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# Recreational physical activity before and during pregnancy and placental DNA methylation—an epigenome-wide association study

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## ABSTRACT

**Background:** Physical activity (PA) prior to and during pregnancy may have intergenerational effects on offspring health through placental epigenetic modifications. We are unaware of epidemiologic studies on longitudinal PA and placental DNA methylation.

**Objectives:** We evaluated the association between PA before and during pregnancy and placental DNA methylation.

**Methods:** Placental tissues were obtained at delivery and methylation was measured using HumanMethylation450 Beadchips for participants in the Eunice Kennedy Shriver National Institute of Child Health and Human Development Fetal Growth Studies—Singletons among 298 participants. Using the Pregnancy Physical Activity Questionnaire, women recalled periconception PA (past 12 mo) at 8–13 wk of gestation and PA since last visit at 4 follow-up visits at 16–22, 24–29, 30–33, and 34–37 wk. We conducted linear regression for associations of PA at each visit with methylation controlling for false discovery rate (FDR). Top 100 CpGs were queried for enrichment of functional pathways using Ingenuity Pathway Analysis.

**Results:** Periconception PA was significantly associated with 1 CpG site. PA since last visit for visits 1–4 was associated with 2, 2, 8, and 0 CpGs (log fold changes ranging from –0.0319 to 0.0080, after controlling for FDR). The largest change in methylation occurred at a site in *TIMP2*, which is known to encode a protein critical for vasodilation, placentation, and uterine expansion during pregnancy (log fold change: –0.05; 95% CI: –0.06, –0.03 per metabolic equivalent of task–h/wk at 30–33 wk). Most significantly enriched pathways include cardiac hypertrophy signaling, B-cell receptor signaling, and netrin signaling. Significant CpGs and enriched pathways varied by visit.

**Conclusions:** Recreational PA in the year prior and during pregnancy was associated with placental DNA methylation. The associated CpG sites varied based on timing of PA. If replicated, the findings may inform the mechanisms underlying the impacts of PA on placenta health. This study was registered at [clinicaltrials.gov](https://clinicaltrials.gov) as NCT00912132. *Am J Clin Nutr* 2022;116:1168–1183.

**Keywords:** physical activity (PA), maternal exercise, epigenome-wide association study, DNA methylation, fetal programming,

epigenetics, intrauterine exposure, pregnancy health, prospective cohort, placenta

## Introduction

Recreational physical activity (PA) during pregnancy has been associated with multiple health benefits by improving physiologic, metabolic, and psychological function (1). Specific benefits, for instance, include enhanced maternal cardiorespiratory function (2) and reduced risk of excessive weight gain, gestational diabetes, preeclampsia, and cesarean delivery (3). Maternal PA during pregnancy may thereby be related to

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The placental genome-wide DNA methylation, gene expression, and genotype data are available through dbGaP with accession number phs001717.v1.p1. All other relevant data are available within the manuscript and its Supporting Information files.

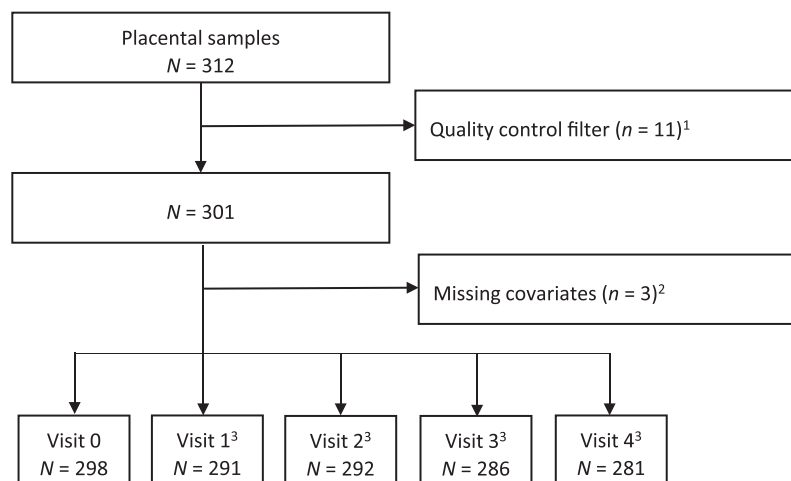
Supplemental Tables 1–4 and Supplemental Figure 1 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/ajcn/>.

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Abbreviations used: FDR, false discovery rate; MET, metabolic equivalent of task; NICHD, National Institute of Child Health and Human Development; PA, physical activity; PC, principal component; PPAQ, Pregnancy Physical Activity Questionnaire; RNA-seq, RNA sequencing.

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**FIGURE 1.** Flowchart for determining analytical cohort for each visit. <sup>1</sup>Details regarding sex and sample identifier mismatch are previously described (25). <sup>2</sup>Missing marital status ( $n = 1$ ), employment ( $n = 1$ ), or prepregnancy BMI ( $n = 1$ ). <sup>3</sup>Sample size varied due to missing physical activity questionnaire.

favorable fetal, neonatal, and childhood health outcomes in the offspring (4–6), although the underlying molecular mechanisms between maternal PA and offspring health remain unclear. Emerging data from *in vivo* and animal studies point to a role for epigenetic changes in placenta (7, 8).

The placenta not only mediates all exchanges between the fetus and mother but also controls the hormonal environment of pregnancy and performs essential physiologic functions (9, 10). Despite being a transient organ with the life span of the gestation it supports, the placenta can affect the offspring's life course through fetal programming (11). Placental development and function are controlled partly by gene expression via epigenetic modifications such as DNA methylation (12). Mice studies suggest that PA before pregnancy can facilitate the maintenance of epigenetic modifications in favor of later embryo development *in vitro* (7). PA before and throughout pregnancy can improve epigenetic regulation of a metabolic master regulator in skeletal muscles for neonatal and 12-mo-old offspring mice exposed to a maternal high-fat diet (13).

Human studies on maternal PA and offspring DNA methylation are scarce. One study using cord blood and another using newborn blood spots identified a handful of methylation differences in imprinting control regions known to be important for fetal development (14, 15) in relation to PA in pregnancy. Both studies mainly assessed PA levels once at enrollment (around the end of the first trimester), capturing different periods before and during early pregnancy, which is an incomplete assessment because women may alter their PA patterns, and time windows beyond the first trimester may be important. Furthermore, neither study measured DNA methylation directly in placental tissue. In the present study, we examined the associations between longitudinal assessments of recreational PA in the year prior and throughout pregnancy and epigenome-wide methylation in placenta among a racially and ethnically diverse cohort.

## Methods

### Study population

The Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Fetal Growth Studies—

Singletons is a prospective cohort established to develop standards for fetal growth (16). Briefly, low-risk participants with ultrasound-confirmed gestational age (between 8 wk and 0 d and 13 wk and 6 d) were enrolled between July 2009 and January 2013 from 12 clinical sites in the United States (17). At enrollment, participants were randomly allocated to 1 of 4 ultrasonography schedules for longitudinal follow-up at 4 visits during which in-person interviews and anthropometric assessments were conducted (18). All participants were followed to delivery. This analysis includes 298 participants with placental DNA methylation data, no missing covariates, and longitudinal measures of PA (Figure 1). This study was approved by institutional review boards at NICHD (IRB number: 09-CH-N152) and each participating clinical site and coordinating center.

### Physical activity measures

Using the Pregnancy Physical Activity Questionnaire (PPAQ) (19), women recalled habitual PA in the periconception period and early pregnancy (past 12 mo, inclusive of first trimester), as well as PA during 8–22, 16–29, 24–33, 30–37, and 34–41 wk of gestation (Supplemental Table 1). These data were obtained at study visits that occurred at enrollment (8–13 wk) (baseline visit) and at targeted follow-up visits at 16–22 (visit 1), 24–29 (visit 2), 30–33 (visit 3), and 34–37 (visit 4) wk. This recall pattern ensured that PA at all time periods before and during pregnancy was captured.

Based on the Compendium of Physical Activity (20), we obtained the estimated energy required for each of the 9 recreational activities reported [in metabolic equivalent of task (MET); Supplemental Table 2]. Total recreational MET-h/wk was obtained by incorporating the MET score and amount (h/wk) for each activity and then summing across activities. Total physical activity was determined *a priori* as our primary exposure. Intensity of activities included light (MET  $\geq 1.5$  and  $< 3.0$ ), moderate (MET  $\geq 3.0$  and  $\leq 6.0$ ), and vigorous (MET  $> 6.0$ ) activities. The American College of Obstetricians and Gynecologists has issued recommendations regarding the benefits of moderate physical activity and encouraged women habitually engaged in vigorous activities to continue during pregnancy (21);

as such, we focus our secondary analyses on activities falling into these intensities. Although some participants performed vigorous activities prior to and during early pregnancy ( $n = 170$ ), few reported any vigorous activities at visit 4 ( $n = 64$ ); we therefore combined METs from moderate and vigorous activities as secondary exposure for analyses. We conducted logic checks for extreme values of all PA measures and did not exclude any observations based on activity levels.

### Placental DNA methylation and gene expression

Placental samples were collected at delivery following a detailed standardized protocol to ensure specimens were comparable and processed within 1 h. More specifically, after the free membranes were trimmed off the placental disc, the cord was cut from its attachment at the insertion site, and the placental disc was inspected, imaged, and sliced transversely at 1- to 2-cm intervals. This uniformed approach resulted in placental parenchymal biopsy specimens measuring  $0.5 \times 0.5 \times 0.5$  cm obtained from the fetal side within 1–2 cm from the cord insertion site. Biopsy samples were placed in RNALater and frozen for molecular analysis, as previously described (22). To profile methylation, extracted DNA was assayed using Infinium HumanMethylation450 Beadchips (Illumina). As our primary outcome, methylation fractions (ratio of methylated and unmethylated signals) were calculated at each CpG site using Genome Studio. To better meet model assumptions (23), methylation fractions ( $\beta$  values) were logit transformed to  $M$  values before analysis. Samples that did not pass quality control filters ( $n = 11$ ) were excluded (24, 25). A total of 409,101 CpGs were available for the analysis (Figure 1).

To quantify RNA in placental samples, RNA was isolated using TRIZOL reagent (Invitrogen) and sequenced using the Illumina HiSeq2000 system. The expression of transcripts was quantified using Salmon, which accounts for experimental attributes and biases (26). Participants with RNA sequencing (RNA-seq) and DNA methylation ( $n = 80$ ) were included in correlation tests between DNA methylation and nearby gene expression levels.

### Covariate measures

Participants self-reported sociodemographic characteristics, reproductive and medical history, and behavioral and lifestyle factors at enrollment. We adjusted for covariates that were related to PA, including maternal age (years), self-identified race/ethnicity (non-Hispanic White, non-Hispanic Black, Hispanic, Asian or Pacific Islander), prepregnancy BMI (in  $\text{kg}/\text{m}^2$ ), parity (0, 1, 2+), education level (high school or less, some college or associate degree, bachelor or advanced degree), marital status (not married, married or living with partner), employment status (not employed, has at least 1 job, or is a full-time student), prepregnancy alcohol use (never, 3 times a month or less, once a week or more), and periconception sleep duration (6 h or less, 7–8 h, 9 h or more). Prepregnancy BMI was calculated from self-reported weight and height, which were highly correlated with weight and height measured by study personnel during enrollment (27). Three participants missing covariates were excluded, resulting in a total of 298 women included in this study (Figure 1).

### Statistical analyses

To test if total recreational PA (MET-h/wk) prior to and during early pregnancy differed by maternal and infant characteristics, we used the Kruskal–Wallis test to compare the differences in medians. Epigenome-wide associations were performed using linear regression to estimate log fold change in DNA methylation for each additional MET-h/wk at each visit. We assessed differential methylation across 409,101 CpGs using the R package limma [i.e., lmFit function for model fitting (least squares) and eBayes function for model summarization (default options; assuming the proportion of genes differentially expressed is 0.01), version 3.48.1] (28). All analyses controlled for false discovery rate (FDR) at 5% at each visit. Analyses controlled for FDR at each visit based on *a priori* hypothesis that the associations can vary by study visits given the dynamics of placental and fetal development during gestation, which was also one of the reasons that longitudinal data on PA were collected across multiple study visits by design. Analyses were performed for moderate recreational PA and moderate or vigorous recreational PA at each visit ( $n = 6$ ).

For all analyses described above, we adjusted for *a priori* covariates, as well as infant sex, 5 methylation plate number (categorical), the first 3 principal components (PCs) from the samples' percent methylation profile using R package pcomp (continuous) (29), and the first 10 genotype-based PCs (continuous) from multidimensional scaling analysis of a pruned set of uncorrelated genome-wide single-nucleotide polymorphisms generated using PLINK to account for population structure (30). Heterogeneity in cell-type composition was accounted for using surrogate variables, as previously described (25). The optimal number of surrogate variables was identified using the “be” method, a permutation-based approach first applied to expression data in 2007 (31), in R package sva (version 3.40.0). Between 20 and 25 surrogate variables were used in analyses related to visits 0–4. Statistical analyses were conducted in SAS (version 9.4; SAS Institute) and R Studio (version 1.3.1073).

In addition, we mapped the top differentially methylated CpG sites to the nearby gene and examined enrichment of molecular pathways using the Ingenuity Pathway Analysis software (IPA; Qiagen). For each visit, the top 100 CpGs (based on FDR-adjusted  $P$  values) that could be mapped to a nearby gene were imported into the proprietary IPA Knowledge Base. If multiple CpGs were tied for the top 100, ties were included if the total number of CpGs did not exceed 500. Using the Core Analysis function in IPA, statistically significant overrepresented canonical pathways were determined by Fisher exact test followed by adjustment for multiple testing using the Benjamini–Hochberg method (32). In the subset of women with both RNA-seq and DNA methylation ( $n = 80$ ), we also estimated the correlation between DNA methylation at significant CpG sites and the mRNA gene expression levels of the annotated genes using Spearman correlations. The analytic codes are available upon request.

### Results

Among 298 participants, most (96%) reported performing recreational PA at baseline, with a median of 11.7 (IQR: 4.1, 21.7) MET-h/wk. The typical women reported engaging in 1.5 h/wk of walking slowly, 0.75 h/wk of walking quickly, 0.25 h/wk

**TABLE 1.** Total recreational PA at baseline and its association with maternal/infant characteristics ( $n = 298$ )<sup>1</sup>

Characteristic	<i>n</i>	PA (MET-h/wk), median (IQR)	<i>P</i> value
Age, <sup>2</sup> y			0.24
18–25	95	8.7 (3.6, 19.6)	
25–34	173	12.5 (4.3, 22.7)	
35–40	30	12.1 (6.0, 23.4)	
Race/ethnicity			<0.01
Non-Hispanic White	76	17.4 (10.9, 27.8)	
Non-Hispanic Black	71	8.3 (3.1, 18.8)	
Hispanic	101	9.7 (3.6, 19.2)	
Asian/Pacific Islander	50	9.4 (2.4, 16.1)	
Prepregnancy BMI, <sup>2,3</sup> kg/m <sup>2</sup>			0.04
Normal weight (19.0–24.9)	194	12.5 (4.7, 24.1)	
Overweight (25.0–29.9)	75	11.0 (3.1, 19.8)	
Obese ( $\geq 30.0$ )	29	8.1 (3.4, 11.7)	
Parity (number of births)			0.01
0	138	13.4 (4.2, 27.0)	
1	108	11.3 (5.5, 18.4)	
$\geq 2$	52	9.0 (2.5, 15.9)	
Education level			<0.01
$\leq$ High school	91	6.3 (2.6, 11.8)	
Some college/associate degree	86	11.4 (4.5, 20.9)	
$\geq$ Bachelor degree	121	16.1 (9.6, 26.2)	
Marital status			0.09
Other	73	8.7 (3.6, 17.4)	
Married/living as married	225	12.5 (4.3, 22.2)	
Employment status			0.02
Unemployed	89	8.1 (3.6, 17.1)	
Employed/student	209	12.5 (4.7, 23.5)	
Alcohol use pre-pregnancy			<0.01
Never	105	5.9 (2.0, 12.6)	
<3 times a month	104	11.4 (5.2, 20.9)	
$\geq$ Once a week	89	18.7 (11.7, 29.1)	
Periconception sleep duration			0.12
$\leq 6$ h	55	13.3 (5.8, 23.8)	
7–8 h	196	11.5 (3.9, 21.3)	
$\geq 9$ h	47	8.6 (2.3, 20.8)	
Infant sex			0.76
Male	150	11.6 (4.3, 22.0)	
Female	148	11.7 (3.7, 20.9)	
Birth weight			0.15
<2500 g	22	7.2 (2.6, 17.7)	
$\geq 2500$ and <4000 g	264	11.8 (4.2, 22.1)	
$\geq 4000$ g	12	9.3 (3.0, 11.8)	
Physical activity			
8–13 wk of gestation (baseline)	298	11.7 (4.1, 21.7)	
16–22 wk of gestation (visit 1)	291	5.2 (1.9, 11.7)	
24–29 wk of gestation (visit 2)	292	5.3 (2.4, 10.4)	
30–33 wk of gestation (visit 3)	286	4.8 (2.0, 11.0)	
34–37 wk of gestation (visit 4)	281	4.3 (1.1, 10.3)	

<sup>1</sup>*P* values from Kruskal–Wallis test of differences in median physical activity at visit 0 (8–13 wk of gestation,  $n = 298$ ). MET, metabolic equivalent of task; PA, physical activity.

<sup>2</sup>Categorized for this table but modeled as continuous variables.

<sup>3</sup>Calculated from self-reported height (meters) and pregravid weight (kilograms). No women were underweight (BMI <18.9 kg/m<sup>2</sup>).

of walking quickly up hills, and 0.75 h/wk of other activities for fun or exercise. The median amount of recreational PA was 5.2 (IQR: 1.9, 11.7), 5.3 (IQR: 2.4, 10.4), 4.8 (IQR: 2.0, 11.0), and 4.3 (IQR: 1.1, 10.3) MET-h/wk for visits 1–4, respectively. Total recreational PA differed by race/ethnicity, with higher levels reported by non-Hispanic Whites than other races/ethnicities.

Greater recreational PA was also associated with having a BMI between 19.0 and 24.9, being nulliparous, having a higher education, being employed, and drinking alcohol more than once a week pre-pregnancy (Table 1).

Total recreational PA was significantly associated with placental DNA methylation for 13 CpG sites (1 at visit

0, 2 at visit 1, 2 at visit 2, 8 at visit 3, and none at visit 4) (**Table 2**; quantile–quantile plots in **Supplemental Figure 1**). Except for 1 CpG (cg06265277), all significant sites were associated with decreased methylation. Several CpG sites, including cg14517108 (*TIMP2*), cg05580512 (*LPCATI*), cg21707521 (*UBE2F*), cg25970266 (*CSK*), cg05074892 (*BRD2*), cg10965041 (*EYA3*), and cg06100552 (*MAGII*), were consistently among the top 100 CpG sites at multiple visits in association with PA. The largest decrease in methylation with respect to higher PA occurred at a site located in a CpG island of *TIMP2* (log fold change:  $-0.05$ ; 95% CI:  $-0.06$ ,  $-0.03$  per MET-h/wk at visit 3). Methylation at one of the sites identified (cg16141228 near *CLINT1*) was negatively correlated with *CLINT1* gene expression in placenta (Spearman  $r = -0.34$ ,  $P = 0.004$ , **Supplemental Table 3**). Methylation at other CpGs was not correlated with gene expression.

Moderate recreational PA was also significantly associated with methylation at 15 CpG sites (1 at visit 0, 11 at visit 1, 1 at visit 2, 2 at visit 3, and none at visit 4) (**Table 3**). Only 1 CpG overlapped with the 13 CpGs associated with total recreational PA (i.e., cg16141228 near *CLINT1*). For cg16141228 (*CLINT1*), higher amounts of both total and moderate PA were associated with decreased methylation at visit 1, and its methylation level was negatively correlated, as described above.

The number of participants performing vigorous activities was small (ranging from 170 at visit 0 to 64 at visit 4). When moderate or vigorous recreational PA was combined, 29 CpGs were identified (1 at visit 0, 20 at visit 1, 2 at visit 2, and 6 at visit 3) (**Table 4**). Eleven of the 13 CpGs identified with total recreational PA overlapped with these 29 CpGs. Of note, cg16141228 (*CLINT1*) was identified regardless of recreational PA intensity modeled. In addition to cg16141228 (*CLINT1*), methylation at cg07424889 near *FOXK1* was negatively correlated with *FOXK1* expression ( $r = -0.29$ ,  $P = 0.015$ ; Supplemental Table 3).

Ten pathways were significantly enriched for genes located near the top 100 CpG sites for total recreational PA (**Table 5**). All 10 pathways were identified at visit 0 (i.e., habitual PA in the periconception period and early pregnancy). Three pathways were tied for being most significant: cardiac hypertrophy signaling (enhanced), B-cell receptor signaling, and netrin signaling. For moderate recreational PA, no pathways were significantly enriched after adjustment for multiple testing (results not shown). For moderate or vigorous recreational PA, 32 pathways were enriched, with 10 identified at visit 0, none at visit 1, 22 at visit 2, none at visit 3, and none at visit 4 (**Table 6**). Among these, the aforementioned cardiac hypertrophy signaling (enhanced) was also the most significant pathway.

## Discussion

In a cohort of women from diverse race/ethnicity groups with longitudinal PA measures from periconception through pregnancy, recreational PA before and during pregnancy was significantly associated with several DNA methylation sites in placenta. Importantly, these associations differed by timing of PA. Furthermore, pathway analysis suggested the impact of PA on placental DNA methylation likely occurs through pathways related to cardiovascular system development and function, cellular development, and skeletal and muscular system development and function.

To the best of our knowledge, we are unaware of studies on PA and placental epigenetic modifications. Biological links have been suggested by previous studies examining epigenetics using blood samples [i.e., cord blood (14) or offspring blood spots (15) at delivery] based on candidate gene approaches. For example, total maternal PA in early pregnancy has been associated with decreased methylation at a differentially methylated region of *PLAGL1*, which is a key regulator of placental and fetal growth (14, 33). No studies have examined methylation in placental tissues using an epigenome-wide association study approach, nor have prior studies assessed PA beyond enrollment (in the first half of pregnancy) (14, 15).

Although the precise underlying mechanisms of the association between PA and placental DNA methylation remain elusive, several lines of evidence suggest the observed associations are biologically plausible. A recent study found that maternal PA could induce the secretion of a placenta-derived protein (i.e., superoxide dismutase 3) that, in turn, regulated glucose metabolic genes in mice fetal liver (8). Maternal PA has also been linked to placentation (34, 35), placental development and growth (36, 37), and placental blood flow to the fetus (38, 39). Given these prior findings, we anticipated maternal PA would influence genes associated with placental function. In our study, the methylation site with the largest fold change was cg14517108, located on a CpG island of *TIMP2*, which is previously known to encode a protein critical for vasodilation, placentation, and uterine expansion during normal pregnancy (40). However, based on experimental data available in the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org), version 21.0) (41), protein expression of *TIMP2* was not detected in decidual or trophoblastic cells (**Supplemental Table 4**).

Other genes significantly associated with total recreational PA were related to cell growth and differentiation, as well as cell assembly and organization. *MOB3B* plays a pivotal role in organ size control and tumor suppression by restricting proliferation and promoting apoptosis (42). *LPCATI* exhibits acyltransferase activity and may synthesize phosphatidylcholine in pulmonary surfactant, thereby playing a role in respiratory physiology (43, 44). *UBE2F* interacts with the E3 ubiquitin ligase and is involved in protein neddylation (45). *CSK* plays an important role in the regulation of cell growth, differentiation, migration, and immune response (46, 47). A meta-analysis shows that *CSK* polymorphism is associated with higher systolic and diastolic blood pressure in Asian populations (48). *C1orf25* may play a role in motor coordination and exploratory behavior and may be involved in postnatal neuronal functions (49). *BRD2* may play a role in spermatogenesis or folliculogenesis and nucleosome assembly, as well as regulate the transcription of other genes (50). *CLINT1* binds to membranes and may have a role in transporting vesicles from the *trans*-Golgi network to endosomes (51). *EYA3* promotes efficient DNA repair and regulates transcription during organogenesis and may be involved in the development of the eye (52, 53). *KPNA4* functions in nuclear protein import as an adapter protein for nuclear receptor and may be involved in cataract formation (54). *RTN3* induces the formation of endoplasmic reticulum tubules (55–57). *MAGII* may play a role as scaffolding protein at cell–cell junctions with tumor suppressive and vascular functions (58).

The most significant pathway for the link between both total and moderate or vigorous recreational PA and placental

**TABLE 2.** Significant CpG sites and nearby annotated genes associated with total recreational physical activity across visits<sup>1</sup>

CpG	Gene	Chromosome	Position	Relation to island	logFC <sup>2</sup>	95% CI	P value	FDR-adjusted P value	Visit number <sup>3</sup>
cg14122102	<i>MOB3B</i>	chr9	27333402	OpenSea	-3.19E-02	-4.15E-02, -2.23E-02	3.53E-10	1.45E-04*	0
cg14517108	<i>TIMP2</i>	chr17	76921504	Island	-4.58E-02	-6.00E-02, -3.17E-02	9.07E-10	3.71E-04*	3
cg05580512	<i>LPCAT1</i>	chr5	1463045	N_Shore	-3.85E-02	-5.62E-02, -2.07E-02	2.78E-05	5.24E-01	4
cg21707521	<i>UBE2F</i>	chr2	238875814	Island	-1.31E-03	-7.92E-03, -2.71E-03	7.64E-05	3.43E-01	0
cg25970266	<i>CSK</i>	chr15	75074299	N_Shore	-1.18E-02	-1.56E-02, -7.96E-03	4.73E-09	9.67E-04*	3
cg07176514	<i>C1orf25</i>	chr1	185126191	Island	-1.61E-02	-2.33E-02, -8.80E-03	1.98E-05	1.42E-01	1
cg05074892	<i>BRD2</i>	chr6	32939292	Island	-1.57E-02	-2.32E-02, -8.22E-03	5.03E-05	3.29E-01	2
cg16141228	<i>CLINT1</i>	chr5	157286335	S_Shore	-2.24E-02	-3.02E-02, -1.45E-02	5.50E-08	7.49E-03*	3
cg10965041	<i>EYA3</i>	chr1	28415294	Island	-1.56E-02	-2.29E-02, -8.42E-03	2.86E-05	2.46E-01	0
cg06265277		chr15	30260973	Island	-2.48E-02	-3.65E-02, -1.30E-02	4.74E-05	3.29E-01	2
cg15787616		chr3	137482006	N_Shore	-3.36E-02	-4.59E-02, -2.13E-02	1.73E-07	1.77E-02*	3
cg21087962	<i>KPNA4</i>	chr3	160283382	Island	-4.09E-02	-5.54E-02, -2.64E-02	8.10E-08	2.17E-02*	2
cg02984023	<i>R7N3</i>	chr11	63448764	Island	-1.43E-02	-2.03E-02, -8.22E-03	5.44E-06	1.24E-01	0
					-2.06E-02	-3.01E-02, -1.11E-02	2.80E-05	1.67E-01	1
					-2.66E-02	-3.62E-02, -1.71E-02	1.06E-07	2.17E-02*	2
					-2.44E-02	-3.48E-02, -1.41E-02	5.78E-06	1.08E-01	3
					-2.65E-02	-3.95E-02, -1.34E-02	8.36E-05	5.61E-01	4
					-1.35E-02	-1.83E-02, -8.77E-03	6.26E-08	2.56E-02*	1
					-1.88E-02	-2.59E-02, -1.17E-02	4.09E-07	3.35E-02*	3
					-1.94E-02	-2.86E-02, -1.02E-02	4.42E-05	5.39E-01	4
					7.95E-03	5.04E-03, 1.09E-02	1.74E-07	3.56E-02*	1
					-2.88E-02	-4.18E-02, -1.58E-02	1.83E-05	2.27E-01	2
					-3.54E-02	-4.90E-02, -2.19E-02	5.70E-07	3.89E-02*	3
					-3.36E-02	-4.68E-02, -2.05E-02	9.09E-07	4.69E-02*	3
					-3.25E-02	-4.52E-02, -1.98E-02	9.17E-07	4.69E-02*	3

<sup>1</sup>Results from epigenome-wide associations performed using linear regression, adjusting for maternal age (years), self-identified race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, Asian or Pacific Islander), prepregnancy BMI (in kg/m<sup>2</sup>), parity (0, 1, 2+), education level (high school or less, some college or associate degree, bachelor or advanced degree), marital status (not married, married or living with partner), employment status (not employed, has at least 1 job, or is a full-time student), pregnancy alcohol use (never, 3 times a month or less, once a week or more), periconception sleep duration (6 h or less, 7–8 h, 9 h or more), infant sex, 5 methylation plate number (categorical), the first 3 principal components (PCs) from the samples' percent methylation profile, the first 10 genotype-based PCs to account for population structure, and surrogate variables to account for heterogeneity in cell-type composition. FDR-adjusted P value <0.05 for any visit are marked with \*, and the corresponding logFC and P values for other visits are also presented if among the top 100 CpGs for that visit. Chr, chromosome; FC, fold change; FDR, false discovery rate; N, north; S, south.

<sup>2</sup>LogFC is the log of fold change (base 2); logFC <0 is associated with hypomethylation and logFC >0 is associated with hypermethylation.

<sup>3</sup>Visits 0–4 are targeted for 8–13, 16–22, 24–29, 30–33, and 34–37 wk of gestation, respectively. The sample size for visits 0–4 is 298, 291, 292, 286, and 281, respectively (Figure 1).

**TABLE 3.** Significant CpG sites and nearby annotated genes associated with moderate recreational physical activity across visits<sup>1</sup>

CpG	Gene	Chromosome	Position	Relation to island	logFC <sup>2</sup>	95% CI	P value	FDR-adjusted P value	Visit number <sup>3</sup>
cg07179329	<i>CDH13</i>	chr16	83171992	OpenSea	-5.02E-02	-6.72E-02, -3.32E-02	2.03E-08	8.32E-03*	2
cg17462962	<i>MRPL22</i>	chr5	154347281	OpenSea	9.46E-03	6.09E-03, 1.28E-02	8.28E-08	1.85E-02*	1
cg16141228	<i>CLINT1</i>	chr5	157286335	S_Shore	-1.92E-02	-2.61E-02, -1.23E-02	1.02E-07	1.85E-02*	1
cg26169320	<i>MYCBP2</i>	chr13	77901010	Island	-3.93E-02	-5.36E-02, -2.51E-02	1.35E-07	1.85E-02*	1
cg13179085	<i>SOC52</i>	chr12	93963345	N_Shore	-3.63E-02	-5.32E-02, -1.95E-02	3.07E-05	5.06E-01	4
cg01106412	<i>OSBPL3</i>	chr7	25020404	N_Shore	-4.55E-02	-6.13E-02, -2.96E-02	5.14E-08	2.10E-02*	3
cg09575314	<i>CPNE1</i>	chr20	34220833	OpenSea	-4.17E-02	-5.71E-02, -2.63E-02	2.38E-07	2.44E-02*	1
cg19596493	<i>PTP4A2</i>	chr1	32404086	Island	-2.39E-02	-3.64E-02, -1.14E-02	2.17E-04	7.32E-01	0
cg16364121		chr17	35291924	Island	-8.02E-03	-1.11E-02, -5.00E-03	3.91E-07	3.20E-02*	1
cg11166466	<i>CDON</i>	chr11	125932389	Island	-6.95E-02	-9.60E-02, -4.30E-02	4.95E-07	3.38E-02*	1
cg13392587	<i>NAGLU</i>	chr17	40689561	S_Shore	-5.06E-02	-7.02E-02, -3.11E-02	6.79E-07	3.47E-02*	1
cg22586884	<i>PPAP2A</i>	chr5	54832140	S_Shore	-5.37E-02	-8.17E-02, -3.62E-02	7.02E-07	3.47E-02*	1
cg12425768	<i>KIF1B</i>	chr1	10269828	N_Shore	-9.44E-03	-1.31E-02, -5.78E-03	1.27E-05	2.48E-01	2
cg01440514	<i>NCAPD3</i>	chr11	134094371	Island	1.63E-02	9.97E-03, 2.27E-02	7.64E-07	3.47E-02*	1
cg00384993		chr20	2630663	N_Shelf	1.41E-02	7.58E-03, 2.06E-02	8.94E-07	3.64E-02*	1
					1.65E-02	1.00E-02, 2.30E-02	2.82E-05	3.21E-01	2
					-4.62E-02	-6.44E-02, -2.81E-02	1.02E-06	8.37E-02	3
					-2.23E-02	-3.03E-02, -1.43E-02	9.79E-07	3.64E-02*	1
					1.18E-02	7.50E-03, 1.62E-02	9.27E-08	3.79E-02*	0
							1.95E-07	3.99E-02*	3

<sup>1</sup>Results from epigenome-wide associations performed using linear regression, adjusting for maternal age (years), self-identified race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, Asian or Pacific Islander), prepregnancy BMI (in kg/m<sup>2</sup>), parity (0, 1, 2+), education level (high school or less, some college or associate degree, bachelor or advanced degree), marital status (not married, married or living with partner), employment status (not employed, has at least 1 job, or is a full-time student), prepregnancy alcohol use (never, 3 times a month or less, once a week or more), periconception sleep duration (6 h or less, 7–8 h, 9 h or more), infant sex, 5 methylation plate number (categorical), the first 3 principal components (PCs) from the samples' percent methylation profile, the first 10 genotype-based PCs to account for population structure, and surrogate variables to account for heterogeneity in cell-type composition. FDR-adjusted P value <0.05 for any visit are marked with \*, and corresponding logFC and P values for other visits are also presented if among the top 100 CpGs for that visit. Chr, chromosome; FC, fold change; FDR, false discovery rate; N, north; S, south.

<sup>2</sup>LogFC is the log of fold change (base 2); logFC <0 is associated with hypomethylation and logFC >0 is associated with hypermethylation.

<sup>3</sup>Visits 0–4 are targeted for 8–13, 16–22, 24–29, 30–33, and 34–37 wk of gestation, respectively. The sample size for visits 0–4 is 298, 291, 292, 286, and 281, respectively (Figure 1).



TABLE 4. Significant CpG sites and nearby annotated genes associated with moderate or vigorous recreational physical activity across visits<sup>1</sup>

CpG	Gene	Chromosome	Position	Relation to island	logFC <sup>2</sup>	95% CI	P value	FDR-adjusted P value	Visit number <sup>3</sup>
cg14122102	<i>MOB3B</i>	chr9	27333402	OpenSea	-3.27E-02	-4.24E-02, -2.30E-02	2.12E-10	8.69E-05*	0
cg05580512	<i>LPCAT1</i>	chr5	1463045	N_Shore	-1.18E-02	-1.57E-02, -7.98E-03	5.94E-09	2.38E-03*	3
					-5.43E-03	-8.04E-03, -2.82E-03	5.73E-05	2.42E-01	0
cg14517108	<i>TIMP2</i>	chr17	76921504	Island	-4.34E-02	-5.79E-02, -2.90E-02	1.16E-08	2.38E-03*	3
					-3.69E-02	-5.50E-02, -1.89E-02	7.46E-05	5.71E-01	4
cg06265277		chr15	30260973	Island	8.77E-03	5.86E-03, 1.17E-02	1.03E-08	4.23E-03*	1
cg16141228	<i>CLINT1</i>	chr5	157286335	S_Shore	-1.40E-02	-1.88E-02, -9.19E-03	2.90E-08	5.92E-03*	1
cg21707521	<i>UBE2F</i>	chr2	238875814	Island	-2.22E-02	-3.01E-02, -1.42E-02	1.11E-07	1.51E-02*	3
					-1.73E-02	-2.45E-02, -1.00E-02	4.47E-06	6.76E-02	1
					-1.58E-02	-2.34E-02, -8.28E-03	4.99E-05	3.39E-01	2
cg21087962	<i>KPNA4</i>	chr3	160283382	Island	-3.59E-02	-4.91E-02, -2.27E-02	2.09E-07	1.90E-02*	3
cg25970266	<i>CSK</i>	chr15	75074299	N_Shore	-3.36E-02	-4.60E-02, -2.11E-02	2.55E-07	1.90E-02*	3
					-1.54E-02	-2.27E-02, -8.08E-03	4.61E-05	2.37E-01	0
cg15787616		chr3	137482006	N_Shore	-2.48E-02	-3.65E-02, -1.31E-02	4.47E-05	3.32E-01	2
					-3.68E-02	-5.04E-02, -2.31E-02	2.78E-07	1.90E-02*	3
					-2.94E-02	-4.24E-02, -1.63E-02	1.47E-05	2.28E-01	2
cg05074892	<i>BRD2</i>	chr6	32939292	Island	-1.65E-02	-2.45E-02, -8.52E-03	6.35E-05	2.53E-01	0
					-2.69E-02	-3.65E-02, -1.72E-02	9.73E-08	3.10E-02*	2
					-1.47E-02	-2.08E-02, -8.62E-03	3.41E-06	1.20E-01	0
					-2.11E-02	-3.05E-02, -1.16E-02	1.73E-05	1.29E-01	1
					-2.45E-02	-3.50E-02, -1.39E-02	7.45E-06	1.34E-01	3
					-2.74E-02	-4.06E-02, -1.42E-02	5.82E-05	5.69E-01	4
cg07176514	<i>C1orf25</i>	chr1	185126191	Island	-4.10E-02	-5.59E-02, -2.61E-02	1.51E-07	3.10E-02*	2
cg16586192	<i>APH1B</i>	chr15	63569620	Island	-5.59E-02	-7.66E-02, -3.51E-02	2.60E-07	3.55E-02*	1
					-4.53E-02	-6.77E-02, -2.29E-02	8.83E-05	3.98E-01	2
cg23923070	<i>ZNF638</i>	chr2	71558899	Island	-2.97E-02	-4.13E-02, -1.82E-02	7.54E-07	4.48E-02*	1
cg07368146	<i>NCRNA00219</i>	chr5	111497076	S_Shore	-2.57E-02	-3.57E-02, -1.57E-02	7.96E-07	4.48E-02*	1
cg14673194		chr17	80132900	OpenSea	7.04E-03	3.84E-03, 1.02E-02	9.39E-07	4.48E-02*	1
cg15467911	<i>CHD3</i>	chr17	7791319	S_Shelf	8.07E-03	4.92E-03, 1.12E-02	8.87E-07	4.48E-02*	1
cg22586884	<i>PPAP2A</i>	chr5	54832140	S_Shore	1.14E-02	6.92E-03, 1.58E-02	9.22E-07	4.48E-02*	1
					1.22E-02	7.17E-03, 1.72E-02	2.89E-06	8.61E-02	3
cg06327998	<i>CDYL</i>	chr6	4892662	OpenSea	1.04E-02	5.63E-03, 1.53E-02	2.82E-05	2.89E-01	2
					1.26E-02	6.36E-03, 1.89E-02	9.66E-05	5.90E-01	4
cg10546562	<i>TNRC18</i>	chr7	5390491	N_Shore	6.82E-03	4.15E-03, 9.49E-03	9.85E-07	4.48E-02*	1
cg19893494	<i>NSMCE2</i>	chr8	126162903	OpenSea	5.92E-03	3.06E-03, 8.79E-03	6.32E-05	3.70E-01	2
					-1.61E-02	-2.25E-02, -9.75E-03	1.22E-06	4.58E-02*	1
					-1.03E-02	-1.44E-02, -6.22E-03	1.34E-06	4.58E-02*	1
					-1.14E-02	-1.58E-02, -6.95E-03	8.64E-07	5.05E-02	3
cg04722155	<i>GAK</i>	chr4	851454	OpenSea	1.29E-02	7.76E-03, 1.80E-02	1.40E-06	4.58E-02*	1
cg01920494	<i>STX18</i>	chr4	4543764	Island	-2.96E-02	-4.14E-02, -1.78E-02	1.45E-06	4.58E-02*	1
					-3.27E-02	-4.61E-02, -1.94E-02	2.35E-06	8.02E-02	3
					-2.95E-02	-4.18E-02, -1.71E-02	4.58E-06	1.25E-01	2
					-1.75E-02	-2.51E-02, -9.80E-03	1.10E-05	1.63E-01	0

(Continued)

TABLE 4. (Continued)

CpG	Gene	Chromosome	Position	Relation to island	logFC <sup>2</sup>	95% CI	P value	FDR-adjusted P value	Visit number <sup>3</sup>
cg07424889	<i>FOXP1</i>	chr7	4768247	S_Shelf	-7.68E-03	-1.08E-02, -4.58E-03	1.85E-06	4.83E-02*	1
cg13392587	<i>NAGLU</i>	chr17	40689561	S_Shore	-6.33E-03	-8.89E-03, -3.77E-03	2.01E-06	4.83E-02*	1
cg08301525	<i>NXN</i>	chr17	867782	OpenSea	1.09E-02	6.51E-03, 1.54E-02	2.11E-06	4.83E-02*	1
cg13631094	<i>ROR2</i>	chr9	94619684	OpenSea	-4.09E-03	-5.75E-03, -2.43E-03	2.19E-06	4.83E-02*	1
cg07312357	<i>ESRRG</i>	chr1	217263805	S_Shore	1.08E-02	6.43E-03, 1.52E-02	2.19E-06	4.83E-02*	1
cg08478283	<i>CD58</i>	chr1	117076803	N_Shore	-4.28E-03	-6.02E-03, -2.54E-03	2.24E-06	4.83E-02*	1
					-3.88E-03	-5.70E-03, -2.06E-03	3.79E-05	3.28E-01	2
cg13951572	<i>SORBS3</i>	chr8	22409000	Island	-4.02E-02	-5.66E-02, -2.39E-02	2.38E-06	4.87E-02*	1

<sup>1</sup>Results from epigenome-wide associations performed using linear regression, adjusting for maternal age (years), self-identified race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, Asian or Pacific Islander), prepregnancy BMI (in kg/m<sup>2</sup>), parity (0, 1, 2+), education level (high school or less, some college or associate degree, bachelor or advanced degree), marital status (not married, married or living with partner), employment status (not employed, has at least 1 job, or is a full-time student), prepregnancy alcohol use (never, 3 times a month or less, once a week or more), periconception sleep duration (6 h or less, 7–8 h, 9 h or more), infant sex, 5 methylation plate number (categorical), the first 3 principal components (PCs) from the samples' percent methylation profile, the first 10 genotype-based PCs to account for population structure, and surrogate variables to account for heterogeneity in cell-type composition. FDR-adjusted P value <0.05 for any visit are marked with \*, and the corresponding logFC and P values for other visits are also presented if among the top 100 CpGs for that visit. Chr, chromosome; FC, fold change; FDR, false discovery rate; N, north; S, south.

<sup>2</sup>LogFC is the log of fold change (base 2); logFC <0 is associated with hypomethylation and logFC >0 is associated with hypermethylation.

<sup>3</sup>Visits 0–4 are targeted for 8–13, 16–22, 24–29, 30–33, and 34–37 wk of gestation, respectively. The sample size for visits 0–4 is 298, 291, 292, 286, and 281, respectively (Figure 1).

epigenetic modifications was the cardiac hypertrophy signaling pathway at the periconception and early pregnancy periods. Cardiac remodeling is an important physiologic adaptation induced by pregnancy, and the placenta can mediate adaptations required for pregnancy (59). Other highly significant pathways associated with total recreational PA include the B-cell receptor signaling pathway and the netrin signaling pathway, which are important for humoral immune response (60–62), as well as involved in nervous system signaling and the development of the skeletal and muscular system (63–65). Moderate or vigorous PA is also strongly associated with the netrin signaling pathway, as well as Gαq signaling, which plays a role in intracellular and second-messenger signaling and lipid metabolism (66–68).

We also observed the association of PA and DNA methylation varied by timing of PA. Although we cannot compare our findings with other studies due to limited longitudinal PA assessment in the literature, we suspect timing of PA is important because temporal modulation of different pathways may influence placental bed blood flow, arterial blood sugar, and oxygen content (37). In addition, a recent study from our group found that timing of moderate or vigorous PA was related to maternal glucose metabolism (69), which has been implicated in the modulation of placenta function. In the present study, our results suggest that timing of PA may be associated with activation of cellular pathways important for fetal programming in specific temporal windows. Importantly, the periconception to early period of PA identified the majority of pathways, despite few individual CpGs becoming FDR significant. These associations continue to emphasize the importance of having information prior to and early in pregnancy, as an important window when methylation patterns are being established.

Strengths of this study include having longitudinal assessment of PA capturing from periconception through pregnancy and implementing a standard protocol for obtaining placental tissues at delivery. We also considered a comprehensive list of covariates that were related to PA. Limitations included that PA was self-reported, which may not necessarily reflect physiologic changes such as changes in oxygen intake and heart rate. However, the PPAQ is a validated questionnaire specifically designed for pregnant women (19). We also have insufficient power to examine sex differences and limited gene expression data to corroborate methylation impact, as well as limited sample size to compare light compared with moderate and vigorous activities separately. In addition, pathways identified are not specific to the placenta, and our interpretation of results is limited by our understanding of how specific pathways influence placental development and function. Last, we may have uncontrolled confounding because of the lack of information on unobserved factors such as paternal lifestyle. We also did not adjust for smoking status because only 1 woman reported smoking in the past 6 mo.

In conclusion, recreational PA both before and during pregnancy was associated with placental DNA methylation with associations varying by activity timing. The impact of PA is potentially related to pathways with functions such as cellular development. These novel findings provide further evidence supporting the significance of pre- and periconception lifestyle on epigenetic changes in the placenta.

**TABLE 5.** Summary of functional pathways enriched for genes near top 100 CpG sites associated with total recreational physical activity at multiple visits<sup>1</sup>

Pathways	Molecule	Ratio <sup>2</sup>	P value	B-H P value	Top functions and diseases	Visit number <sup>3</sup>
Cardiac hypertrophy signaling (enhanced)	CACNA1C, CAMK2B, IL13, JUN, NFATC1, NFATC2, PLCG2, PRKG1, TSC2, WNT10A	0.019	1.02E-04	2.63E-02*	Cardiac enlargement; cardiovascular disease; cardiovascular system development and function	0
B-cell receptor signaling	CAMK2B, CSK, JUN, NFATC1, NFATC2, PLCG2	0.031	1.86E-04	2.63E-02*	Cellular development; cellular growth and proliferation; embryonic development	0
Netrin signaling	CACNA1C, NFATC1, NFATC2, PRKG1	0.056	2.57E-04	2.63E-02*	Cellular assembly and organization; cellular movement; skeletal and muscular system development and function	0
PI3K signaling in B lymphocytes	CAMK2B, JUN, NFATC1, NFATC2, PLCG2	0.035	3.72E-04	2.88E-02*	Cellular development; cellular growth and proliferation; embryonic development	0
B-cell activating factor signaling	JUN, NFATC1, NFATC2	0.070	8.32E-04	2.95E-02*	Cellular development; cellular growth and proliferation; hematologic system development and function	0
Molecular mechanisms of cancer	ARHGEF4, CAMK2B, JUN, LRP1, NOTCH1, PA2G4, PRKDC, WNT10A	0.018	6.46E-04	2.95E-02*	Embryonic development; organismal development; connective tissue development and function	0
APRIL-mediated signaling	APH1B, HDAC4, JAK1, JUN, PRKCZ, PTCHI	0.014	1.38E-02	2.19E-01	Cellular development; cellular growth and proliferation; hematologic system development and function	2
Role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis	JUN, NFATC1, NFATC2	0.071	7.76E-04	2.95E-02*	Cellular development; cellular growth and proliferation; hematologic system development and function	0
Th1 and Th2 activation pathway	CAMK2B, JUN, LRP1, NFATC1, NFATC2, PLCG2, WNT10A	0.022	4.90E-04	2.95E-02*	Cellular development; cellular growth and proliferation; hematologic system development and function	0
Systemic lupus erythematosus in B-cell signaling pathway	AXIN1, CREB5, FZD6, MAP2K3, WNT10A	0.015	4.57E-02	3.12E-01	Cellular development; cellular growth and proliferation; hematologic system development and function	4
	IL13, JUN, NFATC1, NFATC2, NOTCH1	0.029	8.71E-04	2.95E-02*	Cellular development; cellular growth and proliferation; embryonic development	0
	APH1B, HLA-DOB, JAK1, JUN, JAK1, JUN, NOTCH1	0.023	7.08E-03	2.12E-01	Cellular development; cellular growth and proliferation; hematologic system development and function	2
	CSK, IL13, JUN, NFATC1, NFATC2, PLCG2	0.017	4.47E-02	3.20E-01	Cellular development; cellular growth and proliferation; hematologic system development and function	3
		0.022	1.23E-03	3.80E-02*	Cellular development; cellular growth and proliferation; embryonic development	0
	CSK, ISG15, JAK1, JUN, PRKCZ	0.018	7.59E-03	2.12E-01	Cellular development; cellular growth and proliferation; embryonic development	2
	CSK, JAK1, JUN, PRKCA	0.014	3.89E-02	3.19E-01	Cellular development; cellular growth and proliferation; embryonic development	3

<sup>1</sup>Statistically significant overrepresented canonical pathways were determined by Fisher exact test followed by adjustment for multiple testing using the B-H method. BH-adjusted P value <0.05 for any visit are marked with \*. Associated molecules and P values for other visits are also presented if nominal P value <0.05. All pathways presented have 2 or more molecules. APRIL, a proliferation-inducing ligand; B-H, Benjamini-Hochberg (adjustment for multiple testing).

<sup>2</sup>Overlap is the number of molecules in our list over the number of molecules in the pathway of interest.

<sup>3</sup>Visits 0–4 are targeted for 8–13, 16–22, 24–29, 30–33, and 34–37 wk of gestation, respectively. The sample size for visits 0–4 is 298, 291, 292, 286, and 281, respectively (Figure 1).

**TABLE 6.** Summary of functional pathways enriched for genes near the top 100 CpG sites associated with moderate or vigorous recreational physical activity at multiple visits<sup>1</sup>

Pathways	Molecule	Ratio <sup>2</sup>	P value	B-H P value	Top functions & diseases	Visit number <sup>3</sup>
Cardiac hypertrophy signaling (enhanced)	CACNA1C, GNB1, GNG4, IL13, JUN, NFATC1, NFATC2, PDE8A, PLCG	0.020	3.31E-05	9.77E-03*	Cardiac enlargement; cardiovascular disease; cardiovascular system development and function	0
Gaq signaling	CSK, GNB1, GNG4, NFATC1, NFATC2, PLCG2	0.035	1.26E-04	1.86E-02*	Posttranslational modification; cellular assembly and organization; lipid metabolism	0
Netrin signaling	CSK, GNG7, PRKCA CACNA1C, NFATC1, NFATC2, PRKGI	0.018 0.056	4.37E-02 3.24E-04	3.19E-01 3.16E-02*	Cellular assembly and organization; cellular movement; skeletal and muscular system development and function	3 0
Glucocorticoid receptor signaling	FOS, GTF2B, HLA-DOB, HSPA6, JAK1, JUN, NDUFB10, NDUFV1	0.014	1.91E-03	3.39E-02*	Gene expression; embryonic development; organismal development	2
RAR activation	AKR1B1, CSK, FOS, JUN, PRKCZ	0.026	1.00E-03	3.39E-02*	Gene expression; cellular development; cellular growth and proliferation	2
14-3-3-mediated signaling	CSK, JUN, PRKCA, RDH13 FOS, JUN, PRKCZ, TSC2	0.020 0.032	1.26E-02 1.51E-03	2.10E-01 3.39E-02*	Cell death and survival; organismal injury and abnormalities; cell cycle molecules	3 2
Role of PKR in interferon induction and antiviral response	JUN, PLCG2, TSC2, TUBA1B PRKCA, YWHAZ	0.032 0.016	2.69E-03 3.47E-02	5.37E-02 3.49E-01	Cell death and survival; organismal injury and abnormalities; cellular development	0 1
IL-3 signaling	FOS, HSPA6, JAK1, JUN FOS, JAK1, JUN, PRKCZ	0.029 0.051	1.95E-03 2.57E-04	3.39E-02*	Cell signaling; cell-to-cell signaling and interaction; cellular development	2
Thrombopoietin signaling	JAK1, JUN, PRKCA FOS, JUN, PRKCZ	0.038 0.048	5.75E-03 1.91E-03	1.98E-01 3.39E-02*	Cell cycle; cancer; organismal injury and abnormalities	3 2
Molecular mechanisms of cancer	JUN, PLCG2 JUN, PRKCA APH1B, FOS, HDAC4, JAK1, JUN, PRKCZ, PTCHI ARHGEF4, GNB1, GNG4, JUN, NOTCHI, PA2G4, PRKDC APH1B, AXIN1, FZD6, GNG4, HDAC4, ITGB4, PRKCA	0.032 0.032 0.016 0.016 0.016	3.31E-02 3.39E-02 1.74E-03 4.17E-03 2.24E-03	1.78E-01 3.19E-01 3.39E-02*	Embryonic development; organismal development; connective tissue development and function	0 3 2 0 1

(Continued)

TABLE 6. (Continued)

Pathways	Molecule	Ratio <sup>2</sup>	P value	B-H P value	Top functions & diseases	Visit number <sup>3</sup>
iNOS signaling	FOS, JAK1, JUN	0.064	8.13E-04	3.39E-02*	Cell death and survival; organismal injury and abnormalities; respiratory disease	2
nNOS signaling in neurons	JAK1, JUN	0.043	1.95E-02	2.68E-01	nNOS signaling in neurons	3
	CAPN8, GRIN2A, PRKCC	0.064	8.13E-04	3.39E-02*		2
UVB-induced MAPK signaling	FOS, JUN, PRKCC	0.058	1.10E-03	3.39E-02*	Cellular development; cellular growth and proliferation; cell cycle	2
UVC-induced MAPK signaling	JUN, PRKCA	0.039	2.34E-02	2.73E-01		3
	FOS, JUN, PRKCC	0.059	1.02E-03	3.39E-02*	Cell death and survival; connective tissue development and function; cancer	2
EGF signaling	JUN, SMPD3	0.039	2.24E-02	1.41E-01		0
	JUN, PRKCA	0.039	2.29E-02	2.73E-01		3
	FOS, JAK1, JUN	0.055	1.29E-03	3.39E-02*	Cell cycle; DNA replication, recombination, and repair; cancer	2
IL-2 signaling	JAK1, JUN, PRKCA	0.055	2.04E-03	1.98E-01		3
	FOS, JAK1, JUN	0.049	1.74E-03	3.39E-02*	Cellular development; cellular growth and proliferation; embryonic development	2
IGF-1 signaling	JAK1, JUN	0.033	3.16E-02	3.19E-01		3
	FOS, JAK1, JUN, PRKCC	0.039	7.24E-04	3.39E-02*	Cancer; organismal injury and abnormalities; cell death and survival	2
Th2 pathway	JAK1, JUN, SOCS2	0.029	1.20E-02	2.10E-01		3
	APHIB, HLA-DOB, JAK1, JUN	0.029	2.04E-03	3.39E-02*	Hematologic system development and function; tissue morphology; lymphoid tissue structure and development	2
Neuroinflammation signaling pathway	IL13, JUN, NFATC2, NOTCHI	0.029	3.55E-03	5.50E-02		0
	JAK1, JUN, NOTCHI	0.022	2.51E-02	2.73E-01		3
Apelin endothelial signaling pathway	APHIB, FOS, GRIN2A, HLA-DOB, JAK1, JUN	0.019	1.45E-03	3.39E-02*	Cell-to-cell signaling and interaction; hematologic system development and function; immune cell trafficking	2
	FOS, HDAC4, JUN, PRKCC	0.029	2.14E-03	3.39E-02*	Cardiovascular system development and function; organismal development; embryonic development	2
Systemic lupus erythematosus in B-cell signaling pathway	GNB1, GNG4, JUN	0.022	2.51E-02	1.53E-01		0
	GNG7, JUN, PRKCA	0.022	2.57E-02	2.73E-01		3
	CSK, FOS, ISG15, JAK1, JUN, PRKCC	0.022	7.41E-04	3.39E-02*	Cellular development; cellular growth and proliferation; embryonic development	2

(Continued)

TABLE 6. (Continued)

Pathways	Molecule	Ratio <sup>2</sup>	P value	B-H P value	Top functions & diseases	Visit number <sup>3</sup>
	CSK, IL13, JUN, NFATC1, NFATC2, PLCG2	0.022	1.66E-03	4.90E-02*		0
MSP-RON signaling in macrophages pathway	CSK, JAK1, JUN, PRKCA, FOS, HLA-DOB, JUN, PRKCZ	0.014 0.034	3.89E-02 1.12E-03	3.19E-01 3.39E-02*	Cellular movement; hematologic system development and function; immune cell trafficking	3 2
B-cell activating factor signaling	JUN, NFATC1, NFATC2	0.070	9.77E-04	3.72E-02*	Cellular development; cellular growth and proliferation; hematologic system development and function	0
G protein signaling mediated by tubby	FOS, JUN	0.047	1.23E-02	7.94E-02		2
APRIL-mediated signaling	GNB1, GNG4, PLCG2	0.068	1.05E-03	3.72E-02*	Cancer; hematologic disease; immunologic disease	0
Sperm motility	JUN, NFATC1, NFATC2	0.071	9.12E-04	3.72E-02*	Cellular development; cellular growth and proliferation; hematologic system development and function	0
	FOS, JUN	0.048	1.15E-02	7.94E-02		2
	CSK, GNB1, GNG4, NTRK3, PLCG2, PRKG1	0.024	1.07E-03	3.72E-02*	Cellular function and maintenance; molecular transport; amino acid metabolism	0
Estrogen receptor signaling	CSK, GNG7, JAK1, PRKCA, TEC	0.020	6.31E-03	1.98E-01		3
	CACNA1C, CTBP2, GNB1, GNG4, JUN, NOTCH1, PLCG2, PRKDC	0.020	5.13E-04	3.72E-02*	Gene expression; embryonic development; organismal development	0
	FOS, JAK1, JUN, NDUFB10, NDUFV1, PRKCZ	0.015	4.90E-03	5.01E-02		2
	GNG7, JAK1, JUN, NOTCH1, PRKCA	0.012	3.80E-02	3.19E-01		3
Th1 and Th2 activation pathway	IL13, JUN, NFATC1, NFATC2, NOTCH1	0.029	1.12E-03	3.72E-02*	Cellular development; cellular growth and proliferation; embryonic development	0
	APHIB, HLA-DOB, JAK1, JUN	0.023	4.57E-03	5.01E-02		2
IL-10 signaling	JAK1, JUN, NOTCH1	0.017	4.47E-02	3.20E-01		3
	FOS, JAK1, JUN	0.042	2.82E-03	3.98E-02*	Cell signaling; cell-to-cell signaling and interaction; organismal development	2
Corticotropin releasing hormone signaling	JAK1, JUN	0.028	4.27E-02	3.19E-01		3
	FOS, JUN, PRKCZ, PTCHI	0.027	2.75E-03	3.98E-02*	DNA replication, recombination, and repair; cellular development; cellular growth and proliferation	2
	CACNA1C, JUN, PLCG2	0.020	3.02E-02	1.69E-01		0

(Continued)

TABLE 6. (Continued)

Pathways	Molecule	Ratio <sup>2</sup>	P value	B-H P value	Top functions & diseases	Visit number <sup>3</sup>
Hypoxia signaling in the cardiovascular system	JUN, P4HB, UBE2F	0.041	3.02E-03	4.17E-02*	Cell cycle; carbohydrate metabolism; cell death and survival	2
	JUN, UBE2F, UBE2J2	0.041	4.79E-03	1.98E-01		3
	JUN, UBE2O	0.027	4.47E-02	2.12E-01		0

<sup>1</sup>Statistically significant overrepresented canonical pathways were determined by Fisher exact test followed by adjustment for multiple testing using the B-H method. Pathways with BH-adjusted P value <0.05 for any visit are marked with \*. Associated molecules and P values for other visits are also presented if nominal P value <0.05. All pathways presented have 2 or more molecules. APRIL, a proliferation-inducing ligand; B-H, Benjamin-Hochberg (adjustment for multiple testing); EGF, epidermal growth factor; IGF-1, insulin-like growth factor 1; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MSP, macrophage stimulating protein; RON, receptor d'origine nantais; nNOS, neuronal nitric oxide synthase; PKR, protein kinase receptor; RAR, retinoic acid receptor.

<sup>2</sup>Overlap is the number of molecules in our list over the number of molecules in the pathway of interest.

<sup>3</sup>Visits 0–4 are targeted for 8–13, 16–22, 24–29, 30–33, and 34–37 wk of gestation, respectively. The sample size for visits 0–4 is 298, 291, 292, 286, and 281, respectively (Figure 1).

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