Maternal caffeine intake and DNA methylation in newborn cord blood

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ABSTRACT

Background: Epigenetic mechanisms may underlie associations between maternal caffeine consumption and adverse childhood metabolic outcomes. However, limited studies have examined neonate DNA methylation (DNAm) patterns in the context of preconception or prenatal exposure to caffeine metabolites.

Objectives: We examined preconception and pregnancy caffeine exposure with DNAm alterations in neonate cord blood ($n = 378$).

Methods: In a secondary analysis of the Effects of Aspirin in Gestation and Reproduction Trial (EAGeR), we measured maternal caffeine, paraxanthine, and theobromine concentrations from stored serum collected preconception (on average 2 months before pregnancy) and at 8 weeks of gestation. In parallel, self-reported caffeinated beverage intake was captured via administration of questionnaires and daily diaries. We profiled DNAm from the cord blood buffy coat of singletons using the MethylationEPIC BeadChip. We assessed associations of maternal caffeine exposure and methylation β values using multivariable robust linear regression. A false discovery rate (FDR) correction was applied using the Benjamini-Hochberg method.

Results: In preconception, the majority of women reported consuming 1 or fewer servings/day of caffeine on average, and caffeine and paraxanthine metabolite levels were 88 and 36 μmol/L, respectively. Preconception serum caffeine metabolites were not associated with individual cytosine-guanine (CpG) sites (FDR >5%), though pregnancy theobromine was associated with DNAm at cg09460369 near *RAB2A* ($\beta = 0.028$; SE = 0.005; FDR $P = 0.012$). Preconception self-reported caffeinated beverage intake compared to no intake was associated with DNAm at cg09002832 near *GLIS3* ($\beta = -0.013$; SE = 0.002; FDR $P = 0.036$). No associations with self-reported intake during pregnancy were found.

Conclusions: Few effects of maternal caffeine exposure on neonate methylation differences in leukocytes were identified in this population with relatively low caffeine consumption. *Am J Clin Nutr* 2022;115:482–491.

Keywords: DNA methylation, caffeine intake, mother-child dyads, maternal exposures, periconception

Introduction

Caffeine is widely consumed and naturally found in beverages and foods, including coffee, tea, and cocoa products (1). Current guidelines from the American College of Obstetricians and Gynecologists recommend pregnant women limit consumption to less than 200 milligrams per day (2). During pregnancy, the fetus is directly exposed to maternal caffeine intake, as caffeine and its metabolites are readily able to cross the placenta and enter fetal circulation (3). Caffeine is metabolized by the cytochrome P450 1A2 enzyme (CYP1A2) to produce paraxanthine and theobromine, which account for approximately 80% and 12% of caffeine metabolites, respectively (4).

Maternal caffeine exposure has been previously associated with long-term outcomes, including childhood overweight and obesity and liver fat deposits by age 10 years (5–9). These effects of maternal caffeine exposure on childhood outcomes may be mediated through epigenetic mechanisms (10–15). For example, caffeine intragastrically administered from gestational days 9 through 20 (at 30, 60, or 120 mg/kg per day) in pregnant rats is associated with histone acetylation and reduced expression of genes responsible for cholesterol synthesis in male offspring

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Supplemental Figures 1–2 and Supplemental Tables 1–6 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: CpG, cytosine-guanine; CYP1A2, cytochrome P450 1A2 enzyme; DNAm, DNA methylation; EAGeR, Effects of Aspirin in Gestation and Reproduction; EWAS, epigenome-wide association study; FDR, false discovery rate; IPA, Ingenuity Pathway Analysis; LOD, limit of detection.

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liver samples, including cAMP, sirtuin1, and protein kinase A (10). More specifically, differences in DNA methylation (DNAm) patterns, a primary epigenetic mechanism, have been associated with exposure to caffeine (ranging from gestational day 8.5 up until day 20 at 20+ mg/kg per day, or about 2–4 cups of coffee for humans) and its metabolites in animal and in vitro studies (11–15).

Few human studies have examined associations between caffeine exposure and DNAm status, and these studies were limited by only examining self-reported coffee or tea intake in nonpregnant adults (16–18). Chuang et al. (16) identified 11 cytosine-guanine (CpG) probes in blood associated with daily coffee consumption that were linked to lipid metabolism and immune response. Yet, another epigenome-wide association study (EWAS) from Ek et al. (17) found no significant associations between coffee consumption (ranging from 28.8 to 107 cups per month on average) and DNAm among men and women but observed a significant association between tea consumption in women and DNAm at probes cg18192808 (*DNAJC16*) and cg14055589 (*TTC17*). Factors such as the retrospective and self-reported measures of coffee and tea intake may explain the discrepancy in findings. A meta-analysis of 15 cohorts of 15,789 nonpregnant adults in the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium identified coffee-associated differences in DNAm at 11 CpGs (18). Self-reported coffee intake ranged from 0.6 cups per day to 3.5 cups per day. Furthermore, no epidemiologic studies examined these associations in the context of preconception exposure to caffeine or its metabolites. These associations may be important to consider, because DNAm is beginning to be established in early development (19).

Therefore, the aim of this study was to examine the association of maternal caffeine status during preconception and pregnancy with DNAm patterns in cord blood. To do this, maternal caffeine exposure was assessed by both maternal serum biomarkers of caffeine metabolites and self-reported caffeinated beverage intake. Epigenetic markers may provide new mechanistic insights into the impacts of maternal caffeine status.

Methods

Study design

We conducted a secondary analysis of the Effects of Aspirin in Gestation and Reproduction (EAGeR) trial (2007–2011; NCT00467363). EAGeR was a multicenter, double-blind clinical trial that randomized White women with a history of pregnancy loss to low-dose aspirin + folic acid compared with folic acid prior to conception to investigate effects on live birth and pregnancy loss ($n = 1228$) (20, 21). Among them, 48% ($n = 595$) delivered a live birth. The current analysis is nested among newborns from the Salt Lake City, Utah, study site, which recruited >80% of study participants. Cord blood was collected at Utah beginning in 2009 (2 years after enrollment began) and was successful for over 90% of deliveries thereafter ($n = 428$). We removed samples that had insufficient DNA or failed quality control checks ($n = 37$). We also excluded 12 participants of other races/ethnicities [i.e., Black ($n = 4$), American Indian ($n = 1$), Asian ($n = 6$), and unidentified ($n = 1$)] as there have been documented racial/ethnic differences in caffeine consumption and their sources (e.g., more tea consumed in Asian cultures) and genetic ancestry plays a role in establishment of DNAm (22, 23). **Supplemental Figure 1** is a participant flow diagram for the final analytic sample ($n = 378$). The study was approved by the institutional review board at the University of Utah (Salt Lake City, Utah IRB #1,002,521), and all participants provided written informed consent prior to enrolling. We previously observed that randomization to low-dose aspirin had no impact on DNAm in cord blood (24, 25).

Caffeine metabolite assessment

Caffeine and 2 primary metabolites, paraxanthine and theobromine, were measured via LC-MS from serum samples collected from women at the baseline enrollment visit prior to randomization (preconception; average 2.60 ± 1.57 months prior to pregnancy) and again at 8 weeks of gestation (26). Coefficients of variation in the lowest range of detection were 9%–17%. Of the 363 preconception samples available for analysis, 65 (17.9%), 113 (31.1%), and 28 (7.7%) were below the limit of detection (LOD) of 0.04 μg/mL for caffeine, paraxanthine, and theobromine, respectively. Of the 345 eight-week gestation samples available for analysis, 136 (39.4%), 268 (77.7%), and 123 (35.6%) were below the LOD of 0.04 μg/mL for caffeine, paraxanthine, and theobromine, respectively. Values below the LOD were imputed as LOD/$\sqrt{2}$ (27) and were included in the analysis. For this analysis, we categorized serum caffeine metabolites into tertiles due to the skewness of each metabolite toward low concentrations. Preconception categories were defined as follows: caffeine, ≤0.08, 0.08–0.56, or >0.56 μg/mL; paraxanthine, ≤0.04, 0.04–0.18, or >0.18 μg/mL; and theobromine, ≤0.34, 0.34–1.05, or >1.05 μg/mL. Early pregnancy categories were defined as follows: caffeine, ≤0.03, 0.03–0.21, or >0.21 μg/mL; and theobromine, ≤0.03, 0.03–0.22, or >0.22 μg/mL. Early pregnancy paraxanthine was dichotomized according to the LOD (≥0.04 compared with <0.04 μg/mL) because the 33.3 and 66.7 percentiles were both below the LOD.

Caffeinated beverage intake assessment

Self-reported habitual caffeinated beverage intake was captured multiple times during the study. At baseline, prior to pregnancy, study participants completed lifestyle questionnaires capturing intake of coffee (cups/d), tea (cups/d), and caffeinated soda (servings/d) over the past 12 months. Servings were converted to standard servings/d, which corresponds to a 6 oz (177 mL) cup of coffee or tea and a 12 oz (355 mL) can of soda. For the analysis, we categorized each beverage intake by any cups/d or servings/d reported, compared with none. During the first 2 menstrual cycles after study enrollment, 330 (87.3%) women recorded the number of caffeinated beverages consumed on daily diaries completed at home. Similarly, a daily diary capturing total caffeinated beverage intake was also completed between 4 to 8 weeks of gestation. For this analysis, we averaged caffeinated beverage intake from the diaries to get a preconception average (i.e., diaries collected during the first 2 menstrual cycles) and an 8-weeks-gestation average. Intake was categorized as any amount compared with

none. Lastly, questionnaires assessing the usual daily intake of caffeinated beverages over the past month were completed every 4 weeks beginning at 12 weeks gestation until 36 weeks. For this analysis, we categorized self-reported caffeinated beverage average intake over this period as none (i.e., no servings) or any amount.

DNAm measurement and processing

DNA measurement from cord blood samples has been previously described for this study (24). Briefly, prepared cord blood buffy coat was shipped and processed for DNA extraction and analysis at the University of Minnesota. DNA underwent bisulfate conversion (EZ DNA Methylation TM kit, Zymo Research) to differentiate unmethylated and methylated cytosines. DNAm was profiled using the Infinium MethylationEPIC BeadChip microarray (28, 29). Samples were randomly ordered to control for batch effects, and the sample plate and positions were tracked. The minfi package in R was used to process DNAm microarray data, including quantile normalization and background signal and dye-bias adjustment of probes (30). Probes with a detection *P* value greater than 0.01 were filtered and probes on the sex chromosomes were removed, leaving 815,112 probes for analysis. DNAm levels for each CpG probe were reported as β values ranging from 0 (unmethylated) to 1 (methylated). These β values were determined by calculating the ratio of methylated probe fluorescence intensity to the sum of the methylated and unmethylated probe intensities.

Statistical analysis

Linear regression models were used to assess a linear trend between self-reported caffeinated beverage intake and natural log-transformed serum caffeine metabolite concentrations. Spearman correlation coefficients (r_s) were also used to compare within- and between-serum concentrations at preconception and 8 weeks of gestation. To assess the association of methylation β values (dependent variable) and each of the caffeine metabolites, we used multivariable robust linear regression. Separate models were created for each metabolite at each time point, comparing the second or third tertile with the first (lowest) tertile. In a secondary analysis, adjusted robust linear models were used to examine the associations of any self-reported intake of caffeinated beverages (compared with none) with methylation β values. Separate models were created for total caffeinated beverage intake, as well as coffee, tea, and soda beverage intakes. To account for multiple comparisons, we applied a false discovery rate (FDR) correction using the method by Benjamini-Hochberg (31).

Preconception models were adjusted for maternal age in years (continuous), household income of at least $40,000, ever-smoker status, and alcohol consumption obtained from the preconception diary. Pregnancy models were adjusted for maternal age, household income, and ever-smoker status. Alcohol consumption was not included due to few women reporting consuming alcohol (n <5) during pregnancy. Similarly, all women were provided with folic acid supplements as part of the trial, and over 92% of participants reported taking a multivitamin at baseline. In all models, we also adjusted for batch effects, cell count estimation, infant sex, and the infant's epigenetically derived ancestry. Ancestry was inferred using GLINT to generate 4 principle components of ancestry using information from select CpG sites (32). To adjust for batch effects from the DNAm measurement, plate number was included as a covariate in the analysis. The relative proportions of B cells, monocytes, CD4T, CD8T, granulocytes, NK cells, and nucleated red blood cells were estimated using a cord blood–specific reference (33, 34) to account for cellular heterogeneity. The treatment arm (low-dose aspirin or placebo) was not associated with either DNAm (24) or caffeine status (26) in this cohort, so it was not included as a covariate in this analysis. The statistical analysis was conducted in SAS 9.4 (SAS Institute) and R Studio 1.3.

Gene annotations were identified using the Illumina database and Ingenuity Knowledge Database and verified in the University of California Santa Cruz genome browser (GRCh37/hg19). Biologic networks and functional pathways were generated through the use of Ingenuity Pathway Analysis (IPA; QIAGEN Inc.) (35). CpG probes were imported and mapped to algorithmically generated networks and canonical pathways available in the proprietary IPA Knowledge Base. Resulting networks receive scores within IPA for ranking purposes, in which a greater number of molecules in the network results in a higher score. Canonical pathways were determined by IPA based on: *1*) the ratio of the number of molecules from the data set that map to a canonical pathway, divided by the total number of molecules that map to that same pathway; and *2*) a Fisher's exact test *P* value that determines whether the molecules in the data set and pathway overlap by chance alone.

Results

Women were, on average, 28.3 years of age (SD, 4.5 years) with household incomes of at least $40,000 (68.8%). The majority were never smokers (92.1%) and did not report consuming alcohol in their preconception diary (73.1%; **Table 1**). An older age, a higher household income, ever-smoking status, and higher preconception alcohol consumption were significantly associated with increasing caffeine metabolite tertiles. Maternal caffeine metabolite concentrations are reported in **Supplemental Table 1**. Except for preconception theobromine, the first tertile for each metabolite represents serum concentrations below the LOD. Low caffeine exposure was also indicated through maternal self-report. On the baseline questionnaire, 65%, 21%, and 7% reported any soda, coffee, or tea intake, respectively, with the majority consuming 1 or fewer servings/day on average. During the first 2 menstrual cycles of active follow-up, 75.8% reported consuming any amount of any type of caffeinated beverage. The number of caffeinated drinks consumed decreased over early pregnancy; by 8 weeks gestation, only about 23% reported consuming any amount. During the second and third trimesters, about half of all women reported any intake, and caffeinated soda was the primary source.

Self-reported caffeinated beverage intake had a positive correlation with serum caffeine and paraxanthine concentrations at both the preconception and 8-week-gestation assessments (*P* value for linear trend < 0.001) and are presented in **Supplemental Table 2**. Caffeinated beverage intake was not

TABLE 1 Baseline study population characteristics among women with newborn DNA methylation data

Characteristic[1]	Analytic sample	Self-report caffeinated beverage, any intake	Preconception caffeine tertile		
			1 ≤0.08 μg/mL	2 0.08–0.56 μg/mL	3 >0.56 μg/mL
n	378	250	125	119	119
Age, years, mean (SD)	28.3 (4.5)	28.5 (4.6)	27.5 (3.9)	27.9 (4.3)	29.8 (4.8)
Prepregnancy BMI, kg/m^2, mean (SD)	25.2 (5.5)	25.5 (5.8)	24.6 (5.1)	25.2 (5.5)	25.4 (5.2)
Education, >high school	3387 (89.4)	221 (88.4)	115 (92.0)	106 (89.1)	105 (88.2)
Household income, ≥$40k	260 (68.8)	178 (71.2)	74 (59.2)	90 (75.6)	88 (73.9)
Smoking status, never	348 (92.1)	227 (90.8)	124 (99.2)	109 (91.6)	102 (85.0)
Preconception alcohol, any	90 (27.3)	84 (33.6)	7 (6.4)	28 (25.2)	50 (52.1)

[1]All values are *n* (%) unless otherwise stated.

associated with preconception serum theobromine (*P* value for linear trend = 0.52), though a positive association was identified at 8 weeks of gestation (*P* value for linear trend = 0.003). Among preconception serum concentrations, caffeine was highly correlated with paraxanthine (r_s = 0.86; *P* value < 0.001), and theobromine demonstrated low correlations with caffeine (r_s = 0.34; *P* value < 0.001) and paraxanthine (r_s = 0.30; <0.001). Further, serum concentrations at 8 weeks gestation showed low correlations with each other (r_s, 0.35–0.47; *P* values < 0.001). In comparing preconception and 8-week-gestation serum concentrations, the r_s ranged from 0.11 to 0.29, indicating a low correlation between the preconception and gestational-week-8 measures of serum metabolites (*P* values < 0.05).

Serum caffeine metabolites

In the array-wide analysis, preconception serum markers of caffeine, paraxanthine, and theobromine were not associated with individual CpG probes after FDR adjustment. Early pregnancy theobromine concentrations (tertile 2 compared with tertile 1) were associated with differential DNAm at probe cg09460369 (FDR *P* = 0.012) near the *RAB2A* gene on chromosome 8 (**Tables 2** and **3**). A similar trend was observed in comparing tertile 3 to tertile 1 for the same probe (β = 0.017; SE = 0.004; *P* value = 0.005), though statistical significance was not reached after the FDR correction. Caffeine and paraxanthine concentrations at 8 weeks gestation were not associated with individual CpG probes in neonates after FDR adjustment.

Maternal report of caffeinated beverages

Based on maternal self-report of caffeinated beverage intake, 1 significant inverse association with preconception consumption of any type of caffeinated beverage was found involving probe cg09002832 (FDR *P* = 0.036) near the *GLIS3* gene on chromosome 9 (Tables 2 and 3). No FDR-corrected significant associations were found with specific types of preconception caffeinated beverages (i.e., coffee, tea or soda) or with self-reported intake during pregnancy (up to 8 weeks and 12–36 weeks). In an ad hoc analysis, we defined regular or consistent caffeine drinkers using the preconception and gestation week 8 diaries and pregnancy questionnaires (*n* = 54/250), but did not find FDR-significant CpG probes.

Functional enrichment analysis

Next, we imported the top-ranked 100 CpG probes, based on the FDR *P* value identified in the early pregnancy theobromine and preconception intake array-wide analyses, into IPA (**Supplemental Tables 3** and **4**). The resulting networks, along with the top related disease or functions, are provided in the Supplemental Materials (**Supplemental Tables 5** and **6**; **Supplemental Figure 2**). The top network showed that the maternal theobromine at 8 weeks of gestation was related to functions of "cell death and survival, lipid metabolism, small molecule biochemistry" (score = 44). The top IPA network of "cancer, gastrointestinal disease, organismal injury and abnormalities" was associated with the probes from preconception caffeinated beverage intake (score = 59). IPA also returned the overlapping canonical pathways of the top CpG probes identified in the preconception intake and pregnancy theobromine analyses based on the following categories: "intracellular and second messenger signaling"; "cellular growth, proliferation, and development"; "cellular immune response"; and "cellular stress and injury" (**Table 4**).

Discussion

We investigated array-wide methylation profiles in neonatal cord blood in association with maternal caffeine exposure during preconception and early pregnancy. Exposure was examined using 2 approaches: serum markers of caffeine metabolites and maternal report of caffeinated beverage intake. Overall, we found few differences in methylation at individual CpG sites with periconception caffeine exposure. Differential methylation at CpG probe cg09460369 (*RAB2A*) was associated with serum theobromine at 8 weeks of gestation. *RAB2A* encodes a protein required for transport from the endoplasmic reticulum to the Golgi complex and has been implicated in conditions like rheumatoid arthritis (36) and osteoarthritis (37). In addition, cg09002832 (*GLIS3*) was associated with preconception caffeinated beverage consumption. The *GLIS3* gene encodes a protein important in transcription and is involved in the early development of tissues, including pancreatic beta cells and the thyroid, brain, liver, and kidney (38). Both cg09460369 and cg09002832 are located in CpG islands, suggesting a role in the regulation of gene expression, though this needs to be confirmed with gene transcription data (39). Though replication is needed, our study provides novel but limited evidence of

TABLE 2 Top-rank CpG sets from the preconception exposure and cord blood DNAm analysis, $n = 378$

CpG Site	β	SE	*P* Value	FDR *P* Value	Chromosome	Position	Gene	Relation to CpG Island
Preconception Serum								
Maternal serum caffeine (comparing tertile 2 vs. tertile 1)								
cg15058799	−0.02601	0.00497	1.67E-07	0.138984	17	62,257,238	*TEX2*	
cg16968650	−0.01889	0.003896	1.25E-06	0.520046	1	6,599,434	*NOL9*	
cg24229198	−0.01445	0.003046	2.10E-06	0.520962	15	52,785,986	*MYO5A*	
cg02189597	0.0138	0.00296	3.13E-06	0.520962	15	60,881,071	*RORA*	North Shelf
cg05364412	−0.00914	0.00196	3.10E-06	0.520962	19	2,136,934	*AP3D1*	Island
Maternal serum caffeine (comparing tertile 3 vs. tertile 1)								
cg14415456	−0.01267	0.002554	6.97E-07	0.290335	12	5,371,496		
cg01591591	0.0187	0.003699	4.25E-07	0.290335	16	3,921,282	*CREBBP*	
cg09611679	−0.02215	0.004629	1.72E-06	0.476605	8	11,653,098	*FDFT1*	
cg15048078	0.02297	0.005539	3.37E-05	0.683558	4	38,665,599	*KLF3; FLJ13197*	Island
cg24636629	−0.01773	0.004428	6.26E-05	0.683558	4	40,058,917	*LOC344967; N4BP2*	Island
Maternal serum paraxanthine (comparing tertile 2 vs. tertile 1)								
cg08346494	−0.00532	0.001056	4.65E-07	0.19357	11	70,049,962	*FADD*	South Shore
cg22362389	0.03115	0.00614	3.90E-07	0.19357	17	79,619,190	*PDE6G*	South Shelf
cg06726154	−0.01839	0.003751	9.43E-07	0.261841	8	62,669,129		
cg01554873	−0.02448	0.005282	3.58E-06	0.597194	1	33,478,978	*AK2*	
cg21140380	0.03379	0.007379	4.66E-06	0.597194	11	43,938,616	*ALKBH3*	
Maternal serum paraxanthine (comparing tertile 3 vs. tertile 1)								
cg05029100	0.01184	0.00256	3.77E-06	0.522128	5	126,054,138		
cg27054066	−0.01384	0.003033	5.02E-06	0.522128	8	70,905,247		
cg06307802	0.01237	0.002709	4.99E-06	0.522128	10	76,784,752	*KAT6B*	
cg14415456	−0.01152	0.002439	2.31E-06	0.522128	12	5,371,496		
cg01060862	−0.02348	0.005008	2.75E-06	0.522128	12	99,235,137	*ANKS1B*	
Maternal serum theobromine (comparing tertile 2 vs. tertile 1)								
cg25544461	0.009909	0.002102	2.44E-06	0.338786	7	73,894,573	*GTF2IRD1*	North Shore
cg27482594	−0.0153	0.003218	1.98E-06	0.338786	10	98,861,170	*SLIT1-AS1; SLIT1*	
cg08943696	−0.01026	0.002175	2.36E-06	0.338786	12	131,437,802	*GPR133*	
cg16361417	−0.00968	0.002045	2.22E-06	0.338786	14	77,553,212		
cg26022581	−0.01114	0.002283	1.06E-06	0.338786	14	105,832,816	*PACS2*	South Shore
Maternal serum theobromine (comparing tertile 3 vs. tertile 1)								
cg07320628	−0.01531	0.003128	9.91E-07	0.825329	7	28,395,006	*CREB5*	
cg01193403	−0.00237	0.004219	0.5736	0.999954	4	7,736,302	*SORCS2*	
cg09627796	0.003735	0.004211	0.3751	0.999954	4	7,736,472	*SORCS2*	
cg04457631	−0.00213	0.005227	0.6833	0.999954	4	7,736,753	*SORCS2*	
cg14181141	0.001724	0.002884	0.55	0.999954	4	7,738,508	*SORCS2*	
Preconception self-report of caffeinated beverage intake								
Self-reported intake of any cups/d of caffeinated coffee								
cg24571508	−0.01533	0.002902	1.27E-07	0.083732	4	81,094,387		
cg04537283	−0.01474	0.002835	2.01E-07	0.083732	1	41,105,444	*RIMS3*	
cg17377891	−0.01663	0.003407	1.06E-06	0.273555	6	11,851,894		
cg21093052	0.02912	0.006099	1.80E-06	0.273555	6	38,048,627	*ZFAND3*	
cg15057554	0.02294	0.004774	1.55E-06	0.273555	7	157,787,328	*PTPRN2*	

(*Continued*)

TABLE 2 (*Continued*)

CpG Site	β	SE	*P* Value	FDR *P* Value	Chromosome	Position	Gene	Relation to CpG Island
Self-reported intake of any cups/d of caffeinated tea								
cg10610309[1]	−0.1566	0.01065	5.84E-49	4.86E-43	13	100,083,372		
cg14532717[1]	−0.2554	0.02669	1.07E-21	4.45E-16	13	95,897,739	*ABCC4*	
cg05954339	−0.08301	0.01525	5.21E-08	0.01447	7	128,292,014	*LINC01000*	
cg00073010	−0.01684	0.003364	5.52E-07	0.091885	17	17,627,194	*RAI1*	Island
cg06301296	−0.00723	0.001438	5.01E-07	0.091885	4	6,988,743	*TBC1D14*	Island
Self-reported intake of any servings/d of caffeinated soda								
cg24789467	−0.01957	0.004037	1.24E-06	0.454676	5	132,159,003	*SHROOM1*	Island
cg25658464	−0.01012	0.002142	2.28E-06	0.454676	5	150,861,640	*SLC36A1*	
cg05741395	−0.01121	0.002377	2.41E-06	0.454676	15	42,173,869	*SPTBN5*	North Shore
cg25966829	−0.01481	0.003085	1.59E-06	0.454676	1	192,613,150	*RGS13*	
cg19924120	−0.01012	0.002158	2.73E-06	0.45476	18	76,160,108		North Shore
Self-reported preconception intake of any type of caffeinated beverage								
cg09002832	−0.013	0.002	4.39E-08	0.036574	9	4,300,181	*GLIS3*	Island
cg14236539	0.013	0.003	6.26E-07	0.212765	6	47,026,946		
cg04289820	0.023	0.004	7.67E-07	0.212765	14	107,221,194		Island
cg23877720	−0.006	0.001	2.44E-06	0.405877	7	49,815,117	*VWC2*	Island
cg22438063	0.010	0.002	2.38E-06	0.405877	16	75,121,671	*ZNRF1*	

Abbreviations: CpG, cytosine-guanine; DNAm, DNA methylation; FDR, false discovery rate.
[1]Potentially polymorphic.

associations between maternal caffeine exposure and DNAm alterations.

Protein kinase A, AMP-activated protein kinase, and cAMP-mediated signaling were elucidated as potential functions impacted by the methylation patterns in our study. Protein kinase A is essential to regulating metabolic homeostasis and gene expression through its role in phosphorylation of enzymes and transcription factors and is regulated by AMP. For example, it is the primary regulator of acetyl-CoA carboxylase in fatty acid metabolism (40) and its activation by drugs such as metformin decreases blood glucose concentrations (41). Our finding is consistent with an EWAS of habitual coffee consumption among older adults in which genes linked to significant CpG probes (P values $< 10^{-6}$) were related to protein kinase activity in an enrichment analysis (16). Animal models have also provided evidence linking in utero caffeine exposure to gene expression of protein kinase A and AMP (10). This is potentially relevant, as a recent meta-analysis reported a 39% increased risk of childhood overweight and obesity among offspring of mothers with the highest caffeine intake compared to those with the lowest (7). This association was further supported by a significant dose-response relationship in which each 100 mg per day increase of maternal caffeine consumption was associated with a 31% increased risk of childhood overweight and obesity (7). Therefore, DNAm may be one mechanism linking preconception or prenatal caffeine exposure to later disease risks, though the results of this study alone should not be interpreted as a causal mechanism of the developmental effects of maternal caffeine exposure.

Pathways linked to cell cycle function, growth, and development were also prominent in our study. An in vitro experiment demonstrated that 24-hour treatment with theobromine (100–200 μg/mL) stopped preadipocytes in the nondividing, G0/G1 phase (42). Notably, these concentrations of theobromine are higher than the levels observed in this cohort (<1.05 μg/mL) or those that are attainable through typical ingestion of theobromine-rich foods, such as dark chocolate. We were unable to assess this further, as dietary recalls to measure self-reported intake of theobromine-rich foods were not collected in this study. Further research is needed to expand on the potential impacts of caffeine metabolites, particularly theobromine, on cellular function preconceptionally and prenatally. Notably, the pathways identified in IPA are only potential biologic processes that may be impacted by the methylation of CpG probes in that pathway. We cannot rule out that the identification of these potential pathways in our study may be due to the differing number of CpG probes per gene that are present on the MethylationEPIC 850k.

Strengths of this study include the examination of neonate DNAm in the context of preconception and prenatal exposure to caffeine in a well-established, prospective cohort. We uniquely utilized 2 sources of maternal exposure: serum metabolites and self-reported intake. Though exposure misclassification is possible, the prospective design limits potential recall bias of self-reported intake, and findings were similar between self-reported and serum measures. However, this study had some limitations. First, a single preconception marker or a single early gestation marker of circulating caffeine metabolites may not reflect usual intake during these critical periods [caffeine metabolites have half-lives of around 3 hours (43)]. Repeated measures of these metabolites and adjustment of time since

TABLE 3 Top-rank CpG sets from the pregnancy exposure and cord blood DNAm analysis, $n = 378$

CpG Site	β	SE	*P* Value	FDR *P* Value	Chromosome	Position	Gene	Relation to CpG Island
Maternal serum at 8 weeks gestation								
Maternal serum caffeine (comparing tertile 2 vs. tertile 1)								
cg14229247	−0.01007	0.002071	1.16E-06	0.608595	9	100,745,139	*ANP32B*	Island
cg19140548	0.009416	0.002032	3.58E-06	0.608595	10	105,552,406	*SH3PXD2A*	
cg03609636	−0.00767	0.001625	2.37E-06	0.608595	11	102,323,712	*TMEM123*	Island
cg13000180	0.01182	0.002473	1.75E-06	0.608595	16	3,355,450	*ZNF75A*	Island
cg13780046	−0.02103	0.004542	3.65E-06	0.608595	21	43,756,472		
Maternal serum caffeine (comparing tertile 3 vs. tertile 1)								
cg08651856	0.02689	0.005955	6.30E-06	0.397483	4	103,780,165	*UBE2D3*	
cg24441866	0.03135	0.007311	1.80E-05	0.397483	4	175,075,603	*LOC101928509*	
cg17684034	−0.01438	0.003296	1.28E-05	0.397483	5	74,862,658	*POLK*	
cg05164570	0.01445	0.002936	8.60E-07	0.397483	5	140,071,033	*HARS*	Island
cg01252586	−0.00429	0.000952	6.58E-06	0.397483	5	175,815,479	*NOP16; HIGD2A*	Island
Maternal serum paraxanthine (≥LOD vs. <LOD)								
cg03133256	−0.01691	0.003512	1.48E-06	0.61718	22	24,802,889	*SPECC1L*	
cg05112617	0.009442	0.001908	7.46E-07	0.61718	3	93,698,932	*ARL13B*	Island
cg07677273	0.01606	0.003938	4.56E-05	0.621864	4	37,004,924		
cg08679638	−0.01315	0.003147	2.93E-05	0.621864	4	88,883,651		
cg17596409	−0.01198	0.002925	4.23E-05	0.621864	4	153,186,305		
Maternal serum theobromine at 8 weeks gestation (comparing tertile 2 vs. tertile 1)								
cg09460369	0.028	0.005	1.43E-08	0.011927	8	61,429,249	*RAB2A*	Island
cg14651896	−0.012	0.002	2.67E-07	0.111267	9	124,094,730	*GSN*	
cg22906451	−0.014	0.003	4.22E-07	0.117129	19	1,994,824	*BTBD2*	South Shore
cg08151407	0.017	0.003	6.78E-07	0.14123	3	183,171,057	*LINC00888*	
cg15326613	−0.018	0.004	2.56E-06	0.222618	6	114,132,612		
Maternal serum theobromine at 8 weeks gestation (comparing tertile 3 vs. tertile 1)								
cg00282267	0.03608	0.007114	3.95E-07	0.217159	7	36,803,916		
cg20400017	0.01518	0.003026	5.21E-07	0.217159	9	120,535,563		
cg03818021	−0.0116	0.002421	1.66E-06	0.27671	5	1,798,716	*MRPL36*	North Shore
cg27468830	0.01561	0.003248	1.53E-06	0.27671	7	157,227,072		
cg18209808	−0.02923	0.006053	1.38E-06	0.27671	12	27,993,787		
Pregnancy self-report								
Any type of caffeinated beverage (8 weeks gestation)								
cg26341453	−0.0101	0.002095	1.44E-06	0.599156	1	28,593,732		
cg03424508	−0.05267	0.01078	1.02E-06	0.599156	17	86,925		South Shore
cg05164540	−0.01735	0.003965	1.21E-05	0.621971	5	107,896,573		
cg15754587	0.01422	0.003302	1.66E-05	0.621971	6	25,170,368		
cg22149610	−0.00779	0.001812	1.74E-05	0.621971	6	31,857,034		Island
Any type of caffeinated beverage (12–36 weeks gestation)								
cg19954884	−0.00869	0.001737	5.69E-07	0.198177	4	129,775,763	*JADE1*	
cg07791897	−0.01494	0.003027	7.99E-07	0.198177	5	55,897,584		
cg22141918	0.01674	0.003414	9.51E-07	0.198177	14	24,539,803	*CPNE6*	
cg24179591	−0.01066	0.002149	6.96E-07	0.198177	16	66,967,636	*FAM96B*	North Shore
cg20646219	0.01083	0.002318	2.97E-06	0.412843	17	38,490,228	*RARA*	

Abbreviations: CpG, cytosine-guanine; DNAm, DNA methylation; FDR, false discovery rate; LOD, limit of detection.

TABLE 4 Overlapping canonical pathways for CpG sites identified in preconception (intake of any caffeinated beverage) and early pregnancy (serum theobromine), by category

	β-values	SE	*P* Value	CpG sites	Genes
Category 1: intracellular and second messenger signaling					
Protein kinase A signaling					
Theobromine	− 0.019	0.005	6.46E-05	cg12534645	*CAMK2A*
	− 0.012	0.003	9.26E-05	cg14129040	*CREB1*
	− 0.018	0.004	7.97E-06	cg24319143	*DUSP22*
	− 0.013	0.003	0.000102	cg08315283	*NFKBIA*
Caffeinated beverage intake	0.006	0.003	1.79E-05	cg18871670	*ANAPC13*
	− 0.006	0.002	0.000158	cg04459013	*EPM2A*
	0.006	0.002	0.000217	cg10290200	*FLNC*
	0.011	0.003	0.000277	cg11411922	*MYLK2*
	− 0.006	0.001	6.27E-06	cg13552999	*PTPN21*
cAMP-mediated signaling					
Theobromine	− 0.019	0.005	6.46E-05	cg12534645	*CAMK2A*
	0.008	0.002	2.21E-05	cg22426570	*CHRM2*
	− 0.012	0.003	9.26E-05	cg14129040	*CREB1*
Caffeinated beverage intake	0.016	0.004	9.79E-05	cg09028487	*MC1R*
	− 0.009	0.002	9.92E-05	cg19653161	*RGS10*
G-protein coupled receptor signaling					
Theobromine	− 0.019	0.005	6.46E-05	cg12534645	*CAMK2A*
	0.008	0.002	2.21E-05	cg22426570	*CHRM2*
	− 0.012	0.003	9.26E-05	cg14129040	*CREB1*
	− 0.013	0.003	0.000102	cg08315283	*NFKBIA*
Caffeinated beverage intake	0.016	0.004	9.79E-05	cg09028487	*MC1R*
	− 0.009	0.002	9.92E-05	cg19653161	*RGS10*
AMP-activated protein kinase signaling[1]					
Theobromine	0.008	0.002	2.21E-05	cg22426570	*CHRM2*
	− 0.012	0.003	9.26E-05	cg14129040	*CREB1*
	− 0.004	0.001	9.26E-05	cg17287814	*PPP2R2A*
	0.028	0.005	1.43E-08	cg09460369	*RAB2A*
Caffeinated beverage intake	0.004	0.001	0.000236	cg06875318	*ORAI1*
	0.021	0.005	2.68E-05	cg13345558	*PRKAA1*
Category 2: cellular growth, proliferation, and development					
Kinetochore metaphase signaling					
Theobromine	0.004	0.001	0.000033	cg27329848	*BUB1B*
Caffeinated beverage intake	0.006	0.001	1.79E-05	cg18871670	*ANAPC13*
	0.01	0.002	3.81E-05	cg08133816	*CENPT*
	− 0.012	0.003	0.000294	cg21751147	*MAD1L1*
ILK signaling					
Theobromine	− 0.012	0.003	9.26E-05	cg14129040	*CREB1*
	− 0.007	0.002	0.00007	cg18778433	*PARVA*
	− 0.004	0.001	9.26E-05	cg17287814	*PPP2R2A*
Caffeinated beverage intake	− 0.024	0.006	0.000136	cg11689813	*ACTA1*
	0.006	0.002	0.000217	cg10290200	*FLNC*
Category 3: Cellular immune response					
PI3K signaling in B lymphocytes					
Theobromine	− 0.019	0.005	6.46E-05	cg12534645	*CAMK2A*
	− 0.012	0.005	9.26E-05	cg14129040	*CREB1*
	− 0.013	0.003	0.000102	cg08315283	*NFKBIA*
Caffeinated beverage intake	− 0.025	0.007	0.000271	cg02211519	*CD81*
	0.011	0.003	0.000205	cg00150231	*INPP5D*
iCOS-iCOSL signaling in T helper cells					
Theobromine	− 0.019	0.005	6.46E-05	cg12534645	*CAMK2A*
	− 0.013	0.003	0.000102	cg08315283	*NFKBIA*
Caffeinated beverage intake	0.011	0.003	2.05E-04	cg00150231	*INPP5D*
Category 4: cellular stress and injury					
ATM signaling[2]					
Theobromine	− 0.012	0.005	9.26E-05	cg14129040	*CREB1*
	− 0.013	0.003	0.000102	cg08315283	*NFKBIA*
	− 0.004	0.001	9.26E-05	cg17287814	*PPP2R2A*
Caffeinated beverage intake	− 0.007	0.002	2.03E-04	cg24532898	*TLK1*
Hypoxia signaling in the cardiovascular system[3]					
Theobromine	− 0.012	0.005	9.26E-05	cg14129040	*CREB1*
	− 0.013	0.003	0.000102	cg08315283	*NFKBIA*
Caffeinated beverage intake	0.022	0.006	3.27E-04	cg17177602	*UBE2J2*

[1] AMP-activated protein kinase signaling overlaps with the "cellular growth, proliferation, and development" category.

[2] ATM signaling is also part of the "cell cycle regulation" category.

[3] Hypoxia signaling in the cardiovascular system is also part of the "cancer," "cardiovascular signaling," and "ingenuity toxicity list pathways" categories. Abbreviations: CpG, cytosine-guanine; ILK, integrin-linked kinase; PI3K, phosphoinositide 3-kinase; iCOS, inducible costimulator; iCOSL, inducible costimulator ligand; ATM, ataxia-telangiectasia mutated

caffeinated beverage consumption should be considered in future studies. Second, participants in our study population had relatively low reported consumption of coffee and tea intake, which was also reflected in serum caffeine concentrations, and this may limit generalizability. The median concentration in the first tertile of preconception caffeine intake is consistent with a clinical trial of adults (a serum caffeine value of 0.03 μg/mL corresponded to no cups of coffee) (44), and our median value of preconception caffeine was less than what was observed in another low-consumption cohort of premenopausal women (45). Further, our highest preconception tertiles (but not 8-week-gestation tertiles) of caffeine and paraxanthine were similar to the highest quartile of plasma caffeine (>0.66 μg/mL) and paraxanthine (>0.23 μg/mL) in a cohort of pregnant women at gestational weeks 8–13 (46). Additionally, the prevalence of preconception coffee consumption was about 20% in this study. In contrast, the prevalence of coffee consumption among women over 20 years of age was 60.3% based on an examination of NHANES 2011–2016 data (22). Tea consumption was also low in our study population (7%), limiting our ability to examine its intake directly with DNAm. In an EWAS combining 4 European cohorts of older adults, Ek et al. (17) found that tea consumption in women, but not men, was associated with DNAm and mapped to genes related to estradiol. We also did not systematically capture nonbeverage sources of caffeine by self-report in questionnaires/diaries; however, the serum caffeine metabolites would reflect recent exposure regardless of source (e.g., foods, medications). Further, it is also possible that this cohort of women attempting pregnancy may have exhibited different behaviors (i.e., intentionally reducing caffeine consumption) compared to women with unplanned pregnancies (47, 48). Therefore, research among more diverse women of reproductive age with higher levels of caffeine exposure from coffee and tea sources is warranted. Third, the generalizability of results may be further limited, as our study population consisted of White women with a history of 1–2 pregnancy losses who had a live birth delivery during follow-up. Lastly, gene expression data were not available in this study, so we were unable to determine whether differences in methylation correlate with cellular activity. Similarly, genotype data were not available to account for the role of underlying genetics on DNAm, particularly genotypes that might be relevant to caffeine metabolism (i.e., *CYP1A2*). As this discovery study is limited in sample size, the findings should be validated in additional independent studies. Further research in this area would benefit from the integration of genomic and transcriptomic data.

In summary, few differences in cord blood DNAm at individual CpG sites were identified in association with maternal caffeine intake. Future work should examine these associations among women attempting pregnancy, who have a greater variability in caffeinated beverage consumption both within and outside of the recommended ranges. DNAm changes in neonatal cord blood from preconception or early pregnancy theobromine exposure may be linked to energy metabolism, gene expression, and cell cycle function. This suggests that epigenetic mechanisms may underlie the previous associations between maternal caffeine exposure and adverse metabolic outcomes in childhood, including obesity and liver fat deposits. However, additional studies are needed to explore potential underlying mechanisms among women attempting pregnancy with higher caffeine exposure.

This work utilized the computational resources of the NIH High Performance Computing Biowulf cluster (http://hpc.nih.gov).

The authors' responsibilities were as follows—KJP, EFS, and EHY: designed and conducted the research; WG: provided essential materials; KJP, AP-S, and SLR: analyzed data or performed the statistical analysis; KJP: wrote the manuscript; KJP and EHY: have primary responsibility for the final content; SKZ, KCS, RMS, and SLM: provided essential feedback in writing the manuscript; and all authors: read and approved the final manuscript.

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Data Availability

Data described in the manuscript, code book and analytic code will be made available upon request pending application and approval.

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