RESEARCH PAPER

Associations between placental CpG methylation of metastable epialleles and childhood body mass index across ages one, two and ten in the Extremely Low Gestational Age Newborns (ELGAN) cohort

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ABSTRACT

The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that *in utero* and early life conditions can disrupt normal fetal development and program susceptibility to later-life disease. Metastable epialleles are genomic loci in which CpG methylation patterning is responsive to maternal diet and conserved across time and tissues. Thus, these sites could serve as 'signatures' of gestational environment conditions. Here, we sought to determine if methylation of metastable epialleles was associated with changes in childhood body mass index (BMI) z-scores across ages one, two and ten in the Extremely Low Gestational Age Newborns (ELGAN) cohort. CpG methylation of 250 probes (corresponding to 111 genes) within metastable epiallele regions was measured in placental tissue. Linear mixed effects models were fit to evaluate the overall and sex-stratified associations between methylation and changes in BMI z-score over time. In total, 26 probes were associated ($p < 0.05$) with changes in BMI z-score overall, including probes within Mesoderm Specific Transcript (*MEST*) and Histone Deacetylase 4 (*HDAC4*), which have previously been associated with childhood obesity and adipogenesis. Sex-stratified analyses revealed a significant association, after adjusting for multiple comparisons ($q < 0.05$), within female placentas for one probe annotated to the imprinted gene PLAG1 Like Zinc Finger 1 (*PLAGL1*). These findings suggest epigenetic marks may be involved in programming susceptibility to obesity *in utero* and highlight the potential to use placental tissues in predicting growth rate trajectories among premature infants.

ARTICLE HISTORY

Received 30 March 2019
Revised 7 June 2019
Accepted 13 June 2019

KEYWORDS

Metastable epialleles; placenta; epigenetics; CpG methylation; body mass index; child health

Introduction

The placenta plays a critical role in determining fetal health by transferring nutrients, waste, and growth promoting hormones [1]. The placenta also serves as an incomplete barrier to exposures from the maternal environment which can disrupt fetal development and influence susceptibility to later-life health disorders, as posited by the Developmental Origins of Health and Disease (DOHaD) hypothesis [2,3]. Increasing evidence suggests that molecular mechanisms mediating this process include epigenetic mechanisms [1,3–6], such as methylation of cytosines in cytosine-guanine (CpG) dinucleotides. During embryogenesis, CpG methylation patterning

is highly sensitive to environmental insult because of the high rate of DNA synthesis and chromatin regulation required for organismal development [7–10]. Interestingly, genomic loci have been identified in which epigenetic patterning is variable between isogenic organisms and conserved across time and tissues [11].

These sites, termed '*metastable epialleles*,' were first characterized as variably expressed due to persistent, systemic (cross-tissue) epigenetic modifications established prior to gastrulation [11]. In contrast to traditional alleles, in which methylation is erased and re-established in a predictable manner, the establishment of methylation at these sites is stochastic and responsive to environmental factors in a locus-specific

manner. Kessler *et al.* extended the definition to incorporate genomic regions under partial, nondeterministic genetic influence in order to reflect the genetic heterogeneity observed in human populations [12]. Consistency in methylation profiles has been observed across tissues, including the placenta, at imprinted genes [13]. This is of note because many imprinted genes regulate fetal growth and development [10,14,15], placental-specific imprinting preferentially maintains maternal germline-derived DNA methylation [16], and several metastable epialleles overlap imprinted loci. Placental CpG methylation of metastable epialleles could serve both as a molecular biomarker of maternal environmental influences on development as well as a modification that is predictive of growth later in life.

A growing body of literature demonstrates metastable epiallele methylation is a molecular biomarker of environmental influences affecting development. In mouse models, maternal dietary methyl donor supplementation significantly increases methylation of metastable epialleles and is associated with the penetrance of the pseudoagouti coat color, tail kinking, and adult-onset obesity [17–19]. Notably, maternal dietary genistein supplementation reverses BPA-induced hypomethylation of the agouti viable yellow (A^y) metastable epiallele, causing a shift in the coat-color distribution and a decreased incidence of adult-onset obesity among offspring [20]. Limited evidence suggests methylation of candidate human metastable epialleles is also influenced by maternal diet, as methyl-donor nutrient intake and gestational famine have been associated with methylation of these sites [21–23]. Moreover, the elevated rates of obesity observed in the Dutch Hunger Winter study were confined to individuals born to mothers exposed to famine during the early-gestational period [5]. Taken together, these studies suggest that epigenetic programming may establish inter-individual susceptibility to obesity during critical periods of early gestation. Considering these findings, the relationship between methylation of metastable epialleles and later-life changes in body size warrants further investigation.

To address whether CpG methylation of metastable epialleles influences growth rate, we sought to determine if placental methylation of putative metastable epialleles was associated with changes in childhood body mass index (BMI) z-scores across

ages one, two and ten in the Extremely Low Gestational Age Newborns (ELGAN) cohort. Additionally, sex-stratified analyses were used to determine the associations among males and females separately, as sexual epigenetic dimorphism has been observed in the human placenta [24]. The goal of this study was to investigate whether methylation patterns established during early development might be associated with later life health outcomes, in concordance with the DOHaD paradigm. It was hypothesized that placental methylation of metastable epialleles would be associated with growth rate trajectories and that probes would be annotated to genes involved in growth and metabolism. This is among the first studies to examine metastable epialleles in the context of the placenta as it relates to child health across multiple life stages.

Results

Study cohort

Demographic information for the ELGAN placental epigenetics sub cohort ($n = 426$) was collected at childbirth and is largely representative of the full cohort (Table 1). Data on children's BMI were collected during follow-up at ages one, two and ten years. In the sub cohort for the current study, there were 203 (47.7%) females and 223 (52.4%) males, and the average gestational age was 26 weeks. At ages one, two and ten years, the average child's BMI was 16.7, 16.0, and 17.7, respectively. The average maternal BMI and age were 25.4 and 29.4, respectively. Approximately 34% of the sub cohort was covered by public insurance, and mothers self-identified as White (62.1%), Black (28.4%) or other (9.5%). Further details on subject characteristics are presented in Table 1.

Overall association between placental cpG methylation and changes in BMI z-score

In assessing the relationship between CpG methylation of metastable epialleles and changes in BMI z-score, linear mixed effects models were employed to account for within-subject correlation of BMI measurements. These models assessed whether an increase in placental CpG methylation was associated with changes in BMI z-score across ages one,

Table 1. ELGAN (n = 889) and placental epigenetics sub cohort (n = 426) characteristics.

	ELGAN	Sub Cohort
	n (%) Mean (SD)	
Infant Sex		
Male	455 (51.2)	223 (52.4)
Female	434 (48.8)	203 (47.7)
BMI at age 1	17.1 (5.7)	16.7 (2.5) ^a
BMI at age 2	16.2 (3.8)	16.0 (1.9) ^b
BMI at age 10	17.8 (4.1)	17.7 (4.1) ^c
Maternal BMI	25.6 (6.8)	25.4 (7.0) ^d
Maternal Age	29.2 (6.7)	29.4 (6.6)
Gestational Age (Weeks)	26 (1.3)	26 (1.3)
Public Insurance		
Yes	307 (35.1)	143 (34.0)
No	568 (64.9)	278 (66.0)
Missing	14	5
Maternal Race		
White	554 (63.0)	262 (62.1)
Black	227 (25.8)	120 (28.4)
Other	98 (11.2)	40 (9.5)
Missing	10	4
Smoked while pregnant		
Yes	120 (13.8)	46 (11.0)
No	750 (86.2)	372 (89.0)
Missing	19	8
Multiple Gestations		
Yes	313 (35.2)	166 (39.0)
No	576 (64.8)	260 (61.0)

^a38 missing z-scores; ^b39 missing z-scores; ^c7 missing z-scores; ^d14 missing z-scores.

two and ten. In the overall analysis, 26 probes, annotated to 14 genes, displayed significant associations ($p < 0.05$) with changes in BMI z-score (Table 2). The probe (cg13211302) displaying the strongest association ($\beta = 0.0056$, $p = 0.0003$) was not annotated to any genes. After adjusting for multiple comparisons at $q < 0.05$, none of the statistically significant associations between methylation and changes in BMI z-score were conserved. However, several probes were annotated to genes involved in obesity, adipogenesis and development, including PLAG1 Like Zinc Finger 1/Hydatidiform Mole Associated and Imprinted (*PLAGL1/HYMAI*), Mesoderm Specific Transcript (*MEST*), Histone Deacetylase 4 (*HDAC4*), and Engrailed Homeobox 1 (*EN1*).

Sex-stratified analyses reveal imprinted gene *PLAGL1/HYMAI* is associated with changes in BMI z-score among females

To evaluate the association between placental methylation of metastable epialleles and changes in

BMI z-score among males and females separately, sex-stratified analyses were performed. In total, 26 probes, annotated to 15 genes, displayed significant associations ($p < 0.05$) with changes in BMI z-score among placentas derived from female pregnancies (Table 3). After adjusting for multiple comparisons ($q < 0.05$), one association between methylation and changes in BMI z-score was conserved at a probe (cg00702231) annotated to the imprinted gene *PLAGL1/HYMAI* ($\beta = -0.0119$, $p < 0.0001$, $q < 0.01$) (Figure 1, Table 3). Additionally, some probes were annotated to genes related to adipocyte differentiation and obesity, including *MEST*, Fatty Acid Desaturase 3 (*FADS3*), and Regulator of G Protein Signaling 14 (*RGS14*). Among placentas derived from male pregnancies, 18 probes, annotated to 10 genes, displayed significant associations ($p < 0.05$) with changes in BMI z-score (Table 4). However, no associations were conserved after adjusting for multiple comparisons.

Sub-analyses across individual epochs were consistent with findings across ages one, two and ten

As growth is more rapid between ages one and two than two and ten, we evaluated the association between placental methylation of metastable epialleles and changes in BMI z-score across these epochs separately. Fewer associations were identified between ages two and ten ($n = 9$ CpG probes) than ages one and two ($n = 25$ CpG probes), but probes identified with $p < 0.05$ were largely consistent with the primary analysis across ages one, two and ten (Supplementary Table 2–7). After adjusting for multiple comparisons, we identified associations across ages one and two at $q < 0.05$ for cg11324748 (*ANKRD20A11P*) and cg13012494 (*SPATC1L*). Among male-derived placentas, cg11324748 (*ANKRD20A11P*) was significant at $q < 0.05$. Across ages two and ten, we did not identify associations after adjusting for multiple comparisons ($q < 0.05$). However, cg00702231 (*PLAGL1*) was identified in the overall analysis and within placentas derived from female pregnancies ($p = 0.03$ and 0.02 , respectively).

Finally, we explored differentially methylated regions using the DMRScan package, but no significant results were identified (data not shown).

Table 2. Probes associated with changes in BMI z-score overall (n = 26 probes, representing 14 genes).

Probe ID	Gene	Position	CHR	β (95% CI)	p
cg23415434	<i>ARHGEF4</i>	Body	2	-0.0018 (-0.0035, -0.0001)	0.0389
cg10895452	<i>EN1</i>	Body	2	0.0018 (6×10^{-7} , 0.0036)	0.0499
cg11097064	<i>HDAC4</i>	Body	2	0.0024 (0.00005, 0.0048)	0.0456
cg20627174			2	0.0010 (0.0001, 0.002)	0.0256
cg23819411	<i>MCF2L2</i>	Body	3	0.0036 (0.001, 0.0061)	0.0061
cg02447542			3	-0.0011 (-0.002, -0.0002)	0.0127
cg18670801			4	-0.0031 (-0.0052, -0.0009)	0.0057
cg14886255	<i>MGAT4B</i>	Body	5	0.0034 (0.0005, 0.0063)	0.0209
cg00702231	<i>PLAGL1/HYMAI</i>	1stExon/Body/5'UTR	6	-0.0067 (-0.0112, -0.0023)	0.0032
cg04786207	<i>MEST/MESTIT1</i>	TSS1500/5'UTR/TSS1500	7	0.0029 (0.0006, 0.0052)	0.0119
cg13211302			8	0.0056 (0.0026, 0.0085)	0.0003*
cg20088245			8	-0.0014 (-0.0025, -0.0003)	0.0111
cg00797058			10	-0.0024 (-0.0047, -0.0001)	0.0411
cg26328335	<i>AQP5</i>	TSS1500	12	0.0011 (0.0001, 0.0022)	0.0350
cg26573334	<i>TPCN1</i>	Body	12	0.0069 (0.0016, 0.0121)	0.0109
cg04834608	<i>DNASE1</i>	Body	16	-0.0025 (-0.0048, -0.0001)	0.0409
cg04478251	<i>ABR</i>	Body	17	0.0025 (0.0004, 0.0046)	0.0205
cg26536949			17	-0.0008 (-0.0014, -0.0001)	0.0269
cg05528899			17	-0.0011 (-0.002, -0.0001)	0.0292
cg06417478	<i>HOOK2</i>	Body	19	-0.0006 (-0.0011, -0.0001)	0.0257
cg00130086			19	-0.0013 (-0.0025, -0.0001)	0.0409
cg10296238	<i>SPATC1L</i>	TSS1500	21	0.0010 (0.0001, 0.0018)	0.0238
cg13012494	<i>SPATC1L</i>	TSS1500	21	-0.0151 (-0.0291, -0.0011)	0.0345
cg11324748	<i>ANKRD20A11P</i>	Body	21	-0.0108 (-0.0173, -0.0043)	0.0011
cg00566515	<i>ANKRD20A11P</i>	Body	21	-0.0026 (-0.0048, -0.0005)	0.0179
cg27306467	<i>ANKRD20A11P</i>	TSS200	21	-0.0030 (-0.0058, -0.0002)	0.0345

Table 3. Probes associated with changes in BMI z-score within placentas derived from female pregnancies (n = 26 probes, representing 15 genes).

Probe ID	Gene	Position	CHR	β (95% CI)	p
cg10386298			1	0.0012 (0.0002, 0.0023)	0.0219
cg19633822	<i>MGC13005</i>	Body	2	-0.0065 (-0.0124, -0.0006)	0.0296
cg01101448			2	-0.0041 (-0.0078, -0.0004)	0.0292
cg14886255	<i>MGAT4B</i>	Body	5	0.0049 (0.0005, 0.0092)	0.0291
cg25461508	<i>RGS14</i>	TSS200	5	0.0023 (0.0004, 0.0042)	0.0188
cg17597639	<i>SDHAP3</i>	TSS200	5	-0.0019 (-0.0038, -0.0001)	0.0374
cg12301216			5	-0.0018 (-0.0035, -0.00003)	0.0458
cg00702231	<i>PLAGL1/HYMAI</i>	1stExon/Body/5'UTR	6	-0.0119 (-0.0175, -0.0063)	0.00003**
cg12757684	<i>PLAGL1/HYMAI</i>	1stExon/Body/5'UTR	6	-0.0060 (-0.0115, -0.0006)	0.0297
cg04786207	<i>MEST/MESTIT1</i>	TSS1500/5'UTR	7	0.0035 (0.0008, 0.0063)	0.0126
cg20088245			8	-0.0019 (-0.0035, -0.0003)	0.0179
cg13211302			8	0.0046 (0.00003, 0.0091)	0.0486
cg16807687	<i>PCDH21</i>	Body	10	-0.0043 (-0.0076, -0.001)	0.0108
cg02276826			10	-0.0015 (-0.0029, -0.0001)	0.0374
cg04824767	<i>FADS3</i>	TSS1500	11	0.0040 (0.0013, 0.0067)	0.0038
cg24213572	<i>NDUFS8/MIR4691</i>	Body/TSS200	11	-0.0133 (-0.0258, -0.0008)	0.0367
cg13906860			11	0.0024 (0.0004, 0.0043)	0.0168
cg26328335	<i>AQP5</i>	TSS1500	12	0.0019 (0.0004, 0.0034)	0.0141
cg03972466	<i>C16orf72</i>	TSS1500	16	0.0034 (0.0004, 0.0064)	0.0259
cg04478251	<i>ABR</i>	Body	17	0.0033 (0.0001, 0.0066)	0.0447
cg26536949			17	-0.0010 (-0.0019, -0.0001)	0.0313
cg20505444	<i>BCL2</i>	Body	18	-0.0102 (-0.0197, -0.0008)	0.0339
cg00130086			19	-0.0017 (-0.0034, -0.00003)	0.0457
cg10296238	<i>SPATC1L</i>	TSS1500	21	0.0016 (0.0005, 0.0028)	0.0061
cg13732083	<i>SPATC1L</i>	TSS1500	21	0.0498 (0.0036, 0.096)	0.0346
cg27306467	<i>ANKRD20A11P</i>	TSS200	21	-0.0049 (-0.0086, -0.0012)	0.0102

**q < 0.01; CHR: Chromosome.

Table 4. Probes associated with changes in BMI z-score within placentas derived from male pregnancies (n = 18 probes, representing 10 genes).

Probe ID	Gene	Position	CHR	β (95% CI)	p
cg23415434	<i>ARHGEF4</i>	Body	2	−0.0040 (−0.0064, −0.0016)	0.0012*
cg06019884	<i>ARHGEF4</i>	Body	2	−0.0026 (−0.0045, −0.0008)	0.0045
cg02366959	<i>NCRNA00164</i>	Body	2	0.0048 (0.0022, 0.0074)	0.0004*
cg20627174			2	0.0014 (0.0001, 0.0027)	0.0286
cg23819411	<i>MCF2L2</i>	Body	3	0.0053 (0.0019, 0.0088)	0.0026
cg02447542			3	−0.0017 (−0.003, −0.0003)	0.0139
cg17228698	<i>CHST12</i>	5'UTR	7	−0.0046 (−0.0078, −0.0014)	0.0047
cg16247391	<i>ZNF890P</i>	TSS200	7	0.0044 (0.0013, 0.0075)	0.0057
cg13211302			8	0.0056 (0.0015, 0.0096)	0.0067
cg26507521			10	0.0017 (0.0004, 0.0031)	0.0136
cg00797058			10	−0.0037 (−0.0068, −0.0005)	0.0222
cg26573334	<i>TPCN1</i>	Body	12	0.0078 (0.0003, 0.0154)	0.0418
cg15490703	<i>EFNA2</i>	Body	19	0.0170 (0.0007, 0.0333)	0.0405
cg06417478	<i>HOOK2</i>	Body	19	−0.0008 (−0.0016, −0.0001)	0.0269
cg13732083	<i>SPATC1L</i>	TSS1500	21	−0.0707 (−0.1117, −0.0297)	0.0007*
cg13012494	<i>SPATC1L</i>	TSS1500	21	−0.0194 (−0.0355, −0.0033)	0.0179
cg11324748	<i>ANKRD20A11P</i>	Body	21	−0.0111 (−0.0189, −0.0034)	0.005
cg00566515	<i>ANKRD20A11P</i>	Body	21	−0.0030 (−0.0059, −0.0001)	0.0447

* $q < 0.10$; CHR: Chromosome.

Figure 1. Distribution of CpG methylation at probe cg00702231, annotated to *PLAGL1*, within placentas derived from female pregnancies. Females were dichotomized as experiencing a change in BMI z-score that was above (n = 91) or below (n = 91) the median change in BMI z-score across ages one and ten (median = −0.08).

Discussion

In the present study we sought to determine if CpG methylation of metastable epialleles within the placenta was associated with changes in BMI z-score across ages one, two and ten among children born extremely preterm. Metastable epialleles are of interest because CpG methylation at these

genomic loci is responsive to maternal diet and systemically maintained across tissues over time [11]. Several of the identified probes were annotated to genes that have been implicated in development, adipogenesis and obesity. After stratifying the analysis by sex, one probe annotated to the imprinted gene *PLAGL1* was significantly associated with changes in BMI z-score within

placentas derived from female pregnancies. Methylation of this gene has previously demonstrated lability to maternal diet, consistent with the characterization of metastable epialleles. These findings support the DOHaD hypothesis, providing evidence for an association between *in utero* programming and later-life health outcomes in the ELGAN cohort.

Several probes associated with changes in BMI z-score were annotated to genes involved in development, obesity and adipogenesis [25–31]. *MEST* and *HDAC4* were of special interest, as associations between their methylation profiles and obesity-related outcomes have been explored among children and adults. In the present study, DNA methylation of a probe annotated to *MEST* was positively associated with BMI z-score across childhood. In contrast, Nahm *et al.* [32] found that methylation of *MEST* was negatively associated with anthropometric measures among females at ages one and three. Additionally, in assessing later-life associations between DNA methylation of *MEST* and body weight, Hajj *et al.* [33] identified significantly decreased blood methylation of *MEST* among obese adults. Differences in findings between studies may be a result of differences in scientific analyses (e.g. those that focus on single time point associations versus those that examine changes in body size over time). This is further supported in that growth occurs most rapidly during the earliest stages of development. *MEST* is a known imprinted gene which has displayed similar methylation patterning between the placenta and other fetal somatic tissues from all three germ layers [13]. Taken together, these data suggest that early-life epigenetic programming of *MEST*, as indicated by placental methylation, could predict susceptibility to later-life obesity regulated through somatic tissue responses.

A second gene of interest identified in the present study, *HDAC4*, showed increased CpG probe methylation with respect to BMI z-score across childhood. Similarly, in comparing obese and lean pre-school aged children, Li *et al.* [34] showed hypermethylation of *HDAC4* was associated with the obese phenotype, and that *HDAC4* hypermethylation was inversely associated with gene expression. *HDAC4* belongs to a group of histone deacetylases which play a critical role in transcriptional regulation; it is also involved in adipocyte expansion, energy expenditure

regulation and may be protective of obesity [30,35,36]. Thus, the functions of *HDAC4* may shed light on the biological underpinnings of associations between this gene and body size.

Sexual dimorphism has been observed in the human placenta [37–40], and we have recently highlighted sexual epigenetic dimorphism at the level of CpG methylation [24]. Here, sex-stratified analyses revealed two probes annotated to *PLAGL1* and one probe annotated to *MEST* displayed associations to changes in BMI z-score within placentas derived from female pregnancies. Among these, a single probe annotated to the imprinted gene *PLAGL1/HYMAI* displayed a statistically significant negative association. Gonzalez-Nahm *et al.* [32] identified methylation of both *PLAGL1* and *MEST* in cord blood were negatively associated with weight-for-length z-scores among one year old females. In contrast, additional studies examining cord blood have identified that methylation of *PLAGL1* is positively correlated with anthropometric measures at 32 weeks of gestation, birth weight and age one [41,42]. Interestingly, DNA methylation of *PLAGL1* is also influenced by maternal diet, namely alcohol, folate and vitamin B2 intake [41,42]. *PLAGL1* and *MEST* are both maternally-imprinted genes. This is of note because in identifying placenta-specific imprinting, Hanna *et al.* [16] noted that the vast majority of novel DMRs had maternally inherited DNA methylation. Taken together, prior literature and the data from the present study suggest that methylation of *PLAGL1* may be labile to maternal diet and associated with changes in body size over time, specifically among females.

The results from the current study add to the growing body of literature demonstrating associations between CpG methylation of candidate metastable epialleles and later-life outcomes and is among the first to highlight CpG methylation of these genomic loci within the placenta. Still, some factors should be considered in the interpretation of our findings. First, the ELGAN birth cohort is comprised of infants born extremely preterm (prior to 28 weeks gestation), which could limit the generalizability of our results. Second, BMI does not completely account for body composition or changes in body fat related to growth. Still, the use of BMI as a health metric is appropriate as it is largely used in epidemiological studies and clinical settings to define

obesity. Third, weight is influenced by postnatal environmental exposures, genetics, and lifestyle factors. Still, despite the lack of a controlled environment, as with any human population-based study, we demonstrate evidence for a role of epigenetics in the early life programming of later-life body size. Finally, CpG methylation was assessed in the placenta at one time point. While it is hypothesized that methylation of metastable epialleles is stable across time and tissues [11], collection of additional samples across time and tissues and the functional assessment of mRNA expression would be helpful in determining their influence on growth rate trajectories.

This is among the first studies to examine the association between placental methylation of candidate metastable epialleles and longitudinal measures of body weight. Our findings provide support for the DOHaD hypothesis, as several associations were identified among probes annotated to genes involved in growth and metabolism. A major finding from the study was the association between *PLAGL1* and BMI in childhood, as this gene has previously been associated with early life BMI and demonstrated lability to maternal diet [32,41,42]. Future studies should incorporate maternal dietary information and assess the cross-tissue and longitudinal stability of methylation of these sites, as they may be vestiges of intrauterine environmental conditions functionally linked to later-life obesity risk.

Materials and methods

Subject recruitment and sample collection

The ELGAN birth cohort was established to identify factors contributing to the risk of neurological disorders among infants born extremely preterm, which was defined as a live birth before 28 completed weeks of gestation. Study procedures were approved by the Institutional Review Board at each of the 14 participating ELGAN sites [43], and methods of subject recruitment and sample collection have been described elsewhere [43]. Briefly, from 2002 to 2004, women giving birth at one of the 14 participating sites before 28 weeks of gestation were asked to enroll in the ELGAN study. Their consent was provided either upon

hospital admission prior to or shortly after delivery. In total, 1506 infants and 1249 mothers enrolled in the ELGAN study. The cohort consists of both singleton (65%) and non-singleton births (35%), however there are no genetically identical twins in this placental repository. At the time of delivery, placenta specimens were collected from over 90% of study participants. Eighty-two percent of the samples were obtained within 1 hour of delivery. For epigenetic analyses, we focused on placental samples from the subset of study participants who were evaluated at 10 years of age ($n = 889$). Within this subset of study participants, sufficient placental tissue, and thus, DNA methylation data, was available for 426. These 426 placentas and the corresponding offspring comprise the sample for the present analysis.

Participating women gave permission for the collection of a sample of their placenta for the ELGAN study. Upon delivery, placentas were placed into a sterile exam basin and taken to the sampling room, at which point, biopsies of the placentas were collected as previously described [44]. The area sampled was at the midpoint of the longest distance between the cord insertion and the edge of the placental disk. To expose the chorion, the amnion was pulled away from the underlying chorion using a sterile technique. A tissue sample was collected by applying traction to the chorion and the underlying trophoblast tissue and cutting a sample out at the base of this tissue structure. The chorion is comprised of fetal membrane surrounding the developing embryo. Hence, the tissue sample was restricted to the *fetal side* of the placenta. Tissue samples were subsequently placed into a sterile 2 mL cryo-vial that was immediately submerged in liquid nitrogen. Samples were shipped to the University of North Carolina at Chapel Hill and stored at -80°C until processing [44].

DNA extraction and assessment of DNA methylation

DNA sequences >18 nucleotides long were isolated using ALLPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia, CA). Extracted DNA samples were then shipped on dry ice to Wayne State University where bisulfite conversion was performed

using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA). The Illumina Infinium MethylationEPIC BeadChip (Illumina, San Diego, CA) was then used to profile methylation status at more than 850,000 CpG sites across the genome. Average methylation values (β values) were computed to represent the ratio of methylated to unmethylated signal intensities.

As previously described [45], β values were background corrected using the normal-exponential out-of-band (*noob*) correction method and normalized with functional normalization. To evaluate batch effects, a principal component analysis (PCA) was performed. Plate was identified as a significant source of variation; thus, we performed a surrogate variable analysis via the *ComBat* package [46]. PCA was then repeated to ensure this bias was sufficiently corrected.

Identification of illumina probes corresponding to metastable epialleles

Prior to analyses, a list of 252 Illumina probes, annotated to 112 genes, was derived from the publicly available Infinium Methylation EPIC v1.0 B4 Manifest file (<https://support.illumina.com/downloads/infinium-methylationepic-v1-0-product-files.html>) (Supplementary Table 1, Supplementary Figure 1). These probes corresponded to genomic loci identified as metastable epiallele regions and were identified using the chromosome number and map information provided in the most recent genome-wide screening for human metastable epialleles [12]. Probes annotated to the Y chromosome ($n = 2$) were excluded from the analysis to minimize technical artifacts among placentas derived from female pregnancies.

BMI assessment at ages 1, 2, and 10

At ages one, two, and ten, body weight and length were obtained by study personnel. Body mass index (BMI) was then calculated using the following formula: BMI = Weight (in kilograms)/Height (in meters). BMI z-score and percentiles for age and sex were then determined using the Statistical Analysis Software program based on current CDC growth charts [47,48].

Covariates

To identify covariates, a directed acyclic graph (DAG) approach was used. Maternal pre-pregnancy BMI (z-score), maternal race, infant sex, insurance status, multiple gestations, and smoking while pregnant were selected as covariates based on their potential to confound associations between methylation of metastable epialleles and changes in BMI z-score. These indicators were collected at enrollment in the ELGAN study via a structured questionnaire.

Statistical analysis

To test the hypothesis that placental methylation of candidate metastable epialleles is associated with changes in BMI z-score, we implemented a linear mixed effects model at each of the probes. The general model form was as follows:

$$BMI_{z-score_{ij}} = \alpha + X_{i0}\beta + Time_{ij}\beta + X_{i0}(Time_{ij})\beta + Z_{i0}\beta + \mu_{i0} + e_{ij}$$

where $BMI_{z-score_{ij}}$ is the measure of the child's BMI relative to children of the same sex and age for the i th child at time j . X_{i0} is the average methylation level (β value) for a probe located within a metastable epiallele region, as measured in placental tissue. Methylation levels were first multiplied by 100 to estimate the change in BMI z-score associated with a 1% increase in methylation. Time is the duration between baseline and each BMI z-score measurement. The β coefficient for $X_{i0}(Time_{ij})$ is the expected annual change in BMI z-score corresponding to a 1% increase in methylation at the respective CpG probe. Z_{i0} is a matrix of potential confounders, representing adjustments made for fetal sex (male, female), gestational age and maternal pre-pregnancy BMI z-score (continuous variables), maternal race (White, Black, and Other), and insurance (public insurance yes or no). μ_{i0} is a random intercept for each child used to account for the within-subject correlation of the repeated BMI z-score measurements, and e_{ij} is the error term. Due to evidence of sexual epigenetic dimorphism in CpG methylation patterning of the human placenta [24], we conducted sex-stratified analyses to determine the associations among males and females separately.

While our primary hypothesis was that metastable epialleles established a single growth rate trajectory

that would be maintained over the life course, we also evaluated the epochs across ages one and two and ages two and ten separately. Additionally, metastable epiallele probes were analyzed in the context of differentially methylated regions (DMRs) using the DMRScan package (R version 3.6.0) [49]. For all models, we used a post-hoc adjustment for multiple comparisons via the Benjamini-Hochberg (FDR) procedure and controlled for the false discovery rate of 0.05. Apart from the DMR analysis, all analyses were conducted in Stata/SE version 15 (College Station, TX).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Institutes of Health (NIH), including awards funded by the Environmental Influences on Child Health Outcomes (ECHO) program under grants# UG33OD023348 and 5R01HD092374-02, and the National Institute of Nursing Research under grant# 1K23NR017898-01.

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