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Differential methylation of insulin-like growth factor 2 in offspring of physically active pregnant women

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Abstract

Several studies have suggested that maternal lifestyle during pregnancy may influence long-term health of offspring by altering the offspring epigenome. Whether maternal leisure-time physical activity (LTPA) during pregnancy might have this effect is unknown. The purpose of this study was to determine the relationship between maternal LTPA during pregnancy and offspring DNA methylation. Participants were recruited from the Archive for Research on Child Health study. At enrollment, participants' demographic information and self-reported LTPA during pregnancy were determined. High active participants (averaged 637.5 min per week of LTPA; $n = 14$) were matched by age and race to low active participants (averaged 59.5 min per week LTPA; $n = 28$). Blood spots were obtained at birth. Pyrosequencing was used to determine methylation levels of long interspersed nucleotide elements (*LINE-1*) (global methylation) and peroxisome proliferator-activated receptor-gamma (*PPARγ*), peroxisome proliferator-activated receptor-gamma coactivator (*PGC1-α*), insulin-like growth factor 2 (*IGF2*), pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*) and transcription factor 7-like 2 (*TCF7L2*). We found no differences between offspring of high active and low active groups for LINE-1 methylation. The only differences in candidate gene methylation between groups were at two CpG sites in the P2 promoter of *IGF2*; the offspring of low active group had significantly higher DNA methylation ($74.70 \pm 2.25\%$ methylation for low active *v.* $72.83 \pm 2.85\%$ methylation for high active; $P = 0.045$). Our results suggest no effect of maternal LTPA on offspring global and candidate gene methylation, with the exception of *IGF2*. *IGF2* has been previously associated with regulation of physical activity, suggesting a possible role of maternal LTPA on regulation of offspring physical activity.

Introduction

Traditionally, poor diet and physical inactivity have been blamed for the rising obesity epidemic in both children and adults. However, recent evidence suggests that numerous other factors, including the prenatal and maternal environment, may also play a role.[1] Children are increasingly at risk of developing chronic disease risk factors early in life, and data show that cardiovascular disease (CVD) risk factors track moderately well from childhood into adulthood and likely contribute to CVD morbidity.[2] However, interventions designed to reduce CVD risk factors in children and adolescents have been only moderately successful at best.[3] Investigators have begun to consider alternative strategies, such as determining whether a child's CVD risk factor profile is determined before birth, and if so, whether positive genetic alterations in offspring can occur through altered maternal behaviors.

The claim that CVD and obesity risk factors can be traced to fetal origins is a central tenet of the Developmental Origins of Health and Disease (DOHaD) hypothesis.[4] DOHaD posits that critical developmental periods for many components of the metabolic and vascular underpinnings of CVD exist during embryonic and fetal life. If, during these periods, the maternal–fetal unit is unduly stressed, permanent developmental alterations can occur. The alterations that develop may be to preserve the mother's life or health or to provide an ideal environment for the fetus. In particular, low birth weight and/or fetal growth restriction have been associated with increased rates of coronary heart disease and other disorders related to the cardiovascular system in adults, suggesting developmental plasticity of the cardiovascular system during fetal development.[5]

Animal studies of maternal nutrition during pregnancy and epigenetic programming effects in the offspring[6] suggest that the maternal environment during development influences offspring phenotype, particularly in terms of metabolic health. It is reasonable to study

maternal physical activity in the same manner. However, the suggestion to do so[7,8] has not been pursued.

A mechanism by which offspring phenotype might be affected by maternal exposures or behaviors is epigenetics, which are heritable chemical changes in DNA that may influence gene expression. In terms of lifestyle and its influence on the epigenome, the best-studied epigenetic change is DNA methylation,[9] where methyl groups are added to cytosine bases in CpG islands. In general, high levels of methylation are associated with low promoter activity of the particular gene (and lower gene expression). However, fetal and adult methylation do not always coincide.[10] Whether high or low methylation level is advantageous in terms of programming depends on the gene in question and may depend on the tissue being studied as well. In humans, few studies have shown varying levels of DNA methylation in offspring as a consequence of a lifestyle exposure in the mother. Those that we do know of all examined diet.[11,12] For instance, individuals who were in utero during the Dutch Famine of 1944 (exposed to starvation levels of food deprivation) had lower genome-wide DNA methylation than their same-sex siblings not exposed to famine.[11] Others have found that offspring methylation patterns differ in relation to folate supplementation in the mother[13] and mother's diet during pregnancy.[12]

Methylation levels of various genes may be affected in offspring blood via maternal physical activity. These include peroxisome proliferator-activated receptor-gamma coactivator (*PGC1-α*), peroxisome proliferator-activated receptor-gamma (*PPAR-γ*), pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*), insulin-like growth factor 2 (*IGF2*) and transcription factor 7-like 2 (*TCF7L2*). Each of these is either altered epigenetically by exercise/physical activity or has an important role in metabolic function. *PGC1-α* and *PPAR-γ* are genes involved in metabolism of glucose and fat as well as muscle protein synthesis and mitochondrial biogenesis.[14,15] *PDK4* methylation is decreased by an acute bout of physical activity,[16] and *IGF2* is an imprinted gene frequently associated with fetal growth[17] and implicated in regulation of physical activity through haplotype association mapping in mice[18] and humans.[19] *TCF7L2* is a gene with a known role in etiology of type 2 diabetes.[20]

Though the rationale for studying DNA methylation as a mechanism for programming is strong, to date, very few studies in humans have measured methylation differences in offspring of exercising or otherwise physically active mothers compared with less active or inactive controls. Likely reasons for the paucity of data include difficulty controlling for the many factors that may affect DNA methylation (i.e. diet, smoking, age) and the tissue specificity of methylation changes and in particular, the challenges associated with biopsy of muscle and adipose tissue. Still, it is critical that human studies be conducted despite these challenges. The purpose of this study was to determine the relationship between maternal leisure-time physical activity (LTPA) during pregnancy, a commonly utilized term indicating time spent in physical activity during recreation[21] and differentiated from occupational physical activity,[22] and DNA methylation in offspring at birth, both in genome-wide and in metabolism-related candidate genes.

Methods

Study population

The Archive for Research on Child Health (ARCH) Study is a pregnancy cohort study that enrolled participants from 2008–2016 in three mid-Michigan clinics that provide prenatal health care primarily to low-to-middle income women. After enrollment early in pregnancy, women and their offspring are followed longitudinally. Blood spots obtained during newborn genetic screening have been archived and frozen by the Michigan Neonatal Biobank (mnbb.org) since 2009, and we therefore restricted enrollment in this study to ARCH infants born since 2010. At enrollment, at a mean gestational age of 12.7 weeks, researchers obtain mother's date of birth, social security number, race, ethnicity, education level, marital status, household income, home and car ownership, ownership of stocks/bonds, height, pre-pregnancy weight, and whether pregnancy was planned, and urine and blood were archived then and later in pregnancy. ARCH has been approved by the Institutional Review Board at Michigan State University and the Michigan Department of Health and Human Services. Informed consent was obtained from all individual participants included in the study.

At enrollment, study participants were queried regarding their participation in the prior month in moderate and vigorous LTPA and, if active, the number of days per week and minutes per day of activity. Moderate LTPA was defined as an activity that caused a small increase in breathing and heart rate, and vigorous LTPA was activity that caused a large increase in breathing rate and resulted in sweating. Total LTPA participation was calculated as the total number of minutes per week of moderate and vigorous LTPA reported. A nested exposure-control design was used for this study, using ARCH participants. That is, a group of highly active women were matched to women reporting low levels of activity, blood spots were retrieved for offspring of both groups and epigenomics were studied.

We contacted prospective participants by phone to request verbal consent to utilize infant blood spots for this study. Current phone numbers were on hand for 150 of ~300 participants who had completed the physical activity portion of the questionnaire. We called all 150 participants and obtained consent from 59. Women who reported participating in more than 2500 min per week (~6 h per day) of total LTPA were not called due to concerns about reporting accuracy.

From the 59 consented participants, we selected the most active as the 'high active' (HA) group. To be included, participants had to report at least 450 min per week LTPA, and we had to be able to find a suitable less active participant to individually match to the HA participant. There was no specific cutoff for low active (hereafter referred to as LA) but instead the least active consented participant who was within 5 years of age and identified with the same racial group was selected as the LA control. Each individual in the HA group was matched to a control from among the LA women on maternal body mass index (BMI), age and race. We initially further matched a second group of LA women to the HA group, but matched on age and race only, omitting BMI matching. The purpose of matching two separate LA groups was to allow for distinction between the effects of maternal LTPA and the effects of BMI on methylation. However, we found that all three groups had very similar BMI values (26.5 ± 6.0 for HA, 26.3 ± 5.5 for LA with BMI match, 26.9 ± 9.4 for LA no BMI match). Therefore, we opted to combine the two control groups and analyze the data as HA ($n = 14$) compared with LA ($n = 28$).

Matching criteria were selected because these factors have been found to alter DNA methylation in mothers.[23–28] We were concerned that maternal smoking predicts DNA methylation.[29] Five smokers were found among our 42 participants, two in the HA group (14%) and three in the LA group (11%). Due to known

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difficulty with dietary recall, particularly several years after the time frame of interest, we did not assess or match on mother's diet during pregnancy despite evidence that these covariates might influence DNA methylation without affecting birth weight.[30] DNA methylation also varies by gender,[26] but our exposure of interest took place after sex was determined. Finally, we opted not to match on socioeconomic status, despite its relationship with DNA methylation,[25] because the ARCH cohort is fairly homogeneous in this variable (see Table 2).

The Biobank was able to retrieve all 42 of our requested spots, and sent us four 3 mm thawed punches from each blood spot by overnight mail. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen) according to manufacturer recommendations and stored at −20°C.

Laboratory analysis

To study global DNA methylation, we quantified DNA after extraction via spectrophotometry (NanoDrop). We sent isolated genomic DNA to a commercial laboratory (EpigenDX, Worcester, MA, USA) who conducted pyrosequencing analysis for long interspersed nucleotide elements (*LINE-1*) methylation. Four regions of repetitive elements throughout the genome were assayed; methylation of these long regions of repetitive DNA stretches are commonly used as indicators of methylation of the entire genome.[30,31] Methylation of these repetitive elements is similar between mother (peripheral blood) and her newborn (umbilical cord blood); thus we concluded that differences observed between offspring of physically active women *v.* LA can be attributed to maternal exposures.[32]

Table 1 shows the descriptions of each candidate gene and the regions within these genes that were assessed. The same laboratory conducted the second set of analyses. DNA samples were treated with bisulfite and the treated DNA was amplified using polymerase chain reaction (PCR). Primers used for the PCR reactions are considered proprietary information by EpigenDX and thus are unavailable for publication. Pyrosequencing was then used to sequence the regions of candidate genes shown in Table 1. Each pyrosequencing assay has been validated by EpigenDX by use of low, medium and highly methylated DNA as well as a no template control.

Statistical analysis

Descriptive characteristics were calculated for HA and LA women. Methylation data results are provided as percentage methylation of the genome (for *LINE-1*) or a given gene (all candidate genes). When comparing methylation rates between HA and LA groups, no covariates were included due to the matched design and small sample size.

Normality of *LINE-1* data was evaluated using the Kolmogorov–Smirnoff test. The *LINE-1* data were normally distributed and therefore analysis of variance (ANOVA) was used to assess methylation differences between groups. Finally, a Spearman correlation coefficient was calculated to assess the relationship between *LINE-1* data and minutes per week LTPA within the total sample ($n = 42$).

All candidate gene data were checked for normality using Kolmogorov–Smirnoff tests and we found that none met criteria for normal distributions ($P < 0.05$). All data analyses, then, were performed using non-parametric tests. We compared differences in percentage methylation between groups, for each gene, using Mann–Whitney U tests. In addition, Spearman correlations were calculated for each mean methylation value for each gene region in relation to minutes of LTPA per week within the total sample ($n = 42$).

Results

Sample

Table 2 shows the sample characteristics for the two groups of mothers. A total of 42 participants were included in the analyses. Of these, 14 were classified as HA and reported 360–1020 min per week of LTPA, with mean participation of 637.5 min per week. LTPA was significantly higher in the HA group compared with the LA group ($P = 0.001$). There were no other differences in participant characteristics between the two groups of mothers. Although not statistically significant ($P = 0.060$), the HA group tended to be more highly educated than the LA group.

Table 1. Candidate gene descriptions

Gene	Description	PCR Size	#CpGs	Bp location
PPARγ	Promoter	132 bp	14	−91,771 to −91,691 from ATG
PPARγ	Intron 2	281 bp	3	−28,000 to − 28,073 from ATG
PGC1α	Proximal promoter	238 bp	3	−256 to −214 from ATG
PGC1α	Exon 1 to intron 1	174 bp	4	+51 to +94 from ATG
IGF2	P2 promoter	271 bp	8	−30,308 to −30,199 from ATG
IGF2	P4 promoter	130 bp	5	−4792 to −4864 from ATG
IGF2	Distal promoter	270 bp	3	−30,308 to −30,199 from ATG
PDK4	Promoter	149 bp	7	−589 to −532 from ATG
TCF7L2	5'UTR	150 bp	2	−435 to −438 from ATG
TCF7L2	5'UTR to promoter	150 bp	5	−467 to −516 from ATG

PCR, polymerase chain reaction; Bp = base pair; *PPAR-γ,* peroxisome proliferator-activated receptor-gamma; *PGC1-α,* peroxisome proliferator-activated receptor-gamma coactivator; *IGF2,* insulin-like growth factor 2; *PDK4,* pyruvate dehydrogenase kinase, isozyme 4; *TCF7L2,* transcription factor 7-like 2. Description of each candidate gene region assessed for percentage methylation.

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Table 2. Descriptive characteristics for two groups

	HA ($n=14$)	LA ($n=28$)	*P*-value
LTPA per week	637.5 ± 220.7	59.5 ± 79.1	<0.001*
Maternal age (years)	26.4 ± 8.2	27.4 ± 5.2	0.562
Maternal BMI	26.5 ± 6.0	26.6 ± 7.4	0.971
Infant birth weight (grams)	3247 ± 419	3409 ± 470	0.280
Maternal race (% white)	92.9 (13)	71.4 (20)	0.111
Sex (% male)	57.1 (8)	57.1 (16)	1.000
Smoking (% reported)	14.3 (2)	10.7 (3)	0.736
Income (% <$25,000)	57.1 (8)	39.3 (11)	0.273
Marital status (% unmarried)	24.1 (3)	21.4 (6)	1.000
Marital status (% married/living with baby's father)	35.7 (5)	39.3 (11)	0.822
Education (% attended at least some college)	85.7 (12)	64.3 (18)	0.060
Planned pregnancy (% unplanned)	50.0 (7)	53.6 (15)	0.827

HA, high active; LA, low active; LTPA, leisure-time physical activity; BMI, body mass index.
Means ± standard deviations for LTPA age, BMI and birth weight were compared between two groups using analysis of variance. Mean percentages for categorical variables were compared using χ^2 tests, and these data are presented as % (*n*).
*Significant difference between high active group and low active group.

Global methylation (LINE-1)

There were no differences between offspring of HA and LA mothers for *LINE-1* percentage methylation (HA = 77.25 ± 1.44%; LA = 77.54 ± 1.58%; $P=0.558$). We also assessed the continuous relationship between maternal LTPA minutes per week and offspring *LINE-1* percentage methylation, but found no significant relationship based on Spearman correlation coefficients ($r=0.040$; $P=0.801$; data not shown).

Candidate genes

Five candidate genes were analyzed for percentage methylation at various CpG sites. There were no significant differences in methylation between offspring of HA *v.* LA mothers with the exception of *IGF2* (Table 3). Results for *IGF2*, on chromosome 11, are presented in Table 4; other non-significant candidate gene data are not shown. Three regions of interest were analyzed within the *IGF2* gene: the first region we examined is in the P2 promoter and has eight CpGs, another is in the P4 promoter with five CpGs and the last is in the distal promoter with three CpGs studied. There were two sites within the P2 promoter that were significantly different between the two groups; for CpGs 3 and 4, percentage methylation was higher in the LA group compared with HA. The LA group had significantly higher DNA methylation (74.70 ± 2.25% methylation for LA *v.* 72.83 ± 2.85% methylation for HA; $P=0.045$). Although not significantly different, it is worth noting that the same directional effect (LA had higher methylation than HA) was found for CpGs 1 and 4 through 6 for the P2 promoter for *IGF2*. Spearman correlations revealed no significant associations between total LTPA per week and percentage methylation of any gene region (Table 5).

As the trend in education difference between HA and LA groups ($P=0.06$; see Table 2), we used ANOVA to determine mean methylation differences in *IGF2* for each of the three studied promoters by maternal education. We compared those participants with at least some college education to those with none and found no statistically significant difference between offspring's *IGF2* percentage methylation for the P2 promoter ($P=0.256$), P4 promoter ($P=0.285$) or distal promoter ($P=0.236$).

Table 3. Candidate gene mean methylation

Gene	Gene region	HA	LA	*P*-value
PPARγ	Promoter	0.73 ± 0.35	0.64 ± 0.31	0.463
	Intron 2	65.15 ± 7.29	66.13 ± 3.94	0.729
IGF2	P2 promoter	72.83 ± 2.85	74.70 ± 2.25	0.045*
	P4 promoter	1.06 ± 1.29	1.53 ± 2.43	0.724
	Distal promoter	55.31 ± 9.12	52.48 ± 6.14	0.483
PGC1α	Proximal promoter	1.03 ± 0.96	1.16 ± 0.94	0.947
	Exon 1 to Intron 1	5.71 ± 1.12	5.61 ± 1.38	0.683
PDK4	Promoter	0.85 ± 0.58	0.69 ± 0.61	0.445
TCF7L2	5' UTR	0.04 ± 0.16	0.03 ± 0.15	0.638
	5'UTR to promoter	0.11 ± 0.24	0.02 ± 0.09	0.063

HA, high active; LA, low active; *PPAR-γ*, peroxisome proliferator-activated receptor-gamma; *IGF2*, insulin-like growth factor 2; *PGC1-α*, peroxisome proliferator-activated receptor-gamma coactivator; *PDK4*, pyruvate dehydrogenase kinase, isozyme 4; *TCF7L2*, transcription factor 7-like 2.
Percentage methylation across all gene regions within five candidate genes. Data are presented in mean ± standard deviation for each region of the genes. Mann–Whitney U-test *P*-values are presented ($\alpha=0.05$).
*Significant difference between the two groups.

Discussion

We found no effect of physical activity during pregnancy on offspring global DNA methylation. Others have examined the role of chronic physical activity on global methylation in non-pregnant populations; they examined this relationship at an

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Table 4. Insulin-like growth factor 2 (*IGF2*) methylation

Promoters	CpG	HA	LA	*P*-value
P2	CpG1	73.30 ± 4.84	74.66 ± 4.32	0.431
P2	CpG2	87.22 ± 5.24	86.29 ± 4.35	0.393
P2	CpG3	52.98 ±6.67	57.95 ± 5.61	0.020*
P2	CpG4	69.86 ± 3.95	72.35 ± 2.89	0.047*
P2	CpG5	80.78 ± 3.82	82.23 ± 2.63	0.182
P2	CpG6	39.87 ± 3.76	41.68 ± 3.71	0.165
P2	CpG7	98.19 ± 2.41	98.00 ± 2.23	0.581
P2	CpG8	60.05 ± 2.23	60.77 ± 1.75	0.423
P4	CpG1	1.42 ± 1.49	2.10 ± 4.97	0.540
P4	CpG2	1.91 ± 5.24	1.86 ± 3.91	0.604
P4	CpG3	0.30 ± 1.11	1.39 ± 3.67	0.137
P4	CpG4	1.45 ± 2.47	1.43 ± 3.64	0.370
P4	CpG5	0.21 ± 0.78	0.86 ± 2.61	0.477

HA = high active; LA = low active.
Percentage methylation for *IGF2* at each CpG site (column 2) within each studied gene region (column 1). Data are presented in mean ± standard deviation for each group. Mann–Whitney U-test *P*-values are presented (α = 0.05).
*Significant difference between the two groups.

Table 5. Correlation coefficients for leisure-time physical activity (LTPA) and candidate genes

Genes	Gene region	Correlation coefficient	*P*-value
PPARγ	Promoter	0.089	0.574
	Intron 2	−0.008	0.958
IGF2	P2 promoter	−0.176	0.266
	P4 promoter	−0.057	0.720
	Distal promoter	0.165	0.302
PGC1α	Proximal promoter	−0.060	0.706
	Exon 1 to Intron 1	0.005	0.973
PDK4	Promoter	0.110	0.489
TCF7L2	5'UTR	0.058	0.713
	5'UTR to promoter	0.155	0.327

PPAR-γ, peroxisome proliferator-activated receptor-gamma; *IGF2,* insulin-like growth factor 2; *PGC1-α,* peroxisome proliferator-activated receptor-gamma coactivator; *PDK4,* pyruvate dehydrogenase kinase, isozyme 4; *TCF7L2,* transcription factor 7-like 2.
Spearman correlation coefficients between gene regions and LTPA per week. *P*-values are presented (α = 0.05).

individual (not offspring) level only. White *et al.*[31] found that in adult women, higher physical activity levels were associated with higher levels of *LINE-1* methylation in peripheral blood samples. Sample size was $n = 647$ and the median physical activity level in the study was 12.5 h (750 min) per week. The authors adjusted for age, smoking, alcohol consumption and BMI but none of the variables affected the statistical model. Another group found the same positive relationship between DNA methylation and physical activity in 165 participants, but the relationship was attenuated after adjustment for confounders including race, gender and age.[33] However, physical activity levels even in the most active group were low, with the most active group participating in no more than 30 min per day of physical activity. In contrast, physical activity in Swedish older adults ($n = 1016$, all age 70 years) was associated with lower levels of methylation[34] There was no adjustment for confounders. In all of these studies, methylation levels were measured in the individuals who were physically active (or inactive) themselves, but in the present study, methylation levels were only measured in the offspring of those pregnant women who were active/inactive. Unfortunately, we did not collect maternal blood samples for all participants upon study enrollment and thus could not analyze methylation in the pregnant women themselves.

Despite previous results that helped formulate our hypotheses, we found few differences in DNA methylation among groups, and those we did find were the opposite of what was expected. For *IGF2*, we expected the offspring of the active group to have the highest DNA methylation, because *IGF2* is involved in growth regulation, and we know that active women tend to have smaller babies (albeit within a healthy range) compared with sedentary women,[35] particularly if they are active during the second half of pregnancy.[36,37] In the present study, LTPA data were collected in early pregnancy and therefore we do not have data on later pregnancy LTPA in our sample.

Research on dietary folate and its relationship to *IGF2* methylation may provide some insight into the mechanism at play in this study. High *IGF2* methylation should, at least theoretically, result in less gene expression and therefore less growth. A study of folate supplementation in pregnancy resulted in higher methylation levels of *IGF2* in offspring cord blood, suggesting that nutrition and perhaps other maternal lifestyle factors are influencing this gene's methylation status in offspring.[13] However, that same study found no relationship between dietary levels of folate and *IGF2* methylation, suggesting that other factors in addition to dietary consumption may influence folate levels in the blood. Hoyo *et al.*[38] found that folic acid intake both before and during pregnancy was associated with lower methylation of a differentially methylated region near *IGF2*. As *IGF2* dysregulation has been suggested to lead to chronic disease, and colon cancer in particular,[39,40] it is plausible that high levels of physical activity decrease methylation of *IGF2* which then in turn increases expression of the gene and provides a protective effect from chronic disease. This hypothesis, however, suggests that methylation changes in a mother do indeed translate to the fetus, which we do not necessarily know to be the case.

IGF2 is a maternally imprinted gene, and thus the maternal copy of the gene is silenced via methylation. As the known regulation of this gene via methylation and its role in growth, we were surprised to find effects of maternal physical activity on *IGF2* methylation in offspring at only one promoter. Hopkins *et al.* found that exercise training in pregnancy was not related to maternal serum *IGF1* or *IGF2* concentrations, which may mean that exercise is an insufficient stimulus to alter this particular gene's expression.[40] The effect of LTPA at the P2 promoter may have been insufficient to be registered as an overall change in *IGF2* hormone in serum, consistent with the negative finding of Hopkins *et al.* However, this chromosome region has been implicated in other research as a regulator of physical activity in mice[18,41] and in humans.[19] Leamy *et al.* found that a region of the genome on chromosome 11 is regulated by mouse running distance; this is the same region where *IGF2* is found. Moreover, in

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humans, *IGF2* genotype has been shown to be related to grip strength,[42] suggesting a role in muscle mass development leading to strength. Though we cannot exclude the possibility that a mother's genetic (epigenetic) profile is being passed on, our data suggest that maternal physical activity during pregnancy may induce a programming effect on her child that could predispose him/her to be physically active. As fetal and adult methylation patterns do not always coincide,[10] it is not clear how altered *IGF2* methylation in offspring of active women might impact long-term health of said offspring. The role of *IGF2* in growth over time must be explored in further research.

It is plausible that our results might have been different had we measured mRNA levels (indicative of gene transcription) rather than methylation. One study measured both gene expression and DNA methylation in relation to physical activity. Barres *et al.*[16] examined DNA methylation and gene expression of *PGC1-α*, *PDK4* and *PPAR-δ*, in skeletal muscle samples, and they found the expected inverse relationship between methylation and expression for each of these three genes. However, a 3-week exercise training intervention produced no effect on *PGC1-α* DNA methylation but did find increased gene expression. The acute physical activity exposure of the Barres *et al.* study might have different effects than the habitual exposure we studied.

Another possible explanation for lack of differences between groups is that the methylation profile may not transfer from mother to child. We found no studies showing heritability of methylation patterns related to maternal physical activity, even in animal models. Based upon the DOHaD hypothesis, however, it would be expected that such a stimulus might impact offspring health risk by transfer of methylation patterns. Other lifestyle-related stimuli have been shown to induce methylation changes that are passed to offspring. These include smoking (after 18 weeks gestation),[43] mother's BMI[44] and folate intake.[13]

Finally, the physical activity stimulus in this study may have been insufficient to elicit change in DNA methylation in the mother herself, or the timing of measurement, in early pregnancy, may not have been sensitive enough to represent the woman's LTPA throughout the entirety of her pregnancy. The average woman in our HA group reported over 600 min per week of LTPA, and of those participants, eight of 14 reported significant amounts of vigorous physical activity (i.e. more than 1 day per week). In one study that did find a relationship between higher activity and more global methylation, the physical activity stimulus used in our study would be equivalent to their second lowest quartile of activity;[31] the highest active group reported more than 18 h per week of activity. It may be, then, that the total amount of activity was inadequate, or more likely, that the intensity was not great enough to elicit changes in global methylation profiles of the mother and therefore not in her offspring. Still, we did find an effect of maternal LTPA at a genome location that has been associated with physical activity (distance and duration) in mice.[18] This association suggests that the *IGF2* methylation status in humans may be important for long-term health because of a potential role in physical activity regulation.

It is worth noting that the LA group in our study reported an average of ~60 min LTPA per week. Although they did not meet recommendations for physical activity from the Physical Activity Guidelines for Americans (health.gov 2008), they did report doing some activity. Ideally we would have compared highly active women to those who performed no LTPA during pregnancy. However, other researchers have argued that the preferred control group for a high activity model is low as opposed to no activity, given that a LA individual is more representative of naturally occurring activity patterns.[45]

There are several limitations to this study that should be addressed in future research. First, sample size was small. A power analysis for this study was not computed because no human data of a similar nature have been published to date. However, others have performed similar studies using small samples using animal models. For example, Aagaard-Tillery *et al.* found that in age- and weight-matched adult female Japanese macaques, a high-fat diet resulted in an alteration of the fetal chromatin structure.[46] The authors compared 10 experimental primates with nine controls, smaller than our sample size. Thus, we believed that an exploratory analysis of 14 active and 28 controls would provide valuable pilot data on the relationships of interest. Still, there is a possibility that a Type II error was made when we concluded no difference for all gene targets except for *IGF2*, and thus the data should be interpreted cautiously.

The issue of comparative methylation of maternal and offspring DNA could be addressed by taking a maternal blood sample at delivery to compare with infant heel stick blood. Moreover, interesting would be comparisons among maternal, placental and newborn DNA methylation. We also acknowledge that physical activity data were based upon recall in the previous month. However, physical activity recall 6 years *postpartum* has been shown to correlate reasonably well ($r = 0.57–0.86$) to questionnaire data collected during pregnancy.[47] Finally, our conclusions are limited to DNA methylation only in the peripheral blood of infants. Methylation is tissue-specific and therefore it is possible that our results would be different if we had examined another tissue (i.e. placenta or muscle).

We have concluded that this is the one of the first studies to examine the effects of maternal pregnancy physical activity on offspring DNA methylation in humans. Though we found no differences in global methylation levels in offspring of highly active compared with considerably less active women, we have been able to highlight important issues to address in future research in this area. Particularly, future research should focus on understanding how pregnancy physical activity influences the P2 promoter in the *IGF2* gene, and whether offspring *IGF2* methylation status affects growth patterns and/or physical activity levels in childhood, and ultimately long-term chronic disease risk. In the present study, the groups were relatively well matched for known confounders, so future work can emphasize selection of appropriate tissue type for analysis and adequate physical activity stimulus to further understand this issue and whether/how pregnancy LTPA might influence offspring susceptibility to non-communicable disease.

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Conflicts of Interest. None.

Ethical Standards. The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guidelines on human experimentation (United State Department of Health and Human

Services 45 CFR part 46) and with the Helsinki Declaration of 1975, as revised in 2008, and has been approved by the institutional committees (Michigan State University Institutional Review Board and Michigan Department of Health and Human Services).

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