastable epiallele.

association with change in adiposity from birth to 5 months and change in adiposity from 5 months to 5 years for the primary and secondary analyses, yielding a total of four statistical models. A seed of 0.1 was used, indicating a minimum *p*-value per CpG of 0.1 was required to start a region. Peaks/troughs were merged when within 750 bases of the other. We used the full set of 439,532 probes to identify the regions. Following identification of the DMRs through comb-p, the direction of the methylation of the probes within each region was calculated. The top probe within a DMR was defined as that with the smallest *p*-value. This process was conducted for the outcomes, change in adiposity from birth to 5 months and change in adiposity from 5 months to 5 years. The Sidak correction was used to adjust for multiple testing for the number of regions tested [36]. Linear regression models were fit to estimate the association between the predictor variables, the top, most significant CpG site of the DMRs identified by comb-p, and the outcomes of change in adiposity from birth to 5 months and change in adiposity from 5 months to 5 years of age. Covariate adjustments made in the EWAS DMR analysis were similar to those made in the EWAS DMP analysis.

To verify our results, we employed a second DMR identification method, the function bumphunter, as implemented in minfi to the normalized, ComBat-adjusted M-values for all probes. We tested changes in adiposity between birth and 5 months of age and 5 months and 5 years of age, while adjusting for infant sex, race and gestational age at birth. We employed 1000 bootstraps to generate the null distribution to compare our observed regions. We allowed bumphunter to pick the cutoff using the permutation distribution and set the quantile cutoff to 90%. Furthermore, to again mirror our previous comb-p analysis, we specified a maximum location gap to define cluster of 750 bp. We then compared our regions identified from bumphunter with the previous ones identified with comb-p. Using the total sample, we annotated the bumphunter regions with bumphunter's annotate Transcripts function, referencing TxDb.Hsapiens.UCSC.hg19.knownGene (hg19 to

mimic previous analyses which referenced IlluminaHumanMethylation450kanno.ilmn12.hg19). Then we used GenomicRange's subsetByOverlaps function to identify any DMRs from bumphunter that overlapped with our comb-p DMRs. We specified identification of any type of overlap, with a default maxgap of -1 and mingap of 0.

Genomic distance of DMRs and the annotated MEs from previous literature were identified using the R package GenomicRanges. The overlap of those DMRs with MEs identified from the literature (i.e., 927 CpGs) was assessed to identify the presence, if any, of DMRs that were MEs. Supplementary Tables S7 & S8 show the distance that significant DMRs associated with change in adiposity in our study were located from the genomic regions defined as MEs in the literature sources referenced [16,17,35]. A DMR was considered a possible ME if the smallest number of base pairs between the two identified regions was within approximately 10,000 bp [42] and the gene to which the DMR annotated was the same gene that annotated to a ME as referenced from literature sources [16,17,35]. This mapping was done using the documentation for Illumina's 450K array (see R package: IlluminaHumanMethylation450kanno.ilmn12.hg19).
The tables present both the comb-p annotation for such DMRs and the ME annotation from the literature sources alluded to above [16,17,35]. The reference gene was defined according to the annotation provided for in the Illumina manifest and annotation information provided in the R package IlluminaHumanMethylation450kanno.ilmn12.hg19 (v 0.6.0). Further review of the methods used for the targeted DMP and DMR analyses conducted is provided in Supplementary Figure S2. A link to the Github repo is provided here: https://github.com/CIDA-CSPH/Waldrop_P21002Borengasser_CBMethylationDMRsMEs. This details the EWAS and DMR (comb-p) analyses as well as select QC plots reflecting the adequacy of batch correction with ComBat.

3. Results

3.1. Study participants

Characteristics of the 588 mother-offspring dyads in our study are shown in Table 1 and are compared with the Healthy Start Cohort in Supplementary Table S1. Offspring adiposity data were available for 572 infants (97%) at birth, 396 infants (67%) at 5 months and 339 children (58%) at 5 years (Figure 1). Differences in sample size across the analyses are due to missing adiposity data at the 5 month visit (n = 176) or the 5 year visit (n = 233). Maternal and infant characteristics for offspring with complete adiposity data at each study time point were similar despite attrition (Supplementary Table S2).

3.2. EWAS of DMPs/MEs with offspring adiposity at birth

Of the total 10,722 known CpGs within MEs [16,17,35], 927 were included in the Illumina 450K and were retained following processing and quality control of DNAm cord blood data as shown in Figures 1 & 2. We found no association of DMPs within MEs with adiposity at birth, change in adiposity from birth to 5 months of age or change in adiposity from 5 months of age to 5 years of age (all false discovery rate-adjusted $p \geq 0.05$).

3.3. Association of MEs & several DMRs with offspring adiposity in infancy & childhood using comb-p

Supplementary Figure S2 explains the sequence of DNAm bioinformatic statistical analyses. From a total of 10,722 CpGs within MEs identified from the literature [16,17,35], there were a total of 1100 CpGs within MEs present on the Illumina Infinium 450K array and of those, 927 CpGs within those MEs were present on both the array as well as in the cord blood data. We identified two potential MEs, *ZFP57* and *B4GALNT4* [16], and three significant DMRs which annotated to the same genes that annotated to MEs from the literature [17,35] as associated with change in adiposity from birth to 5 months of age in the primary analysis which adjusted for infant characteristics. Three of these remained significant after further adjustment for maternal characteristics (i.e., the secondary analysis) (Supplementary Tables S3 & S4). There were five DMRs which annotated to ME genes [17,35] identified as associated with change in adiposity from 5 months to 5 years of age in the primary analysis. Three of these remained significantly associated with change in adiposity during this age interval in the secondary analysis (Supplementary Tables S5 & S6). The DMR annotated to *B4GALNT4* that was significantly associated with the change in offspring adiposity from birth to 5 months of age, was also significantly associated with the change in offspring adiposity from 5 months to 5 years of age.

3.4. DMRs associated with change in adiposity from birth to 5 months

The association of DNAm at birth with offspring change in adiposity from birth to 5 months of age in the primary analysis revealed five DMRs, including *B4GALNT4, TOP1MT, ZFP57, CBFA2T3, and CYP26C1* as shown in Table 2. Three of these remained significant in the secondary analysis that adjusted for maternal characteristics and included *ZFP57*, *CBFA2T3*, and

Table 1. Study participant characteristics[†].

Maternal characteristics	Total N = 588
Pre-pregnancy body mass index (ppBMI (kg/m^2)	26.00 ± 6.68
Gravidity	
<3 pregnancies	480 (83%)
>3 pregnancies	99 (17%)
Gestational diabetes	29 (5.2%)
Age at delivery (years)	27.56 ± 6.2
Prenatal smoking (n)	52 (9%)
Mode of delivery	
Vaginal	450 (78%)
Cesarean	129 (22%)
Race/ethnicity (self-reported), N (%)	
White, non-Hispanic	315 (54%)
Hispanic	144 (24%)
Black, non-Hispanic	90 (15%)
All others combined	39 (6.6%)
Household income past year, N (%)	
Less than $40,000	164 (28.3%)
$40,000 to $69,999	106 (18.3%)
$70,000 or more	195 (33.8%)
Missing or do not know	114 (19.6%)
Neonatal characteristics	*Total N = 572*
Sex: male	292 (52%)
Gestational age at birth (weeks)	39.48 ± 1.22
Birth weight (kg)	3.13 ± 0.42
Breastfed	212 (39%)
Percent fat mass (%)	9.01 ± 3.85
Infant characteristics	*Total N = 396*
Percent fat mass (%)	24.64 ± 5.49
Child characteristics	*Total N = 339*
Percent fat mass (%)	20.08 ± 6.66
Adiposity birth – 5 months	15.7 ± 5.8%
Adiposity 6 months – 6 years	-4.10 ± 8.0%
Adiposity birth – 6 years	10.6 ± 7.6%
Adiposity 5 months – 5 years	15.7 ± 5.8%
Adiposity 6 months – 6 years	-4.10 ± 8.0%
Adiposity birth – 6 years	10.6 ± 7.6%

[†]Data are mean ± SD, unless otherwise stated.
SD: Standard deviation.

CYP26C1. A graphical depiction of the relationship between change in offspring adiposity from birth to 5 months of age and DNAm of the most significant CpG (i.e., with the lowest Sidak *p*-value) within the DMR for select genes is shown in Supplementary Figure S3. For the CpGs within the DMRs annotated to genes *B4GALNT4* and *TOP1MT*, every 10% change in methylation at the CpG site resulted in a 4.6% and 2.4% decrease in adiposity change, respectively. For every 10% change in methylation at the most significant CpG site within each of the other aforementioned DMRs, we found a 4.1% decrease in adiposity change for *CYP26C1*, a 4.3% decrease for *CBFA2T3* and a 1.6% decrease for *ZFP57*. For those DMRs that remained significantly associated with the outcome in the secondary analysis, the beta estimates were minimally changed when maternal BMI, gravidity and smoking were added as covariates to the model (Table 2). None of the DMRs identified contained any of the 927 CpGs, but *ZFP57* was within base pair range consistent with ME criteria (10,360 bp) as was *B4GALNT4* (10,150 bp) (Supplementary Tables S7 & S8).

3.5. DMRs associated with change in adiposity from 5 months to 5 years

Five DMRs noted to be annotated to ME genes were significantly associated with change in adiposity from 5 months to 5 years in the primary analysis (Table 2 & Supplementary Table S5). These included *ANO7*, *SBK1*, *B4GALNT4*, *DLGAP2*, as well as *HLA-DPA1* and *HLA-DPB1*, the latter two of which both annotated to the same DMR. Only one of these was also previously identified in the analysis from birth to 5 months of age (i.e., *B4GALNT4)* (Table 2 & Supplementary Table S3). Of these five DMRs, three DMRs remained significantly associated with change in offspring adiposity with further adjustment for maternal characteristics (secondary analysis) as shown in Table 2 & Supplementary Table S6. The graphical relationship between change in offspring adiposity from 5 months of age to 5 years of age and DNAm of the most significant CpG within the DMR for select genes is shown in Supplementary Figure S3. For the most significant CpGs within the DMRs annotated to genes *ANO7* and *SBK1*,

Table 2. Reference genes annotated to 'top CpGs' of differentially methylated regions associated with change in adiposity and to metastable epialleles from literature sources[†].

Reference gene for DMR (top CpG)[‡]	Beta estimate ± S.E. (adjust *p*-value) (Sidak *p*-value) for the primary analysis[¶]	Beta estimate ± S.E. (adjust *p*-value) (Sidak *p*-value) for the secondary analysis[§]	#Chr	Reference gene location	DMR start site to end site in base pairs	CpG location	Reference gene description
Birth to 5 months of age							
B4GALNT4 (cg21996245)	-4.59 ± 1.35 (0.71) (0.005)	N/A	chr11	Intergenic	368351 to 368898 (547 bp)	N/A	Enzyme involved in the biosynthesis and transfer of N-acetylgalactosamine residues to N- and O-glycans present on mammalian glycoproteins such as proopiomelanocortin (POMC), a regulator of energy intake and expenditure(45, 46).
TOP1MT (cg2324402)	-2.42 ± 0.721 (0.73) (0.013)	N/A	chr8	intron+utr5	144437314 to 144437592 (278 bp)	N/A	Enzyme responsible for catalyzing reactions that break and rejoin DNA within the mitochondrial genome during replication and transcription. https://www.genecards.org/cgi-bin/carddisp.pl?id+116447
ZFP57 (cg12644888)	-1.72 ± 0.698 (0.74) (5.02E-04)	-1.60 ± 0.70 (0.73) (0.001613)	chr6	TSS+intron +exon+utr5	29648161 to 29648952 (791 bp)	N/A	Associated with parental methylation marks in the early embryo and with methylation of MEs; reported to be in the proximal vicinity (within 10 kb) of MEs (16). https://www.genecards.org/cgi-bin/carddisp.pl?id+346171
CBFA2T3 (cg04220636)	-4.33 ± 1.16 (0.71) (1.62E-06)	-3.94 ± 1.15 (0.71) (1.39E-04)	chr16	Intron	89033895 to 89034343 (448 bp)	island	Transcriptional corepressor; down-regulates expression of glycolytic genes; inhibits glycolysis and stimulation of mitochondrial respiration (65).
CYP26C1 (cg05219493)	-4.08 ± 1.16 (0.71) (9.60E-07)	-4.11 ± 1.15 (0.71) (7.03E-07)	chr10	utr5+cds	94820892 to 94821136 (244 bp)	island	A cytochrome P450 enzyme involved in the catabolism of all-*trans*- and 9-*cis*-retinoic acid. Regulates retinoic acid levels in cells and tissues (58).
5 months–5 years of age							
ANO7 (cg13339454)	-11.0 ± 3.62 (0.993) (0.004)	N/A	chr 2	intergenic	242127690 to 242127999 (309 bp)	N/A	Prostate specific gene. Associated with AR10 Spinocerebellar Ataxia, and Gnathodiaphyseal Dysplasia. https://www.genecards.org/cgi-bin/carddisp.pl?id=31677. Associated with change in BMI, but not adiposity, in a population of 374 preschoolers (7).
SBK1 (cg06897606)	-9.91 ± 2.83 (0.993) (0.004)	N/A	chr16	intergenic	28270490 to 28270805 (315 bp)	island	May play a role in metabolic adaptation to obesity through regulation of lipid metabolism in the liver and insulin sensitivity (68).
B4GALNT4 (cg20846508)	6.86 ± 1.96 (0.993) (0.001)	6.22 ± 1.94 (1.00) (0.02)	chr11	intergenic	368351 to 368763 (412 bp)	N/A	See above.
DLGAP2 (cg12133423)	3.69 ± 1.23 (0.993) (0.002)	3.70 ± 1.20 (1.00) (0.001)	chr 8	intron	1094484 to 1094955 (471 bp)	island	*A* maternally imprinted gene; methylation associated with maternal insulin resistance and smoking.(69–71) Expressed in brain tissue within postsynaptic neurons modulates glutamate signaling (72). May be associated with feeding behavior traits (73).

[†]DMRs associated with percent fat mass change and their annotated genes based on "top CpGs" within the DMR. Annotation of the identified DMRs to genes also annotated to MEs was based upon the following literature sources [16].

[‡]The "top DMP" or "top CpG" is denoted as that with the lowest Sidak *p*-value, the largest effect estimate and the lowest proportion of standard error) localized within cord blood DMRs associated with change in adiposity. Beta estimates and standard errors are shown for primary (adjustment for infant characteristics).

[§]Secondary (adjustment for infant and maternal characteristics).

[¶]Analyses performed.

CDS: Coding region sequence; DMR: Differentially methylated region; ME: Metastable epiallele.

Table 2. Reference genes annotated to 'top CpGs' of differentially methylated regions associated with change in adiposity and to metastable epialleles from literature sources[†] (cont.).

5 months–5 years of age							
HLA-DPA1 (cg25511667)[5]	-4.10 ± 1.39 (0.993) (1.93E-07)	-4.46 ± 1.35 (1.00) (1.868E-11)	chr 6	TSS+intron +exon+utr5	33048254 to 33048970 (716 bp)	island	Encodes the Major Histocompatibility Class II Antigen DPA1 which presents peptides from foreign extracellular proteins on antigen presenting cells to the immune system.
HLA-DPB1 (cg25511667)[5]	-4.10 ± 1.39 (0.993) (1.93E-07)	-4.46 ± 1.35 (1.00) (1.868E-11)	chr6	intron+cds	33048254 to 33048970 (716 bp)	island	Encodes the Major Histocompatibility Class II Antigen DPB1 which presents peptides from foreign extracellular proteins on antigen presenting cells to the immune system. Associated with intrauterine growth restriction in an analysis restricted to CD3+T cells (74).

[†]DMRs associated with percent fat mass change and their annotated genes based on "top CpGs" within the DMR. Annotation of the identified DMRs to genes also annotated to MEs was based upon the following literature sources [16].
[‡]The "top DMP" or "top CpG" is denoted as that with the lowest Sidak *p*-value, the largest effect estimate and the lowest proportion of standard error) localized within cord blood DMRs associated with change in adiposity. Beta estimates and standard errors are shown for primary (adjustment for infant characteristics).
[§]Secondary (adjustment for infant and maternal characteristics).
[¶]Analyses performed.
CDS: Coding region sequence; DMR: Differentially methylated region; ME: Metastable epiallele.

every 10% change in methylation at the CpG site resulted in a 11.0% and 9.9% decrease in adiposity change, respectively. For every 10% change in methylation at the most significant CpG within each of the other aforementioned DMRs, we found a 6.9% increase in adiposity change for *B4GALNT4*, a 3.7% increase for *DLGAP2* and a 4.1% decrease for *HLA-DPA1/HLA-DPB1*. For those DMRs that remained significantly associated with the outcome in the secondary analysis, the beta estimates were again minimally changed when maternal BMI, gravidity and smoking were added as covariates to the model (Table 2). *B4GALNT4* was the only DMR within range of a ME (10,150 bp) significantly associated with change in offspring adiposity from 5 months to 5 years of age.

3.6. Genomic loci distribution of DMRs identified

The genomic location of the DMRs that annotated to ME genes associated with offspring change in adiposity is shown in Supplementary Tables S3 & S4 for the primary analysis. Two DMRs were localized to a transcription start site (TSS) region and four were localized to the five prime untranslated region (5'UTR). The most commonly noted genomic locations in order included the intronic region, the intergenic region, and the 5'UTR. The genomic distances from MEs established from the literature of the identified DMRs associated with change in adiposity in our study are shown in Supplementary Tables S7 & S8.

3.7. Verification of DMRs with bumphunter

DMRs identified by bumphunter were not significant in their association with our outcomes of change in adiposity from birth to 5 months of age nor change in adiposity from 5 months to 5 years after multiple correction testing (*p*-value of family-wide error rates < 0.05) (data not shown). We found overlap of DMRs significantly associated with our outcomes using comb-p, with four DMRs listed among the top 100 genomic regions ranked by bumphunter permutation *p*-value (Supplementary Tables S9 & S10). These DMRs annotated to *B4GALNT4* and *ZFP57* for the analysis evaluating change in offspring adiposity from birth to 5 months of age and to *HLA-DPB1* and *DLGAP2* for the analysis evaluating change in offspring adiposity from 5 months to 5 years of age. All four of these DMRs were of the same directionality using both DMR identification methods (Supplementary Tables S9 & S10).

4. Discussion

This was an exploratory analysis examining differential methylation of DMPs within MEs and DMRs potentially representative of MEs in cord blood and their novel association with offspring adiposity at birth and change in adiposity as measured via ADP from birth through early infancy and early childhood, respectively. We found no DMPs within MEs that were associated with adiposity at birth. We found one DMR that represents a potential ME associated with change in offspring adiposity from birth to 5 months of age (*ZFP57*) and one DMR that represents a potential ME associated with change in offspring adiposity from birth to 5 months of age and 5 months to 5 years of age (*B4GALNT4*). Focusing on those DMRs annotating to genes which annotated to MEs from the literature, we found five DMRs that were associated with change in adiposity from birth to 5 months, three of which remained significant in their association after

adjustment for maternal characteristics (i.e., secondary analysis). We also found five DMRs associated with change in adiposity from 5 months to 5 years, one of which was significantly associated with change in adiposity from birth to 5 months of age as well as from 5 months to 5 years of age. Of those, three remained significant in their association after adjustment in the secondary analysis. Approximately 3% of the total DMRs identified in our study and judged to be significantly associated (Sidak $p \leq 0.05$) with change in adiposity outcomes in the primary analysis (i.e., adjustment for infant characteristics only) were localized to the TSS or 5'UTR regions (data not shown). In general, we found increased DNAm of the DMRs to be associated with decreased change in adiposity from birth to 5 months of age for all associated annotated genes. For the change in adiposity evaluated from 5 months of age to 5 years of age, however, increased DNAm of three DMRs was associated with decreased change in adiposity (i.e., *HLA-DPA1/HLA-DPB1*, *ANO7*, *SBK1*) while methylation of two DMRs was associated with increased change in adiposity (i.e., *DLGAP2* and *B4GALNT4*). The genes identified play roles in nutrient metabolism [43,44], obesity risk [7,8], hypothalamic and pituitary hormone processing [44–47] and immune function [48–51], supporting the biological plausibility of our findings.

We aimed to explore DMRs potentially representative of MEs documented from the literature as novel potential biomarkers of adiposity at birth and adiposity change in infancy and childhood and attempted to identify a persistent association of these in cord blood with adiposity change throughout infancy and early childhood. Our *a priori* research interest was to understand persistent epigenetic marks associated with adiposity from birth to 5 months and 5 months to 5 years. We believed these two particular time periods to be important given that rate of growth/weight gain in the first several months of life has been shown to contribute to risk of obesity later in childhood [52]. Furthermore, early adiposity rebound at 3 - 5 years of age has been shown to increase risk of obesity in later childhood, adolescence and young adulthood [53]. We found methylation of one DMR and a potential ME, *ZFP57* [54], to be associated with decreased change in adiposity from birth to 5 months only. The gene is proposed to be intimately involved with the Tripartite Motif Containing 28 gene (TRIM28) in promoting obesity as well as dysregulation of imprinting gene networks and body weight control [55]. It is also involved in maintaining parental methylation marks in the early embryo [16]. *ZFP57* in our study was localized within a TSS and the 5'UTR. These findings find credence in work by Kessler et al. in which it is suggested that ME regions are typically associated with TSS regions and zinc finger genes [16] as well as work by Harris et al., where authors defined ME regions as those containing two or more CpG sites residing within an approximate 10 kilobase pair distance (Supplementary Tables S7 & S8) [42]. Thus, the identification of *ZFP57* in our study potentially supports our approach to investigating MEs as early life biomarkers for adiposity change in the first 5 years of life using a nonspecific platform such as the Illumina 450K array.

We further identified another DMR and potential ME annotated to *B4GALNT4* that associated with change in offspring adiposity from birth to 5 months and from 5 months to 5 years of age. This was the only gene annotated to a DMR that was associated with adiposity change in both age intervals in our analysis. Overall, all CpGs within the *B4GALNT4* DMR demonstrated an inverse relationship with change in adiposity from birth to 5 months of age but showed a direct relationship with change in adiposity from 5 months to 5 years of age (Supplementary Tables S3, S5 & S6, Supplementary Figures S3 & S4). The change in directionality of association of *B4GALNT4* with change in offspring adiposity may be due to the 14–16% positive change in adiposity from birth to 5 months but a 4–5% negative change in adiposity noted from 5 months to 5 years in our study population. The persistent association of *B4GALNT4* with change in adiposity is supported by potential biological plausibility, given its role in the biosynthesis and transfer of N-acetylgalactosamine residues to *N*- and *O*-glycans present on glycoproteins found on mammalian pituitary and hypothalamic hormones and peptides such as *POMC*, a regulator of energy intake and expenditure [45,46]. *B4GALNT4* annotated to the same gene as referenced in Gunasekara et al. [17] and met ME base pair distance criteria as denoted by Harris et al. [42].

We found the highest number of DMRs to be associated with change in adiposity from birth to 5 months of age. This is particularly notable given the literature suggesting the importance of rate of growth/weight gain in the first several months of life in contributing to risk of obesity later in childhood [52,56,57]. Early infancy (i.e., the first 6 months of life) is a period of low fatty acid oxidation and rapid growth, and in the presence of positive energy balance, fat deposition occurs. In the context of relatively constant dietary fat intake, deposition of fat mass during this time period (e.g., an average of ~15% increase) may be more related to physiologic "programmed" mechanisms developed in utero [58]. Of the DMRs associated with change in offspring adiposity from birth to 5 months of age (*CYP26C1*, *CBFA2T3*, *ZNF57*, *B4GALNT4 and TOP1MT*), all are novel in their association with change in infant adiposity in our study, and four are known to play roles in either obesity risk, energy homeostasis, nutrient metabolism or

hypothalamic/pituitary hormone processing. We discuss select ones in turn below.

We have previously identified the association of *CYP26C1* methylation, a cytochrome p450 gene implicated in retinoic acid metabolism, with adiposity [8] within the first 5 months of life in the Colorado Healthy Start population [51] as well as with rate of adiposity gain (increase in %FM/day) from birth to 5 months (unpublished work). Within our present investigation, lower methylation of *CYP26C1* was associated with an increased change in adiposity that occurred during this time period. The role of *CYP26C1* methylation in retinoid X receptor (RXR) and retinoic acid receptor (RAR) induced transcription and in regulating the retinoic acid (RA) level has been suggested by Lee et al. because binding sites for the RAR and the RXR dimer can be found within the *CYP26C1* promoter region [43]. Thus, expression of *CYP26C1* may be regulated by RA-linked transcription factors and hypomethylation of its promotor region [43,59]. Our findings are further supported in part by Godfrey et al. who noted increased umbilical cord blood DNAm of the RAR gene to be positively associated with FM and percent FM in children at age 9 years of age [8]. In our analysis, the DMR that annotated to *CYP26C1 was* localized to the 5′ UTR as well as the coding region sequence, suggesting effects on gene expression.

Lower methylation of *CBFA2T3*, a gene involved in regulation of glycolysis, transcription and mitochondrial aerobic respiration [60] was found to be associated with the overall increase in percent adiposity in our study population from birth to 5 months of age and annotated to a DMR localized to a CpG island and intronic region. In adult patients with obesity undergoing weight loss, DNAm of this gene (specifically CpG cg00035197) has been associated with ketosis induced by very low-calorie diets and appears to be consistent with the methylome present in normal weight patients [60]. As noted above, we identified increased methylation of the DMRs, *ZFP57* and *B4GALNT4*, to be associated with lower offspring change in adiposity from birth to 5 months. Although validation in other cohorts would be needed, these DMRs may represent novel new target biomarkers for later adiposity in childhood. *TOP1MT* was another gene annotated to a significant DMR in our study and was found to be associated with decreased offspring change in adiposity during this age interval as well. This gene, however, did not appear to have any role in obesity risk, nutrient metabolism, nor energy homeostasis and is more well known for its role as a mitochondrial DNA topoisomerase.

The average offspring change in adiposity (i.e., fat mass as a percentage of total body mass) from 5 months to 5 years in our study was negative, likely in part due to the well-known "adiposity rebound" [61]. During this time period (i.e., after ~6 months of age) and assuming a relatively constant dietary fat intake, growth of fat free mass relative to body weight increases more than fat mass [58]. Given this, the DMRs and annotated genes identified as associated with adiposity change during this age interval may be more reflective of changes in percent lean body mass from a functional standpoint. This is particularly pertinent in considering our findings with respect to *ANO7*, which annotated to a DMR associated with change in adiposity from 5 months to 5 years in our study and was shown in the European Childhood Obesity Project (CHOP) Study to contain one of the top ten differentially methylated probes associated with change in BMI, but not absolute fat mass nor fat free mass, in a population of 374 preschoolers [7]. In our study, we identified the same CpG identified in the CHOP study (cg17810765) but found a 10% change in methylation of this CpG within the *ANO7* DMR to be associated with an approximate 6% decrease in adiposity change, whereas in the CHOP Study they found a 1% change in methylation of the CpG was associated with a 0.14 kg/m^2 increase in childhood BMI at 5.5 years of age [7]. The difference in our results may be related to our ability to look at change in adiposity (i.e., change in %FM) over infancy and early childhood versus the CHOP study, which assessed absolute BMI, fat mass and fat free mass at only approximately 5 years of age, in addition to their use of bioelectrical impedance analysis to assess body composition outcomes. We did not, however, find associations of other cord blood DMPs or DMRs noted in other studies to be associated with BMI in early childhood [62].

Similar to other studies, we found one DMR annotated to MHC genes, such as *HLA-DPA1 and HLA-DPB1*, to be significant in their association with the change in adiposity during this age interval, which is consistent with the methylation of many major histocompatibility genes being linked to obesity and metabolic disease in adults and adolescents [48–50]. The implications of the association of these immune function genes with change in adiposity within early infancy and childhood is not well defined. We have published work, however, showing the association of HLA immune genes with adiposity in infancy and early childhood even with adjustments for cellular heterogeneity [51]. The association noted may be attributable to the influence of maternal periconceptional nutritional exposures not evaluated in our study (e.g., maternal glucose, maternal dietary fat intake and maternal BMI >30 kg/m^2). These maternal metabolic and nutritional exposures have been shown to

adversely impact offspring immune system development through epigenetic changes, placental lipid accumulation and inflammation and to also be associated with offspring adiposity [63].

Despite the novelty of our investigation given our focus on DMPs and DMRs potentially representing MEs as documented from the literature, and our outcome of change in adiposity measured via ADP in a large prospective birth cohort, our study has limitations and thus we must express some skepticism with respect to our results. We identified nine DMRs associated with offspring change in adiposity in infancy and early childhood and two DMRs are potential MEs. From a statistical software analysis perspective, while comb-p is well accepted in the field, a limitation of our study is that our results did not achieve statistical significance after multiple correction testing when analyzed with bumphunter. We otherwise did not evaluate our data with other DMR identification methods that may have supported our results or produced alternate findings. Second, we only assessed DNAm at one-time point and we were not able to assess changes in gene expression to validate the presumed downstream effects based on methylation patterns and DMR genomic location. This latter evaluation, however, was not an aim of our exploratory work though we hope to be able to evaluate such downstream effects with future investigations when the data are available. We recognize our statistical approach may be less powerful than linear mixed models or a repeated measures model, however, the latter methods posed greater challenges in interpretation of effect. Trajectory clustering analyses (i.e., clustering participants based on similar adiposities over time) and modeling cluster as the categorical outcome in a multinomial regression model were considered. However, trajectories in adiposity have been explored for other studies in this cohort and there was insufficient data to derive meaningfully different trajectories [64]. Significantly distinct clusters in the adiposity data were also not evident (data not shown). Further, due to sample attrition, statistical power to detect significant associations varied across the analyses. The use of the Illumina 450K Human Methylation Bead Chip array platform significantly limited our interrogation of MEs reported in the literature and the proximity of the DMRs to MEs identified using the Illumina 450K is inherently dependent upon the array alignment, which is predetermined by the manufacturer. We also acknowledge the Illumina 450K array is biased toward probes localized within promoter regions, potentially overestimating the identification of DMRs that may be involved in regulation of gene expression. Lastly and ideally, we would need to validate our findings within another cohort.

5. Conclusion

In summary, we found associations between newborn cord blood DNAm of DMRs that annotated to biologically relevant genes related to obesity risk, nutrient metabolism, energy homeostasis and pituitary and hypothalamic hormone processing with offspring change in adiposity at different developmental growth periods in infancy and childhood. We identified one DMR and potential ME, *ZFP57*, associated with change in adiposity from birth to 5 months of age. We identified one DMR and potential ME, *B4GALNT4*, which associated with change in adiposity from both birth to 5 months and 5 months to 5 years of age. We believe our investigation is novel given our *a priori* focus on DMRs potentially representative of MEs and their association with offspring adiposity as measured by ADP as opposed to BMI, particularly within infancy. However, we acknowledge that we found very limited evidence of ME associations with offspring adiposity using the Illumina 450K array. Nonetheless, in this exploratory investigation using cord blood, we have identified novel and potentially, biologically plausible, epigenetic biomarkers at birth for adiposity change during two separate critical periods of development considered important for assessing growth and potential risk of obesity that may guide future investigations.

Article highlights

Background
- Differences in obesity risk may appear as early as infancy, suggesting that developmental factors driving obesity are operating very early in life.
- There is emerging evidence from human studies of the potential for epigenetic signatures, namely DNA methylation (DNAm), to serve as predictive biomarkers of obesity risk.
- Human metastable epialleles (MEs) are unique genomic regions established during early embryogenesis that show systemic interindividual variation and stability across different tissues and may be influenced by preconceptional exposures.
- DNAm of MEs has been suggested to play a role in energy balance and has previously been associated with obesity in adult and pediatric populations.

Methods
- We explored differentially methylated regions potentially representative of MEs from the literature as potential biomarkers of adiposity at birth and adiposity change in infancy and childhood and attempted to identify a persistent association of these in cord blood with adiposity change throughout infancy and early childhood.

Results
- We found associations between offspring change in adiposity in infancy and childhood and newborn cord blood DNAm of nine DMRs that annotated to genes, which also annotated to MEs referenced in the literature. The annotated genes are biologically relevant and related to obesity risk, nutrient metabolism and neuroendocrine energy balance.
- We found lower methylation of one DMR and a putative ME, *ZFP57*, to be associated with an overall increased change in adiposity from birth to 5 months only. The gene is proposed to promote dysregulation of imprinting gene networks and body weight control and has been identified as a metastable epiallele by others.

- We identified one DMR annotated to *B4GALNT4* that associated with change in offspring adiposity from birth to 5 months and from 5 months to 5 years of age. This was the only gene annotated to a DMR that was associated with adiposity change in both age intervals in our analysis. *B4GALNT4* has a role in the biosynthesis and transfer of N-acetlygalactosamine residues to *N*- and *O*-glycans present on glycoproteins found on mammalian pituitary and hypothalamic hormones and peptides such as *POMC*, a regulator of energy intake and expenditure. Furthermore, the kilobase distance of *B4GALNT4* from the ME annotated to the same gene as referenced in Gunasekara et al. may support its potential as a ME, though more studies would be needed.

Conclusion

- Using cord blood, we have identified potential, biologically plausible, epigenetic biomarkers for adiposity change during two separate critical periods of development considered important for assessing rapid growth and risk of obesity that may be of use in future investigations.
- However, skepticism of our results is due given lack of validation with the alternative DMR identification method employed (i.e., bumphunter) in addition to the inability to replicate findings using a different cohort.

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Author contributions

SW Waldrop, SJ Borengasser and KA Sauder conceived and designed the current analysis. D Dabelea conceived, designed and conducted the parent Healthy Start study. D Dabelea, W Perng, AP Starling provided expertise in study design and analysis. KJ Kechris and IV Yang supervised data collection and processing. SS Niemiec, KJ Kechris, and IV Yang supported data base management, provided statistical expertise and conducted the statistical analyses. SW Waldrop drafted the manuscript with critical input from all authors for subsequent revisions. All authors read and approved the final version of the manuscript.

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Competing interests disclosure

The authors have no competing interests or relevant affiliations with any organization or entity with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Writing disclosure

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

Ethical conduct of research

The authors obtained appropriate institutional review board approval (Colorado Multiple Institutional Review Board) and/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, written informed consent has been obtained from the participants involved. The COMIRB approval number for the Healthy Start Study is 09-0563.

Data availability statement

Datasets on which the conclusions of this manuscript rely are available on request. The precise dataset used in this study subset of participants does not have a DOI.

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