Maternal gestational weight gain and DNA methylation in young women: application of life course mediation methods

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Aim: To investigate the role of maternal gestational weight gain (GWG) and prepregnancy BMI on programming offspring DNA methylation. **Methods:** Among 589 adult (age = 32) women participants of the Jerusalem Perinatal Study, we quantified DNA methylation in five candidate genes. We used inverse probability-weighting and parametric g-formula to estimate direct effects of maternal prepregnancy BMI and GWG on methylation. **Results:** Higher maternal GWG, but not prepregnancy BMI, was inversely related to offspring *ABCA1* methylation ($\beta = -1.1\%$ per quartile; 95% CI: -2.0, -0.3) after accounting for ancestry, parental and offspring exposures. Total and controlled direct effects were nearly identical suggesting included offspring exposures did not mediate this relationship. Results were robust to sensitivity analyses for missing data and model specification. **Conclusion:** We find some support for epigenetic programming and highlight strengths and limitations of these methods relative to other prevailing approaches.

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Increasing focus has been placed on aspects of intrauterine environment (IUE) as important determinants of offspring cardiometabolic health. For example, extremes of maternal gestational weight gain (GWG) and prepregnancy BMI may have important consequences on fetal environment [1,2] including macro- and micronutrients deficiencies [3], glucocorticoid excess [4] or insulin and leptin imbalance [5]. Developmental Origins of Health and Disease (DOHaD) theory posits that these cues may change early embryonic development leading to life-long differences in energy balance, inflammation, sympathetic nervous system activity and other biologic pathways important to cardiometabolic health [1–3]. Several epidemiologic studies have demonstrated positive associations between maternal GWG and prepregnancy BMI on offspring cardiometabolic risk factors such as BMI, waist circumference, blood pressure [6], weight trajectory [7] and fat mass [8]. Recent epigenetic epidemiologic studies suggest low maternal prepregnancy BMI [9,10] and GWG [11] to be related to differential peripheral blood DNA methylation in offspring, however findings were inconsistent [12]. Importantly, whether associations between prepregnancy BMI and GWG and offspring DNA methylation are causal [10] or persist through the life course remains unclear [9], though such evidence may support the plausibility of epigenetic programming as a mechanism for life course health [13,14].

We investigated the associations between two measures of intrauterine environment and offspring adult peripheral blood DNA methylation in a previously-identified set of candidate genes [14] using several approaches for modeling the total effect and controlled direct effects (i.e., by intervening to fix the values of mediators) of IUE, including standard covariate-adjusted regression, inverse probability-weighted (IPW) marginal structural models (MSM) [15,16], and g-computation by parametric g-formula [17]. The latter two methods may be able to better account for the complex relationships between life course exposures including avoiding over-adjustment for

mediators [18], an issue identified in recent epigenetic investigations [10], and estimate quantities of greater interest to DOHaD researchers. We apply several sensitivity analyses to test for residual biases, including from mediator mismeasurement, a critical issue in estimating mediated effects [19,20]. We discuss and compare the respective interpretations of each method, highlight the relevant assumptions and limitations, and comment on why this approach may be useful for investigations in the context of the Developmental Origins of Health and Disease and complementary to other proposed approaches [10,21,22].

Methods

Study setting

This study was conducted in the setting of the Jerusalem Perinatal Family Follow-Up Study, a sub-cohort of 1400 young adult study participants born in West Jerusalem between 1974 and 1976 who, along with their mothers, were re-contacted between 2007 and 2009 (offspring mean age = 32 years) [23]. Mother-offspring pairs were oversampled for maternal prepregnancy BMI ≥ 27 kg/m² and offspring birth weight ≤ 2500 g or ≥ 4000 g. Pairs were eligible if the offspring was a singleton born ≥ 36 weeks of gestation without congenital malformations. At the time of delivery in 1974–76, maternal medical history and pregnancy course and index subject's birth weight were abstracted from birth certificates or maternity ward logs. Additional sociodemographics and medical history were collected by maternal interview 1 or 2 days postpartum. Between 2007 and 2009, adult offsprings were interviewed by telephone about demographics, health behaviors and medical history. At a separate visit, study staff conducted physical exams to collect anthropometrics and a peripheral blood sample [23]. In 2014, all female offsprings with sufficient stored blood ($n = 589$) were included in a candidate gene methylation pilot study [14]. The current study includes all participants of this pilot.

All protocols were approved by the University of Washington (WA, USA) and Hadassah-Hebrew University Medical Center (Jerusalem, Israel) Institutional Review Boards and all mothers and offspring (at age 32) provided written informed consent.

Data collection

Intrauterine environment exposure variables (A)

In line with past studies of IUE [6,7,9], we used data from birth records and maternity logs to characterize four measures: First, maternal prepregnancy BMI was calculated from weight (in kilograms) divided by squared height (in meters) and also grouped by category (<18.5 ; $18\text{--}24.9$; $25\text{--}29.9$; ≥ 30); second, maternal GWG was calculated by subtracting weight at delivery from prepregnancy weight (in kilograms) and grouped into quartiles. For descriptive purposes, we also categorized GWG as 'inadequate,' 'adequate' and 'excessive' based on Institute of Medicine guidelines [24].

DNA methylation outcome variables (Y)

Adult (mean age = 32 years) peripheral blood (buffy coat) and the EpiTYPER MassARRAY Compact System (CA, USA), a tandem mass spectrometry-based assay, were used to profile DNA methylation in five candidate genes related to growth/energy balance (*ABCA1*, *INS-IGF2*, *LEP*) and stress response (*HSD11B2* and *NR3C1*). These genes were chosen to represent the body of early studies investigating prenatal exposure – offspring blood DNA methylation associations. Sites failing $>25\%$ of calls were excluded; no subjects were excluded by this criterion. Control samples of known methylation (0, 50 and 100%) were run for quality control. Samples were randomly plated, though no batch corrections were performed in analyses. Gene regions, a literature review including effect estimates, primer sequences and protocol details, are given in Supplementary Materials.

Parental characteristics (C)

We included the following parental characteristics measured as of the time of the subject offspring's birth as time-invariant confounders (i.e., assuming they are independent of all exposures and mediators): maternal age, parity (excluding subject offspring) and years of education; paternal years of education and any paternal smoking during the course of the index pregnancy (yes/no). Maternal smoking was excluded as it was not associated with the outcomes and consequently had limited influence on estimates.

Figure 1. Hypothesized life course causal model. To identify a direct effect of intrauterine environment on adult DNA methylation through metabolic and physiologic programming, we might be interested in the effect of A on methylation through B and O, but not E, P and S. However, adjustment for E, P and S may induce confounding through B or O (collider stratification bias).

A: Intrauterine environment exposure (maternal prepregnancy BMI or gestational weight gain); B: Birth weight; O: Adolescent overweight; E: Educational attainment; P: Parity; S: Smoking.

Precision & control variables (C)

Since DNA methylation can be sequence dependent [25,26], we attempted to control for population stratification by adjusting all analyses for offspring ethnic origin using dummy variables indicating the proportion of biological grandparents (0–4 out of 4) from Ashkenazi, Israel, Morocco, Other Africa, Iraq, Iran, Kurdistan, Yemen and/or Other Asia origins [6]. Although several pairwise associations were observed, there was no strong patterns of association by either origin or gene region (Supplementary Table 1). Though all participant offspring had blood drawn at similar ages (interquartile range: 31.7–33.3 years), we adjusted for offspring age at blood draw in all models to control for age-related variation in DNA methylation [27].

Offspring life course variables (M)

The following variables were included as offspring life course variables, some of which plausibly mediate the relationship between maternal characteristics and offspring adult DNA methylation: Birthweight (≤ 2500 , 2501–3999 or ≥ 4000 g) from medical records; self-report of being overweight in grades 4–6 (~ 10 –12 years); completed education (≤ 12 ; 13–16; 17+ years); parity (any children/no); and any smoking (yes/no) at the time of follow-up interview in 2007–2009. For a subset of the population ($n = 285$), objectively measured BMI at age 17 (kg/m^2) was available from military intake records.

When estimating direct effects of maternal characteristics on adult methylation, we may wish to know what association would remain if we could control for one or more life course exposures, such as education, parity or smoking, as they may not be related to the developmental programming mechanism of interest. On the other hand, birthweight and adolescent weight status are potential mediators of early development [13] and we only wish to eliminate their role in confounding other mediators (Figure 1). We do this by standardization via inverse probability weighting or g-computation [14,17,18].

Statistical analyses

First, we described the study population using means or proportions and mean methylation stratified by quartile of maternal GWG and categories of prepregnancy BMI (< 18.5 , 18.5–24.9, 25–29.9, ≥ 30). Next, we estimated associations between each of the maternal exposures (continuous BMI, BMI categories, continuous GWG and GWG quartiles) and mean methylation at each of the five candidate gene regions using the following three approaches to estimate *total effects* (TE), the effect of maternal exposures on adult DNA methylation including all mediating pathways by which IUE may operate, and *controlled direct effects* (CDE), the effect of maternal exposures on adult DNA methylation if certain modifiable mediator(s) can be fixed at a given level in the population. Those models were estimated as follows (target effect given in parentheses):

Multivariable linear regression Mean methylation in each gene was estimated by a linear regression model including as covariates: the specific maternal exposure measure (A) and

Maternal GWG or ppBMI:	$w_i^A = \frac{Pr(A=a_i)}{Pr(A=a_i C=c_i)}$
Offspring birth weight category:	$w_i^B = \frac{Pr(B=b_i C=c_i)}{Pr(B=b_i A=a_i, C=c_i)}$
Offspring adolescent overweight:	$w_i^O = \frac{Pr(O=o_i C=c_i)}{Pr(O=o_i A=a_i, B=b_i, C=c_i)}$
Offspring educational category:	$w_i^E = \frac{Pr(E=e_i C=c_i)}{Pr(E=e_i A=a_i, B=b_i, O=o_i, C=c_i)}$
Offspring parity:	$w_i^P = \frac{Pr(P=p_i C=c_i)}{Pr(P=p_i A=a_i, B=b_i, O=o_i, E=e_i, C=c_i)}$
Offspring smoking status:	$w_i^S = \frac{Pr(S=s_i C=c_i)}{Pr(S=s_i A=a_i, B=b_i, O=o_i, E=e_i, P=p_i, C=c_i)}$
Overall weight:	$W_i^{overall} = w_i^A * w_i^B * w_i^O * w_i^E * w_i^P * w_i^S * w_i^{oversample}$

Figure 2. Inverse probability weighting equations. A = GWG quartile or prepregnancy BMI category; B = birth weight category; O = self-reported overweight in adolescents (yes/no); E = educational attainment category; P = parity (yes/no children) and C = vector of parental confounders and control variables as defined in the text. Each individual in the marginal structural model is ultimately weighted by the overall weight ($W_i^{overall}$) which accounts for the oversampling weight ($W_i^{oversample}$).

- 1.1 parental confounders and control variables (C) only (TE)
- 1.2 both (C) and offspring life course mediating variables (M) (CDE)

Observations were weighted only by the inverse of the sampling probabilities to account for oversampling. Under assumptions of no exposure-mediator interactions, no unmeasured confounding, a linear relationship between all variables and correct model specification, Model 1.1 would give the TE of the exposure and Model 1.2 would give the CDE of the exposure if the mediators could be fixed to baseline values of normal birth weight (2501–3999 g), no overweight in adolescence, high school (12–16 years) education, no children and no current smoking. We discuss the tenability of these assumptions below. Multiple imputation by chained equations was used to estimate the influence of missingness (n = 43 subjects with any missing values; maternal education was the most frequent, missing in 19 subjects; Supplementary Table 2). Models for GWG were also adjusted for prepregnancy BMI.

Marginal structural models estimated by inverse probability weighting

Mean methylation at each gene was estimated by a linear regression model including as covariates: the specific exposure measure (A) and:

- 2.1 No control variables (TE)
- 2.2 Offspring educational attainment (<12, 12–15, 16+ years) only (CDE)
- 2.3 Offspring has any children (yes/no) only (CDE)
- 2.4 Offspring any current smoking (yes/no) only (CDE)
- 2.5 All three life course mediating variables (CDE)

Observation in the MSM was weighted by the joint inverse probability of: being exposed to each level of the given exposure (A), for example, having a mother in the first quartile of GWG; each of the mediators (M); and being oversampled (Figure 2).

Under assumptions of no unmeasured confounding and correct model specification, estimates of exposure-outcome associations including no other covariates in the model (model 2.1) using this weighted sample can be

Target effect	Intervention group (Y1)		Comparison group (Y0)		Contrast ^a
	Value of A	Value(s) of M	Value of A	Value(s) of M	Y1 – Y0
Total effect (TE)	0	M(0)	x	M(x)	$Y(A = 0, M(0)) - Y(A = x, M(x))$
Controlled direct effect (CDE)	0	M(m)	x	M(m)	$Y(A = 0, M(m)) - Y(A = x, M(m))$
Natural direct effect (NDE)	0	M(0)	x	M(0)	$Y(A = 0, M(0)) - Y(A = x, M(0))$
Natural indirect effect (NIE)	x	M(0)	x	M(0)	$Y(A = x, M(0)) - Y(A = x, M(x))$

Figure 3. Target contrasts estimated by parametric g-formula (g-computation).

^a The *gformula* program in Stata [17] actually gives the reverse comparison. This was done for comparability to other models.

A = IUE exposure variable; M = mediator variable(s); Y = outcome variable, estimated as an expectation (i.e., mean). 0 = the baseline level that the hypothetical intervention forces A to take.

x = the observed level of exposure.

Thus, M(0) = the value of a mediator if A were set to the baseline level, etc.

interpreted as the *total effect* of one unit increase in exposure (e.g., 1 kg higher GWG) in a population where the exposure and mediators are randomly assigned [15,19]. Under the same assumptions, including mediators (models 2.2–2.5) as covariates estimates the *controlled direct effect* of one unit increase in the IUE exposure on adult methylation if the respective mediators can be controlled at their baseline levels.

G-computation by the parametric g-formula G-computation using the Stata *gformula* command [17] comprises two main steps: First, we modeled the relationships between each exposure, mediator and outcome and their respective predictors (Figure 1) using parametric models. For example, a multinomial logistic regression model was fit predicting birth weight category by the set of parental characteristics (C) and IUE exposure value (A). Missing values were replaced by a single stochastic imputation by chained equations [17]. Second, the coefficients from each of these equations are used to predict the outcome values (Y) for each subject via simulation. Specifically, 100 datasets of identical size to the observed population (n = 589) were generated by sampling the original dataset with replacement. For each subject in each bootstrapped dataset, IUE exposure (A) values are assigned and potential outcomes are predicted for mediators (M) and then adult methylation level (Y), based on the target effect estimates (Figure 3). From these simulated data, total, direct (controlled and natural) and indirect effects were estimated. The process, code and definitions for effects (contrasts) are detailed in Supplementary Table 3.

Sensitivity analyses

Because the experimental treatment assumption, or nonpositivity, is important for the IPW method, we looked for extreme weights and changes in estimates using truncated probability weights. Because mediator mismeasurement is a critical source of bias in mediation analyses [19,20], we compared our results with models using alternative mediator measures and parameterizations.

Exploratory analyses

For any regions where DNA methylation was directly and consistently associated to a maternal measure of IUE, we subsequently explored whether methylation in the region was (cross-sectionally) associated with cardiometabolic biomarkers, whether there were longitudinal associations between the IUE measure and the biomarker, and whether methylation may mediate this relationship using a product-of-coefficients approach with bootstrapped confidence intervals.

We conducted all data analyses using Stata MP 13.1 (StataCorp, TX, USA).

Results

In general, mothers in our study were of normal BMI before pregnancy (mean = 24.3 kg/m²). Approximately equal proportions of mothers (~38%) gained an ‘insufficient’ or ‘appropriate’ amount of weight [24] during pregnancy with the subject offspring (Table 1). Higher quartiles of GWG were associated with higher maternal and paternal

Table 1. Study population characteristics, by maternal gestational weight gain quartile.

	Overall (n = 589)	GWG quartile			
		Quartile 1 -6 to 8 kg (n = 170)	Quartile 2 9 to 10 kg (n = 113)	Quartile 3 11 to 13 kg (n = 157)	Quartile 4 14 to 33 kg (n = 143)
Intrauterine environment					
Prepregnancy BMI (kg/m ²)	24.3 (3.9)	25.0 (4.2)	24.4 (3.9)	23.8 (3.9)	23.9 (3.6)
GWG (kg/m ²)	10.9 (4.7)	5.8 (2.6)	9.6 (0.5)	11.8 (0.8)	16.8 (3.5)
IOM (2009) category of GWG	37.9%	73.2%	49.5%	26.1%	-
– Insufficient (%)	38.4%	26.8%	45.9%	47.1%	36.6%
– Appropriate (%)	23.7%	-	4.5%	26.8%	63.4%
– Excessive (%)					
Parental confounders/control variables (C)					
Any maternal smoking (%)	15.4%	7.6%	9.7%	13.5%	20.3%
Any paternal smoking (%)	46.9%	42.9%	44.2%	48.1%	53.1%
Maternal parity	2.0 (2.0)	2.3 (1.9)	1.8 (2.1)	1.9 (2.1)	1.7 (1.9)
Maternal education (years)	11.8 (3.4)	11.6 (3.5)	11.7 (3.0)	11.9 (3.6)	12.0 (3.3)
Paternal education (years)	12.2 (4.0)	12.2 (4.0)	11.9 (3.7)	12.5 (4.1)	12.2 (4.0)
Maternal age (years)	28.3 (5.8)	28.9 (5.5)	27.3 (5.4)	29.0 (5.8)	28.1 (5.8)
Paternal age (years)	31.9 (6.7)	32.7 (7.1)	30.7 (6.2)	32.7 (6.7)	31.2 (6.3)
Maternal ethnic origin					
– Israel (%)	12.4%	8.8%	14.2%	11.5%	15.4
– Asia (%)	28.4%	30.6%	31.9%	24.2%	27.3
– Africa (%)	22.8%	21.2%	23.9%	24.8%	22.4
– West (%)	36.5%	39.4%	30.1%	39.5%	35.0
Age at blood draw (years)	32.5 (1.1)	32.6 (1.1)	32.4 (1.1)	32.5 (1.1)	32.5 (1.1)
Offspring life course (M)					
Birth weight (grams)	3297 (601)	3160 (544)	3206 (579)	3327 (596)	3517 (626)
Adolescent overweight (%)	20.0%	19.4%	13.3%	19.7%	27.3%
Education (years)	15.0 (2.6)	14.8 (2.6)	14.6 (2.4)	15.6 (2.6)	14.8 (2.9)
Parity	2.3 (2.1)	2.6 (2.2)	2.2 (1.8)	2.0 (2.0)	2.3 (2.1)
Current smoker (%)	18.8%	21.8%	15.0%	18.2%	19.7%
GWG: Gestational weight gain.					

GWG: Gestational weight gain.

smoking. Parental education, age and ethnic origins did not differ substantially by GWG. Offspring of mothers with higher GWG had higher mean birthweight, with extreme GWG quartiles (1 and 4) differing by approximately 350 g (Table 1). Of the life course exposures, only adolescent overweight appeared to differ by GWG quartile, with offspring of mothers who gained the most weight during pregnancy (Quartile 4) having the highest proportion (27%) of overweight in grades 4–6.

Except for *ABCA1*, which showed an inverse relationship, methylation in candidate regions did not exhibit clear linear relationships with GWG (Tables 2 & 3). Inverse associations of 1%-point lower mean *ABCA1* methylation per higher maternal GWG quartile were consistently observed across regression models (Table 3; Table 4, row 1; & Table 4, row 2) and following multiple imputation (Table 4, row 3). The magnitude of association was also unchanged in an analysis restricted to individuals with objective BMI measured at age 17 (n = 285), though precision was predictably reduced (Table 4, row 4).

Likewise, when IPW was used to estimate the association between GWG quartile and mean *ABCA1* methylation within a standardized population with similar chances of exposure to different levels of GWG and life course mediators (total effect, *TE*), the magnitude and direction of associations were similar, but with less precision (Table 4, row 5). The estimate of the *CDE* fixing a woman to be high school-educated, childless and a nonsmoker was nearly identical to the *TE* (Table 4, row 6). Truncating weights at different thresholds (1/99 percentile; 5/95 percentile; 10/90 percentile) had minimal influence on estimates (results not shown). Using the parametric

Table 2. Methylation (%) distribution, by candidate gene region and gestational weight gain quartile (mean/SD).

Gene	Location (GRCh37)	# CpG sites	Overall (N = 589)	Q1 -6 to 8 kg (n = 170)	Q2 9 to 10 kg (n = 113)	Q3 11 to 13 kg (n = 157)	Q4 14 to 33 kg (n = 143)
<i>ABCA1</i>	Chromosome 9: 107 690 502–107 690 821	27	20.0 (7.3)	20.9 (7.6)	20.1 (7.8)	19.6 (6.2)	19.1 (7.5)
<i>HSD11B2</i>	Chromosome 16: 67 464 230–67 464 442	6	5.7 (2.1)	6.0 (2.4)	5.3 (1.7)	5.6 (1.9)	5.8 (2.3)
<i>INS-IGF2</i>	Chromosome 11: 2 182 336–2 182 640	4	77.3 (5.4)	77.4 (4.7)	78.1 (4.7)	77.2 (5.9)	76.6 (5.9)
<i>LEP</i>	Chromosome 7: 127 881 051–127 881 408	32	22.2 (11.7)	23.0 (11.4)	21.2 (12.5)	21.9 (11.7)	22.3 (11.4)
<i>NR3C1</i>	Chromosome 5: 142 783 506–142 783 905	47	6.5 (2.7)	6.6 (3.8)	6.3 (1.7)	6.6 (1.8)	6.7 (2.8)

Table 3. Associations between maternal gestational weight gain and mean DNA methylation, adjusted for ethnic origin and age at blood draw (β [95% CI]; shaded = $p < 0.01$).

GWG	Per kg	Per quartile	Quartile 2 (vs lowest)	Quartile 3 (vs lowest)	Quartile 4 (vs lowest)	F-test for Δ (p-value)
<i>ABCA1</i>	-0.2 [-0.4, -0.01]	-1.0 [-1.8, -0.3]	-0.2 [-3.3, 2.9]	-1.1 [-3.3, 1.1]	-3.2 [-5.5, -0.9]	0.032
<i>HSD11B2</i>	-0.008 [-0.05, 0.03]	0.03 [-0.2, 0.2]	-0.4 [-1.0, 0.2]	-0.07 [-0.7, 0.5]	-0.01 [-0.6, 0.6]	0.356
<i>INS-IGF2</i>	-0.01 [-0.1, 0.1]	0.1 [-0.3, 0.6]	0.6 [-1.0, 2.2]	0.8 [-0.5, 2.2]	0.4 [-1.2, 1.9]	0.646
<i>LEP</i>	0.05 [-0.3, 0.4]	0.3 [-0.9, 1.5]	-1.8 [-5.6, 1.9]	0.6 [-3.0, 4.3]	0.2 [-3.5, 3.8]	0.614
<i>NR3C1</i>	-0.01 [-0.1, 0.1]	-0.2 [-0.8, 0.3]	-0.8 [-2.5, 1.0]	-0.6 [-2.1, 0.9]	-0.8 [-2.6, 1.1]	0.411

GWG: Gestational weight gain.

Table 4. Adjusted estimates of association between maternal gestational weight gain and mean *ABCA1* methylation, by modeling strategy (β [95% CI]; shaded = $p < 0.01$).

	Per kilogram	Per quartile
Multivariable regression		
Adjusted for (C)	-0.2 [-0.4, -0.03]	-1.1 [-1.9, -0.3]
Adjusted for (C) and (M)	-0.2 [-0.5, -0.03]	-1.1 [-2.0, -0.3]
Adjusted for (C) and (M), multiple imputation	-0.2 [-0.4, -0.02]	-1.2 [-2.0, -0.4]
Adjusted for (C) and (M), objective BMI @ age 17	-0.2 [-0.4, -0.02]	-1.3 [-2.5, -0.1]
Marginal structural model by IPW		
Total effect (TE)	–	-1.3 [-2.5, -0.1]
Controlled direct effect (CDE)	–	-1.3 [-2.5, -0.05]
Parametric g-formula	11 kg vs Obs. [†]	Q3 vs Obs. [†]
Total effect (TE)	-0.004 [-0.07, 0.06]	-1.4 [-2.4, -0.4]
Controlled direct effect (CDE)	0.004 [-0.05, 0.06]	-1.5 [-2.4, -0.5]
Natural direct effect (NDE)	0.04 [-0.05, 0.1]	-1.4 [-2.4, -0.4]
Natural indirect effect (NIE)	-0.04 [-0.1, 0.02]	0.04 [-0.2, 0.2]

[†]Associations for the parametric g-formula are expressed as the contrast between the observed distribution of methylation versus a counterfactual distribution where maternal gestational weight gain is fixed to 11 kg (left column) or Quartile 3 (11–13 kg; right column).

IPW: Inverse probability-weighted.

g-formula to compare the distribution of *ABCA1* methylation observed in the population (mean ~20%) to what it might be if every mother were forced to gain between 11 and 13 kg during pregnancy, estimates were virtually identical when life course mediators were (*CDE*, *NDE*) or were not (*TE*) fixed (rows 7–9). Moreover, the relatively small estimate of natural indirect effect (*NIE* = 0.04, ~3% of total effect's magnitude), suggested little of effect of GWG on adult *ABCA1* methylation was mediated through offspring education, parity or smoking. Using continuous versus dichotomized mediators did not change estimates (i.e., *TE* = *CDE* = *NDE* = -1.4; Supplementary Table 4).

With respect to prepregnancy BMI, *ABCA1* methylation appeared to be highest among offspring of underweight mothers (BMI < 18.5) and lowest among offspring of obese mothers (BMI ≥ 30) when compared with offspring of normal weight (BMI 18.5–24.9) mothers (7% higher and 1.7% lower, respectively; Supplementary Tables 5–7). However, these estimates of association were imprecise and inconsistent following multiple imputation (Supplementary Table 7). Given previous findings regarding prepregnancy BMI and offspring DNA methylation [9], we explored whether prepregnancy BMI may modify the observed association between GWG and *ABCA1* methylation. We found maternal GWG may be more strongly related to offspring adult DNA methylation among women with prepregnancy BMI < 19.5 kg/m² (-1.4% per kg higher GWG) than ≥ 19.5 (-0.1% per kg higher GWG), though there was not strong statistical evidence of additive interaction (Supplementary Table 8), possibly due to sample size. No other associations between candidate regions and GWG or BMI were consistently observed and only the results for maternal GWG and offspring *ABCA1* associations are presented here. Complete results for maternal prepregnancy BMI are given in the Supplementary Materials (Supplementary Tables 5–7).

We were also interested in assessing whether adult methylation itself mediates relationships between IUE exposures and adult phenotype. Thus, we conducted further analyses to explore the potential role of adult peripheral blood *ABCA1* methylation in mediating the relationship between maternal GWG and offspring adult cardiometabolic phenotype (Supplementary Tables 9 and 10). We found each higher percent *ABCA1* methylation to be associated with 0.8% (95% CI: 0.05%, 1.6%) higher fasting plasma insulin and therefore estimated beta cell function (HOMA-B), adjusted for ethnic origin, age at blood draw, parental characteristics and life course exposures (Supplementary Table 10). We found maternal GWG to be directly related to offspring adult weight and hip circumference independent of *ABCA1* methylation, corroborating previous findings [7]. Moreover, we found some evidence for an indirect effect of 0.6% (95% CI: -1.0%, -0.1%) lower offspring beta-cell function (HOMA-B) for each kilogram higher maternal GWG mediated through *ABCA1* methylation (Supplementary Table 11). At the recommendation of reviewers, we conducted further sensitivity analyses eliminating adjustments for offspring age, adding adjustments for maternal diabetes (4.9% of pregnancies) and hypertension (2.0% of pregnancies), and adding adjusting for duration of breastfeeding. None of these model changes affected estimates (Supplementary Table 12).

Discussion

In our study, we found evidence that offspring whose mothers gained more weight during pregnancy had lower average *ABCA1* methylation as adults while demonstrating different strategies to control for the effects of life course exposures. For example, each higher quartile of maternal GWG was associated with about a 1%-point lower mean *ABCA1* methylation. This magnitude remained consistent across modeling strategies and even when accounting for the small amount of missing data and mediator mismeasurement. We found less consistent evidence for associations between maternal prepregnancy BMI and methylation at our chosen genes. We found preliminary evidence that *ABCA1* methylation may mediate relationships between maternal GWG and offspring glucose homeostasis.

Several candidate gene studies have demonstrated relationships between IUE-related maternal exposures and offspring *ABCA1* methylation [28,29]. Notably, Tobi *et al.* found prenatal exposure to the Dutch Famine to be related to 0.7% higher *ABCA1* methylation, in the same promoter region we interrogated [28]. We found maternal GWG, which presumably is restricted during famines, to be inversely related to mean *ABCA1* methylation (e.g., one quartile lower maternal gestational weight gain was associated with a 1% higher *ABCA1* methylation), supporting a similar relationship. While Tobi *et al.* used sibships to control for shared family and environmental characteristics, their study did not further investigate whether the associations may be due to metabolic programming or differential health behaviors. While this study controlled for offspring smoking, the same approach could be applied elsewhere for other potentially mediating behaviors such as eating patterns.

A more recent Epigenome-Wide Association Study investigated the relationships between maternal prepregnancy BMI and GWG on offspring peripheral blood DNA methylation at prespecified CpG sites [9]. While investigators

found numerous associations with maternal BMI, particularly maternal underweight status, they found no false discovery rate (FDR) adjusted associations with GWG. We found some associations between extremes of prepregnancy BMI and *ABCA1* methylation, particularly higher methylation in offspring of underweight (vs normal) women (Supplementary Table 7) and some indication that the association between GWG and *ABCA1* methylation may be stronger in offspring of mothers with BMI <9.5 kg/m² (Supplementary Table 8). However, these associations were not consistent. Relevantly, however Sharp *et al.* noted that differences in methylation at several CpG sites associated with maternal BMI appeared to be minimized through late adolescence due to differing rates of change, potentially explaining our lack of strong associations. These findings highlight the necessity of quantifying how life course factors contribute to observed, or failure to observe, longitudinal associations [14,30]. Notably, Sharp *et al.* [10] indicated adjustments for certain confounding variables such as sex may be inappropriate if they represent a mediating component (e.g., circulating sex hormones) to the observed relationship. Our methods proposed here could address this directly through standardization and complement their findings which employed a negative control approach, and thus a distinct set of identifying assumptions [10].

Interestingly, we found no association between methylation levels in *ABCA1*, which facilitates intracellular cholesterol transport, with offspring total cholesterol, HDL-C, or LDL-C (Supplementary Table 10), which has been described in a previous study of familial hypercholesterolemia [31]. However, our study enrolled mainly healthy women and assessed methylation at a different set of CpG sites limiting comparability (27 CpGs, mean = 20% vs 8 CpGs, mean = 36%). Instead, in exploratory analyses we found *ABCA1* to be positively associated with plasma insulin and beta-cell function (HOMA-B). The cross-sectional nature (measures assayed from the same blood sample) limits interpretation of the observed association. However, the role of cholesterol in beta-cell dysfunction [32] and observed associations between offspring *ABCA1* methylation and maternal glucose tolerance [29], indicates follow-up study to better understand this association is warranted.

Strengths: comparison of mediation methods

A major strength of the current study over previous investigations of fetal environment – adult DNA methylation associations – is the comparison of estimates using several methods to control for the effects of life course exposures on adult methylation measures. It is well known that DNA methylation varies over time due to both stochastic and environmental changes and must be accounted for when measured in adulthood [33]. A substantial methodological challenge remains in adjusting for exposures over the life course that affect DNA methylation such as attained obesity [34] which may also be a consequence of early development. To estimate direct effects of maternal exposures, for example, researchers often employ traditional mediation and path analyses, adjusting for life course mediators as covariates in regression models [8]. However, these conditional models may not be able to estimate associations that are most of interest with respect to fetal or developmental programming hypotheses [14]. Specifically, including intermediates such as birth weight or adolescent obesity as covariates over adjusts for important consequences of early development, in other words, some of the variation in adult outcomes due to the trajectory set by putative fetal programming would be eliminated by the adjustment [18,35]. On the other hand, DOHaD researchers may wish to eliminate the influence of mediators on other life course social processes and behaviors, for example, the higher adoption of smoking by overweight adolescents [36,37]. To accomplish this, we used mediation approaches that employ standardization, in other words, IPW marginal structural models [15,22] and g-computation by parametric g-formula [17]. Under standard assumptions for causal inference in observational data [19], such methods would facilitate the estimation of direct effects of IUE on adult outcomes without ‘adjusting away’ important mediating pathways [17,38]. Doing so, we found that putative mediators did not substantially reduce the associations observed between maternal gestational weight gain and offspring adult *ABCA1* methylation under any model. This would not have been the case in the presence of strong intermediate confounding by measured mediators. The use of multiple methods also allowed us to mutually address the limitations of respective approaches: G-computation is superior to inverse probability weighted approaches in accommodating continuous exposures. Conversely, inverse probability weights can quickly reveal violations of the experimental treatment assumption (c.f. the presence of large weights) that is less apparent in g-computation.

Another notable strength of these approaches is the attempt to quantify the remaining ‘direct’ effect of early development on adult health despite potential intermediate interventions to alleviate them within a defined population. Such estimates have potential relevancy to investigating early life interventions of interest to DOHaD researchers [39]. G-computation, in particular, is gaining traction as a way of represented potential benefits of public health policies and interventions [40]. Moreover, these methods may complement negative control [10]

and instrumental variable approaches (Mendelian Randomization) to investigating mechanisms within causal frameworks [21], as they offer flexibility in the choice of exposures and estimated effects (e.g., rather than life-long difference in gene expression) at the cost of stronger assumptions regarding unmeasured confounding.

Limitations

Nonetheless, standard assumptions in observational epidemiology such as no unmeasured confounding remain a challenge even when employing the proposed methods. To the extent that offspring life course characteristics are explicitly included in these models and mediator-outcome confounding is handled appropriately, our analyses hold an advantage over other studies which may only include parental, prenatal characteristics. However, we acknowledge we may have omitted relevant mediators such as child breastfeeding. Additionally, exposure and mediator mismeasurement may overestimate mediated [20] or direct [19] effects, respectively. We note that mediated effects were nearly absent in this population and alterations in mediator parameterization had little influence on effect estimates. Further, the consistency assumption may not hold for our choice of maternal GWG and prepregnancy BMI exposures, as various interventions to ‘fix’ maternal weight or weight gain to certain levels would likely have drastically different effects [41]. Nonetheless, we observed a similar magnitude and direction of association with differences in quartiles of maternal GWG as was observed by past studies of maternal famine exposure, suggesting the effect, if real, may be quite robust [28]. Finally, our results based on a cohort of Israeli women with unique life trajectories [23] may not be readily generalizable to males or other populations.

There are several other technical challenges related to DNA methylation assays that are worth highlighting. First, we used an aggregate measure of DNA methylation in recognized gene promoter regions to mimic past and likely continuing candidate gene studies in existing cohorts. More recent studies have favored agnostic epigenome-wide association study approaches to identify associations with tag CpG sites recognizing that functional variations may occur in many coding and noncoding regions across the genome [8]. Nonetheless, replication and targeted follow-up studies for mechanisms could benefit from employing these methods. Additionally, we did not adjust for batch effects, which may mask true associations through nondifferential measurements error. Finally, we did not adjust for blood cell composition. Though Sharp *et al.* [9,10] found no clear patterns of offspring peripheral blood composition relative to maternal prepregnancy BMI and GWG, adjustments for composition in Sharp *et al.* reduced magnitudes of association. Further investigation of the causal role of cellular heterogeneity in observed associations is necessary. Notably, we cannot exclude the possibility that associations are mediated by compositional changes. Given these challenges and the limited current literature on IUE and offspring methylation, replication of findings using these and other epidemiologic approaches coupled with supporting functional studies will be essential.

Conclusion

In general, we found mean *ABCA1* methylation appeared to be directly related to both maternal gestational weight gain and some markers of glucose homeostasis. This relationship was not confounded or strongly mediated by some predictors of adult DNA methylation, such as adolescent overweight or adult smoking. Given the current lack of literature demonstrating causal relationships between IUE and offspring DNA methylation and the technical challenges detailed above, replications of these findings using these and complementary causal inference techniques are essential. Additionally, more work is needed to build on the strengths of these approaches to assess potential causal effects of early life environment, including quantitative bias analyses for unmeasured confounding in the epigenetic epidemiologic context. Nonetheless, we feel the approaches outlined herein may be a useful complement to other approaches to strengthen causal inference in life course epigenetic investigations.

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Summary points

- For investigators interested in the long-term effects of intrauterine environment on epigenetic marks and adult phenotype (i.e., Developmental Origins of Health and Disease), appropriate accounting for life-course (postnatal) environment is key.
- Recent studies have demonstrated some evidence for associations between measures of intrauterine environment such as prepregnancy BMI and gestational weight gain (GWG) on offspring peripheral blood DNA methylation.
- However, associations vary over time likely due to differential postnatal environment yet traditional conditional models may ‘adjust away’ effects of mediating mechanisms resulting in unclear causal interpretations.
- Using data from the Jerusalem Perinatal Family Follow-Up Study, we modeled the relationship between maternal GWG and prepregnancy BMI on offspring adult peripheral blood DNA methylation at several candidate cardiometabolic and stress response genes.
- We used inverse probability weighting (marginal structural models) and simulation (parametric g-formula) approaches to estimate the direct effects of intrauterine environment under different hypothetical interventions to control life course exposures reducing the need for direct adjustment.
- We found evidence for a similar total and direct effect of greater GWG on reduced ABCA1 gene methylation across different models, suggesting differential methylation would persist despite interventions on offspring exposures such as smoking.
- When carefully applied, these methods can be useful not only for investigating longer-term effects hypothesized by Developmental Origins of Health and Disease, but also provide estimates that are interpretable as the result of population interventions.
- By providing flexibility in modeling different causal structures, these methods complement other prevailing approaches such as negative control exposures and instrumental variables (e.g., Mendelian Randomization) to strengthening causal inference in epigenetic epidemiology.

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-2017-0085

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