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Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans

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ABSTRACT **The *in utero* availability of methyl donors, such as choline, may modify fetal epigenetic marks and lead to sustainable functional alterations throughout the life course. The hypothalamic-pituitary-adrenal (HPA) axis regulates cortisol production and is sensitive to perinatal epigenetic programming. As an extension of a 12-wk dose-response choline feeding study conducted in third-trimester pregnant women, we investigated the effect of maternal choline intake (930 *vs.* 480 mg/d) on the epigenetic state of cortisol-regulating genes, and their expression, in placenta and cord venous blood. The higher maternal choline intake yielded higher placental promoter methylation of the cortisol-regulating genes, corticotropin releasing hormone (*CRH*; $P=0.05$) and glucocorticoid receptor (*NR3C1*; $P=0.002$); lower placental *CRH* transcript abundance ($P=0.04$); lower cord blood leukocyte promoter methylation of *CRH* ($P=0.05$) and *NR3C1* ($P=0.04$); and 33% lower ($P=0.07$) cord plasma cortisol. In addition, placental global DNA methylation and dimethylated histone H3 at lysine 9 (H3K9me2) were higher ($P=0.02$) in the 930 mg choline/d group, as was the expression of select placental methyltransferases. These data collectively suggest that maternal choline intake in humans modulates the epigenetic state of genes that regulate fetal HPA axis reactivity as well as the epigenomic status of fetal derived tissues.—Jiang, X., Yan, J., West, A. A., Perry, C. A., Malysheva, O. V., Devapatla, S., Pressman, E., Vermeylen, F., Caudill, M. A. Maternal choline intake alters the epigenetic state of fetal cortisol-regulating genes in humans. *FASEB J.* 26, 000–000 (2012). www.fasebj.org**

Key Words: methyl donor · prenatal nutrition · DNA methylation · HPA axis

Nutrients such as choline, betaine, and folate provide methyl groups for cellular methylation reactions. In animal models, the maternal consumption of these methyl donors modifies fetal epigenetic marks (*e.g.*, promoter region DNA and histone methylation; refs. 1–4) and leads to sustainable functional alterations throughout the life course (5, 6). Nevertheless, evidence of fetal epigenetic modification by maternal consumption of methyl donors in humans is lacking.

Our research group recently conducted a 12-wk dose-response choline feeding study in which pregnant women (gestational age wk 26–29) were randomized to choline intakes of 480 [approximating the adequate intake (AI) for choline] or 930 mg/d throughout their third trimester. We found that a higher maternal choline intake increased the use of choline as a methyl donor, as evidenced by elevations in maternal and fetal plasma dimethylglycine, the metabolite produced when choline is used as a methyl donor (7). The increased use of choline as a methyl donor may alter the epigenetic state of genes regulated by methylation as previously shown in animal studies (2, 3).

The methylation state of the cortisol-regulating genes, corticotropin-releasing hormone (*CRH*), and nuclear receptor subfamily 3, group C, member 1 (*NR3C1*), may be particularly sensitive to prenatal and early postnatal exposures (1, 8–10). These genes en-

Abbreviations: 5mdC, 5-methyl-2′-deoxycytidine; AI, adequate intake; BHMT, betaine-homocysteine *S*-methyltransferase; CpG, cytosine-phosphate-guanine; CRH, corticotropin releasing hormone; DNMT1, DNA (cytosine-5-)-methyltransferase 1; DNMT3A, DNA (cytosine-5-)-methyltransferase 3 α; DNMT3B, DNA (cytosine-5-)-methyltransferase 3 β; EHMT2, euchromatic histone-lysine *N*-methyltransferase 2; GLM, general linear model; GNAS-AS1, guanine nucleotide binding protein, α-stimulating, antisense transcript; H3K4me2, dimethylated histone H3 at lysine 4; H3K9me2, dimethylated histone H3 at lysine 9; H3K27me3, trimethylated histone H3 at lysine 27; HPA, hypothalamic-pituitary-adrenal; IGF2, insulin-like growth factor 2; IL10, interleukin 10; IUGR, intrauterine growth restriction; LC, liquid chromatography; LEP, leptin; MS, mass spectrometry; NGFI-A, nerve growth factor inducible protein A; NR3C1, nuclear receptor subfamily 3, group C, member 1; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; SUV39H1, suppressor of variegation 3–9 homolog 1

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code components of the hypothalamic-pituitary-adrenal (HPA) axis, which have important roles in stress response, immunity, and glucose metabolism. In response to stress, the hypothalamus produces CRH, which stimulates the secretion of adrenocorticotropic hormone from the anterior pituitary and subsequently glucocorticoids (*e.g.*, cortisol) from the adrenal gland. *NR3C1* encodes the glucocorticoid receptor, which plays a critical role in central HPA inhibition *via* glucocorticoid negative feedback (11). The programming of the HPA axis through promoter region methylation of *CRH* and *NR3C1* may have implications for susceptibility to stress-related (*i.e.*, autoimmune disorders and mental health disturbances; refs. 12, 13) and metabolic (*e.g.*, hypertension and diabetes) diseases later in life (14, 15).

As an extension of our 12-wk choline feeding study conducted in third-trimester pregnant women (7), this study investigated the influence of maternal choline intake (480 *vs.* 930 mg/d) on the epigenetic state of cortisol-regulating genes, and their expression, in placenta and cord venous blood. As varied maternal choline intake may systematically affect a wide array of placental genes regulated by DNA and histone methylation, 4 additional genes for site-specific methylation measurements were included: insulin-like growth factor 2 (*IGF2*), leptin (*LEP*), guanine nucleotide binding protein, α-stimulating, antisense transcript (*GNAS-AS1*), and interleukin 10 (*IL10*). Selection of these genes was based on prior evidence of methylation regulation, susceptibility to nutritional exposures, and associations with chronic disease risk (16, 17). Finally, given that prenatal choline exposure alters epigenomic readouts in animal models (2, 4), global DNA and histone methylation were measured.

MATERIALS AND METHODS

Study participants

Healthy third-trimester (wk 26–29 gestation) singleton pregnant women aged ≥21 yr were recruited from Ithaca, NY, USA, and surrounding areas between January 2009 and October 2010. During the screening phase, all study volunteers completed a questionnaire that queried participants on their age, education, work status, ethnicity and race, prepregnancy body mass index, parity, health history, medication and nutritional supplement use, and physical activity. Entry into the study was contingent on good health status (*i.e.*, no chronic diseases, normal kidney and liver function, and no anemia). Additional inclusion criteria included no tobacco or alcohol product use and a willingness to comply with the study protocol. For those who were included in the study, information on gestational weight gain, health insurance, mode of delivery, obstetrical complications, and newborn sex and health characteristics was obtained from medical charts after delivery. Twenty-six of the 29 pregnant women who started the study completed it. Of the 26 women completing the study, placental samples were retrieved from 24 participants (n=12/choline intake group) and cord blood samples were retrieved from 23 participants. The study protocol was approved by the Institutional Review Board for Human Study Participant Use at Cornell University and at Cayuga Medical Center (Ithaca, NY, USA). Written informed consent was obtained from all the participants before study entry. The study was registered at ClinicalTrials.gov as NCT01127022 (http://clinicaltrials.gov/). Additional information regarding the study participants is described elsewhere (7).

Study design and diet

This was a 12-wk controlled feeding study in which pregnant women were randomized to either 480 (approximating the choline AI; n=12) or 930 (n=12) mg choline/d. The choline was derived from the diet (380 mg/d) plus supplemental choline chloride (either 100 or 550 mg choline/d for the 480 or 930 mg/d intake levels, respectively). During the last 6 wk of the study, ~20% of the total choline intake was provided as deuterium-labeled trimethyl d9-choline (Cambridge Isotope Laboratories, Andover, MA, USA). Throughout the 12 wk study, the participants consumed a 7-d cycle menu as detailed in Yan *et al.* (7). All food and beverages were provided by the investigators, and 1 meal/d was consumed onsite throughout the week. All other meals and beverages were provided as takeaways. In addition to the supplemental choline, a prenatal multivitamin (Pregnancy Plus; Fairhaven Health, Bellingham, WA, USA) and DHA (200 mg; Neuromins; Nature's Way Products, Lehi, UT, USA) were provided daily; a potassium and magnesium supplement (General Nutrition Corp, Pittsburgh, PA, USA) was provided 3×/wk. The supplements were consumed with the onsite meal under the supervision of the investigators. At the end of the study, participants continued on their choline supplement until the delivery of their babies.

Sample collection

Fasting (10 h) venous blood samples were obtained at baseline (wk 0) and at the end of the study (wk 12) in 10-ml EDTA tubes (Vacutainer; Becton-Dickinson, Franklin Lakes, NJ, USA). In addition, maternal EDTA-blood was obtained within 24 h of delivery and cord venous EDTA-blood was collected immediately after delivery (7). The placenta samples (n=21) were processed within 10–30 min of delivery, except for 3 cases: n = 2 in the 930 and n = 1 in the 480 mg/d groups, which were processed within 60–90 min of delivery. After the amniotic sac was removed, the placenta was visually divided into 4 quadrants. Full-thickness biopsies (0.5 cm × 0.5 cm × placenta depth) were obtained from the center of each quadrant, rinsed with PBS, and either flash-frozen in liquid nitrogen or immersed in RNAlater (Qiagen, Valencia, CA, USA). The samples were stored at −80°C until subsequent analysis. For DNA, protein, and metabolite measurements, 1 tissue biopsy from each quadrant was pooled and ground in liquid nitrogen. For RNA extraction, 1 tissue biopsy from one of the quadrants was homogenized. As the placenta is a heterogeneous tissue, the results obtained from our experiments represent an average effect of maternal choline intake on a subset of the placental cells.

Analytical measurements

Cortisol concentration

The relative intensity of maternal and umbilical cord plasma cortisol was initially measured by liquid chromatography (LC)-mass spectrometry (MS) as part of a metabolite panel by a diagnostics and services company Metabolon (Durham, NC, USA). The actual concentrations of cortisol (μg/dl) were later quantified in-house using a commercially available

ELISA kit (Diagnostic Automation, Calabasas, CA, USA) according to the manufacturer's instructions.

Site-specific methylation

Site-specific methylation was analyzed using base-specific cleavage and MS (18). The loci of interest included regions in the promoters of *CRH*, *GNAS-AS1*, *LEP*, and *IL10* (16); the 5′ untranslated exon 1F (and flanking regions) of *NR3C1* (8); and a part of the differentially methylated region 0 (DMR0) of *IGF2* (19, 20). Genomic DNA was extracted *via* the DNeasy blood and tissue kit (Qiagen), and 1 μg extracted DNA was bisulfite treated using the EZ 96-DNA methylation kit (Zymo, Irvine, CA, USA). DNA sequences of interest were PCR amplified using the bisulfite-treated DNA as the template and published primers (8, 16) that incorporate the T7 tag (Supplemental Table S1). The amplification products were analyzed with the MassArray EpiTyper system (Sequenom, San Diego, CA, USA) at the Cornell Life Sciences Core Laboratories Center (Cornell University). Briefly, the PCR products were *in vitro* transcribed by T7 polymerase, digested by RNase A, and measured *via* matrix-assisted laser desorption ionization/time of flight mass spectrometry. Percentage methylation of the cytosine residues was determined for each cytosine-phosphate-guanine (CpG) unit, which may contain 1 or multiple adjacent CpG dinucleotides (CpG sites) that cannot be resolved individually by EpiTyper. The CpG sites belonging to a particular CpG unit of each gene are shown in Supplemental Table S2. Only CpG units with measurement success rates of >75% were included in the final analyses (Supplemental Table S3 and ref. 16).

RNA extraction and quantitative real-time PCR

RNA was extracted *via* a commercially available kit (RNeasy Mini kit; Qiagen) from placental tissues. The concentration and purity of the isolated RNA were assayed with NanoDrop ND-1000 instrumentation (Thermo, Wilmington, DE, USA). All of the samples had RNA concentrations >300 ng/μl and A260/280 ratios of >2.0, indicating adequate quantity and purity, respectively. The quality of RNA samples was verified by agarose gel electrophoresis, which showed discreet 18S and 28S ribosomal RNA bands. Reverse transcription was performed *via* the ImProm-II Reverse Transcription System (Promega, Madison, WI, USA). The reaction conditions were as follows: 25°C for 10 min, 42°C for 40 min, 95°C for 5 min, and quenching at 4°C for 5 min. Gene transcript abundance of *CRH* and *NR3C1*, as well as certain DNA and histone methyltransferases [*i.e.*, DNA (cytosine-5-)-methyltransferase 3 α (*DNMT3A*), DNA (cytosine-5-)-methyltransferase 3 β (*DNMT3B*), DNA (cytosine-5-)-methyltransferase 1 (*DNMT1*), euchromatic histone-lysine *N*-methyltransferase 2 (*EHMT2*), and suppressor of variegation 3–9 homologue 1 (*SUV39H1*)] were analyzed by quantitative real-time PCR with the SYBR Green system in a Roche LightCycler480 machine (Roche, Basel, Switzerland). The reaction conditions were as follows: 95°C for 5 min, followed by 40 cycles with 15 s at 95°C, 20 s at 60°C, and 20 s at 72°C. Data were expressed as the fold change of the gene of interest relative to the housekeeping gene (21) glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). All primers were designed using GeneRunner 3.01 (http://www.softpedia.com/). The primers were as follows: *CRH*, F: 5′-*TGAAACATAGAGGGAGAGAGGG*-3′, R: 5′-*ACCTGGAAACGGAAACTAAACG*-3′; *NR3C1*, F: 5′-TACCTAACGCCCTATTTTTGCA-3′, R: 5′-AGCTGGTTATCTGGAATCACAA-3′; *DNMT3A*, F: 5′-ACGGCAAATTCTCAGTGGTG-3′, R: 5′-GGACCTCGTAGATGGCTTTG-3′; *DNMT3B*, F: 5′-CAAACCCAACAACACGCAAC-3′, R: 5′-CAGCTGTGCGTCTTCGAGTC-3′; *DNMT1*, F: 5′-GTGTGGGAAATGTAAAGCCTGC-3′, R: 5′-TCATCGTCATCTGCCTCCTTCA-3′; *EHMT2*, F: 5′-AGACAGAGCGAGGGTTTGAGG-3′, R: 5′-AGCTCTCCGTCCACACTCTCA-3′; *SUV39H1*, F: 5′-ATAGACAACCTTGACGAGCGG-3′, R: 5′-GGGTCCACTTGCATGTTGTAA-3′; *GAPDH*, F: 5′-TGTTGCCATCAATGACCCCTT-3′, R: 5′-CTCCACGACGTACTCAGCG-3′.

Global DNA methylation

Global DNA methylation was measured in placental tissues, cord blood leukocytes, and maternal leukocytes (wk 0 and 12) *via* LC-MS/MS, as described by Song *et al.* (22) with modifications based on our instrumentation (23). Briefly, DNA was extracted with the DNeasy blood and tissue kit (Qiagen) and digested with nuclease P1 (Sigma-Aldrich, St. Louis, MO, USA), phosphodiesterase 1 (Sigma-Aldrich) and alkaline phosphatase (Sigma-Aldrich). Hydrolyzed DNA was separated and analyzed with a LC-MS/MS system consisting of a TSQ Quantum mass spectrometer with electrospray ionization source operated in positive ion mode (Thermo), a refrigerated Accela autosampler (Thermo), and an Accela pump with degasser (Thermo). Global DNA methylation [5-methyl-2′-deoxycytidine (5mdC)] is expressed as a percentage of 2′-deoxyguanosine (representing total 2′-deoxycytidine). Isotopic enrichment (d3-5mdC) is expressed as a percentage of total 5mdC (labeled and unlabeled metabolite). Natural isotopic enrichment and background noise were addressed by subtracting the signal obtained from control placental samples that were not exposed to labeled choline.

Global histone methylation

Global histone methylation marks dimethylated histone H3 at lysine 4 (H3K4me2), dimethylated histone H3 at lysine 9 (H3K9me2), and trimethylated histone H3 at lysine 27 (H3K27me3) were measured using Western blot analysis. Histone proteins were extracted from 100 mg frozen placental tissue and 200 μl of buffy coat. Samples were incubated in 1 ml lysis buffer (40 mM sodium citrate and 1% Triton X-100) for 10 min and then centrifuged at 2000 rpm for 10 min at 4°C. The pellet was resuspended in 0.2 N hydrochloric acid at a cell density of 4×10^7 /ml and incubated at 4°C overnight. Samples were centrifuged again. Total protein concentrations of the supernatant were quantified by the Bradford assay (Thermo), and histone bands were visualized by SDS-PAGE and Coomassie blue staining to ensure the presence of histones before Western blot analysis. The extracts were separated by SDS-PAGE and transferred to Immobilon-FL polyvinylidene fluoride transfer membranes (Millipore, Billerica, MA, USA). Membranes were blocked in Odyssey blocking buffer (LI-COR, Lincoln, NE, USA) and incubated overnight with anti-H3K4me2 (GeneTex, Irvine, CA, USA), anti-H3K9me2 (Abcam, Cambridge, MA, USA), anti-H3K27me3 (GeneTex), and anti-Histone 3 (Abcam) antibodies. Secondary antibodies were either IRDye 800CW goat anti-rabbit or IRDye 680 goat anti-mouse (LI-COR), depending on the primary antibodies. Target protein bands were visualized and quantified using the LI-COR Odyssey imaging system (LI-COR) and expressed as the ratio of intensity of the histone epigenetic mark to total histone 3 protein (4).

S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) measurements

SAM and SAH measurements were performed using the method described by Kim *et al.* (24) with modifications based on our LC-MS/MS instrumentation. Briefly, 50 mg of placen-

TABLE 1. *Baseline characteristics of singleton third-trimester pregnant women before randomization to 480 or 930 mg/d maternal choline intake groups*

Baseline characteristic	480 mg/d	930 mg/d
Subjects (*n*)	12	12
Age (yr)[a]	29 (25–33)	28 (22–34)
Prepregnant body mass index (kg/m^2)[a]	23.6 (20.2–31.9)	23.4 (19.9–29.8)
Parity, primiparas/multiparas	8/4	3/9*
Gestational age (wk)[a]	27 (26–29)	27 (26–28)
Ethnicity, Caucasian/African American/Latino/Asian/Other	8/0/2/1/1	6/1/2/3/0
Physical activity, usual daily activity/exercise >3× per wk/unknown	4/8/0	1/9/2
Education, high school/college or university level	1/11	4/8
Work status, not employed/employed	2/10	2/10
Health insurance, government subsidized/private/unknown[b]	2/8/2	4/8/0

[a]Values are means, with range in parentheses.[b]This parameter served as a surrogate of income. Eligibility for government subsidized insurance implied a relatively lower income. *$P < 0.05$ *vs.* 480 mg/d group, χ^2 test.

tal tissue was mixed with d3-SAM (CDN Isotopes, Pointe-Claire, QC, Canada) and d4-SAH (Cayman Chemical, Ann Arbor, MI, USA) as internal standards and homogenized in 300 μl of 25 mM ammonium acetate. The homogenate was treated with 250 μl of 1 N perchloric acid and centrifuged at 12,000 *g* for 10 min at 4°C. An aliquot of the supernatant (550 μl) was neutralized with 300 μl of 1 M ammonium hydroxide. The neutralized samples were applied to the Sep-Pak Vac C18 cartridge (Waters, Milford, MA, USA) preconditioned with 1 ml methanol, 750 μl of 0.1 N NaOH, and 1 ml water. The samples were washed with 1 ml water and eluted with 1 ml 85:15 water:methanol (vol/vol) with 0.1% formic acid. The eluent was dried in a speed vacuum and redissolved in 150 μl 0.05 M ammonium formate with 0.1% formic acid. Analyses were conducted with the LC-MS/MS instrumentation described in the global DNA methylation section. Compounds of interest were separated by HPLC using a Luna C18(2) column (250×4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA) with matching guard column (4×3 mm). The mobile phase containing acetonitrile and 0.1% formic acid in 0.05 M ammonium formate was run at a rate of 500 μl/min with 2% acetonitrile for 4 min, a linear gradient from 2 to 80% acetonitrile for 7 min, followed by a return to 2% acetonitrile over the next 4 min. The mass spectrometer was operated in positive-ion mode using electrospray ionization. The metabolites of interest were detected in a multiple reaction monitoring mode with the following transitions: SAM, *m/z* 399→250; d3-SAM 402→250; d4-SAH, *m/z* 385→136; SAH-d4, *m/z* 389→136. Quantification of SAM and SAH was performed by comparing samples with the signals obtained from the standards using Xcalibur software (Thermo).

Statistical analysis

Student's *t* tests (for continuous dependent variables) and χ^2 tests (for categorical variables) were performed to test for differences between the choline intake groups at baseline and at delivery.

General linear models (GLMs) were constructed to assess the effects of maternal choline intake (independent variable) on the dependent variables (*e.g.*, cord plasma cortisol concentration, placental gene transcript abundance, and global DNA and histone methylation).

For the site-specific CpG methylation data, GLMs were constructed to analyze the effects of maternal choline intake on the average CpG methylation of each gene (*i.e.*, the mean methylation levels of all the CpG units). To explore the effects of maternal choline intake on individual CpG units, mixed models were run. The mixed models included choline intake, all the CpG units (*e.g.*, CpG unit A, CpG unit B, *etc.*) within the region of interest, and the 2-way interaction between the CpG units and choline intake as fixed factors. Participant identifier was included as a random factor. The analyses were followed by individual contrasts assessing the effect of choline on each CpG unit.

Candidates for entry as covariates into the GLMs and mixed models are listed in **Table 1** and **Table 2**. To adjust for the potential influence of circadian rhythm, time of delivery was included as an additional covariate. The covariates not achieving a statistical significance of $P < 0.1$ were removed from the models in a stepwise process. Baseline measures (wk 0) were also entered into the models as covariates for maternal DNA and histone methylation. To account for batch effect, batch was included in the models as a covariate if the measurements of a variable were done in multiple runs. All 2-way interactions between the covariates and maternal choline intake were tested in the models. Covariates retained in the final statistical models are specified following the presentation of the *P* values.

In addition, paired *t* tests were employed to assess differences in global DNA methylation among tissues. Pearson's correlation coefficient (*r*) was used to examine the associations between select variables (*e.g.*, maternal and fetal cortisol concentrations).

Plots and histograms of the residuals were used to assess normality and variance homogeneity in the models. Dependent variables that deviated from the normal distribution (*e.g.*, placental gene transcript abundance) were logarithmically transformed to meet the assumption of normality. Differences between choline intake groups were considered to be significant at $P \leq 0.05$; $P < 0.10$ was considered to be indicative of trends. Values are presented as means ± SE. If covariates were retained in the final model, the values presented are predicted mean; *P* values are 2-tailed. All analyses

TABLE 2. *Maternal and neonatal characteristics at delivery for pregnant women consuming 480 or 930 mg choline/d throughout the third trimester*

Characteristic	480 mg/d	930 mg/d
Mode of delivery, caesarean section/ vaginal	3/9	2/10
Gestational age at birth (wk)	40 (39–42)	40 (39–40)
Infant sex, female/male	3/9	4/8
Infant birth weight (kg)	3.4 ± 0.1	3.4 ± 0.1
Infant Apgar score	8–9; 9	8–9; 9

were performed using SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA).

RESULTS

Mother and neonate characteristics

At baseline, maternal characteristics of the third-trimester pregnant women did not differ (P=0.14–0.99) between the choline intake groups (*i.e.*, 930 *vs.* 480 mg/d) with the exception of parity (*i.e.*, there were more primiparous women in the 480 mg/d intake group; P=0.04; Table 1). All babies were delivered without major complications and were apparently healthy. Mode of delivery, gestational age at birth, infant sex, infant birth weight, and Apgar score did not differ (P=0.11–0.79) between the choline intake groups (Table 2).

Cord plasma cortisol

Cord plasma cortisol concentrations of both choline intake groups (range: 5.9–56.3 μg/dl) were consistent with previous observations in uncomplicated pregnancies (25). Both vaginal birth (P=0.004) and primiparous pregnancy (P=0.04) positively affected the concentration of cord plasma cortisol. Because the number of women giving birth through caesarean section was small (n=2–3/choline intake group), only cases of vaginal birth were further analyzed. In this subset of samples, cord plasma cortisol concentrations tended to be 33% lower (P=0.07, controlled for parity) in babies born to mothers consuming 930 (20.9±3.6 μg/dl) *vs.* 480 (31.1±3.6 μg/dl) mg choline/d (**Fig. 1*A***). Maternal plasma cortisol did not differ (P=0.17) between the choline intake groups (930 mg/d: 46.1±8.3 *vs.* 480 mg/d: 61.1±6.4 μg/dl; Fig. 1*B*), nor did it correlate with cord plasma cortisol (Pearson's correlation r=0.13; P=0.60; Fig. 1*C*). As expected (25, 26), maternal plasma cortisol was higher (P<0.001) than cord plasma cortisol.

Site-specific CpG methylation

CRH and NR3C1

We assessed CpG methylation of the proximal promoter region of *CRH*. This region interacts with several transcription factors that regulate *CRH* expression (**Fig. 2*A*** and ref. 27). Five CpG units in this region met our quality control criteria and were included in the final analyses. In placental tissue, the higher maternal choline intake (930 *vs.* 480 mg/d) yielded higher (P=0.05, controlled for infant sex) average CpG methylation of the *CRH* promoter region (Fig. 2*B*). In addition, although statistical significance was not achieved, methylation of each individual CpG unit was consistently higher in the 930 mg/d group (Fig. 2*C*). The higher maternal choline intake also resulted in lower (P=0.04) placental *CRH* gene transcript abundance (Fig. 2*D*). In cord leukocytes, the higher maternal choline intake yielded lower (P=0.05) average promoter methylation of *CRH* (Fig. 2*E*) and lower (P=0.02) methylation of CpG unit A (containing CpG site 1; Fig. 2*F*). Maternal choline intake did not affect maternal blood leukocyte average CpG methylation of *CRH* (480 mg/d group: 62.4±0.7%; 930 mg/d group: 61.8±0.8%; P=0.39).

We also examined CpG methylation of the CpG-rich region of *NR3C1* including exon 1F (**Fig. 3*A***). In rats, hippocampal CpG methylation of the homologue of this region (*Nr3c1* exon 1_7) is altered by maternal care with downstream effects on HPA axis reactivity (9). As rat *Nr3c1* exon 1_7 (and presumably human *NR3C1* exon 1F) contains a binding site for nerve growth factor inducible protein A (NGFI-A), the methylation state of this region may modify NGF1-A binding with subsequent effects on *NR3C1* expression. In the present

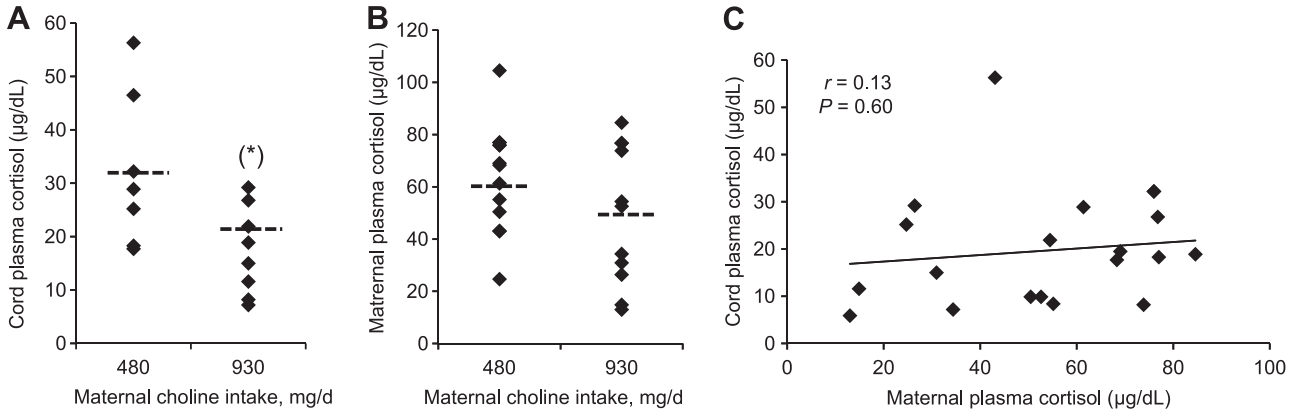

Figure 1. Effect of maternal choline intake (930 *vs.* 480 mg/d) on maternal and fetal cortisol. *A*) For neonates born vaginally, cord plasma cortisol tended to be lower (P=0.07 controlled for parity; n=7–8/treatment group) in the 930 *vs.* 480 mg/d maternal choline intake group. *B*) No differences (P=0.17; n=10–11/group) were detected in maternal plasma cortisol at delivery between the choline intake groups. *C*) No correlation (Pearson's correlation r=0.13; P=0.60) was detected between cord and maternal plasma cortisol concentrations. Dashed lines (*A*, *B*) represent the predicted mean or mean cortisol concentrations, respectively, for each choline intake group.

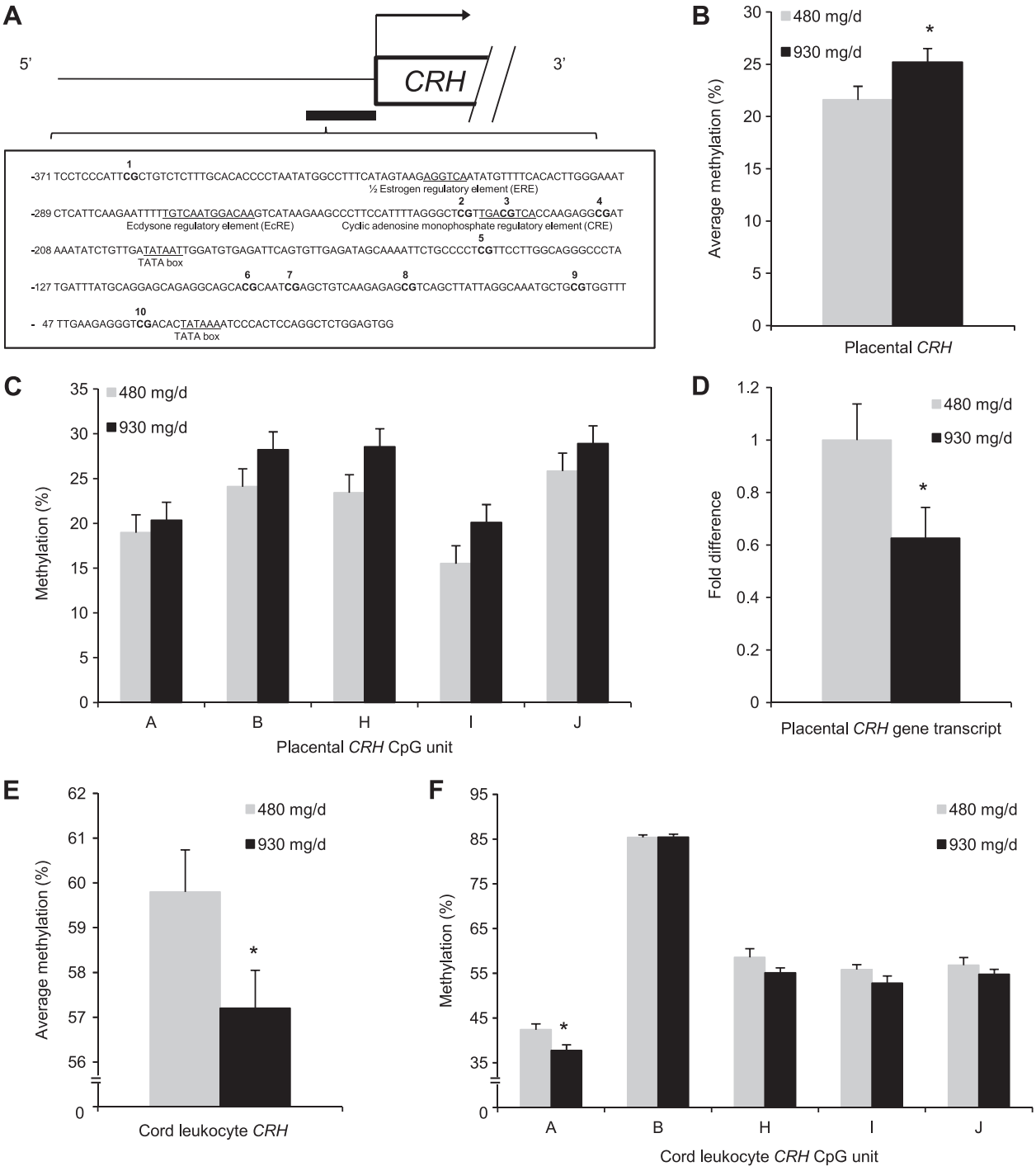

Figure 2. Effect of maternal choline intake (930 *vs.* 480 mg/d) on *CRH* promoter region CpG methylation and gene expression. *A*) Sequence map of the individual CpGs analyzed by EpiTyper and the binding sites of select transcription factors (27). *B*) Average percentage of CpG methylation across the placental *CRH* target promoter region. *C*) Percentage of methylation of each CpG site within the target promoter region of placental *CRH*. *D*) Placental *CRH* gene transcript abundance relative to the 480 mg/d group. *E*) Average percentage of CpG methylation across the cord leukocyte *CRH* target promoter region. *F*) Percentage of methylation of each CpG site within the target promoter region of cord leukocyte *CRH*. Each CpG unit contains 1 CpG site. Only CpG units with measurement success rates > 75% were included in the analyses. Shaded bars, 480 mg/d group; solid bars, 930 mg/d group. Values represent means ± SE, except for *B* and *C*, which are predicted means controlled for infant sex; n = 11–12/group. *$P \leq 0.05$ between choline intake groups.

study, 15 CpG units within this region were included in the analyses. The higher maternal choline intake (930 *vs.* 480 mg/d) yielded higher (P=0.002) average placental *NR3C1* promoter methylation (Fig. 3*B*), as well as higher placental methylation of CpG unit C (containing CpG sites 5–7; P=0.01), F (containing CpG site 12; P=0.001), M (containing CpG site 29; P=0.003), and N (containing CpG sites 30–32; P=0.05;

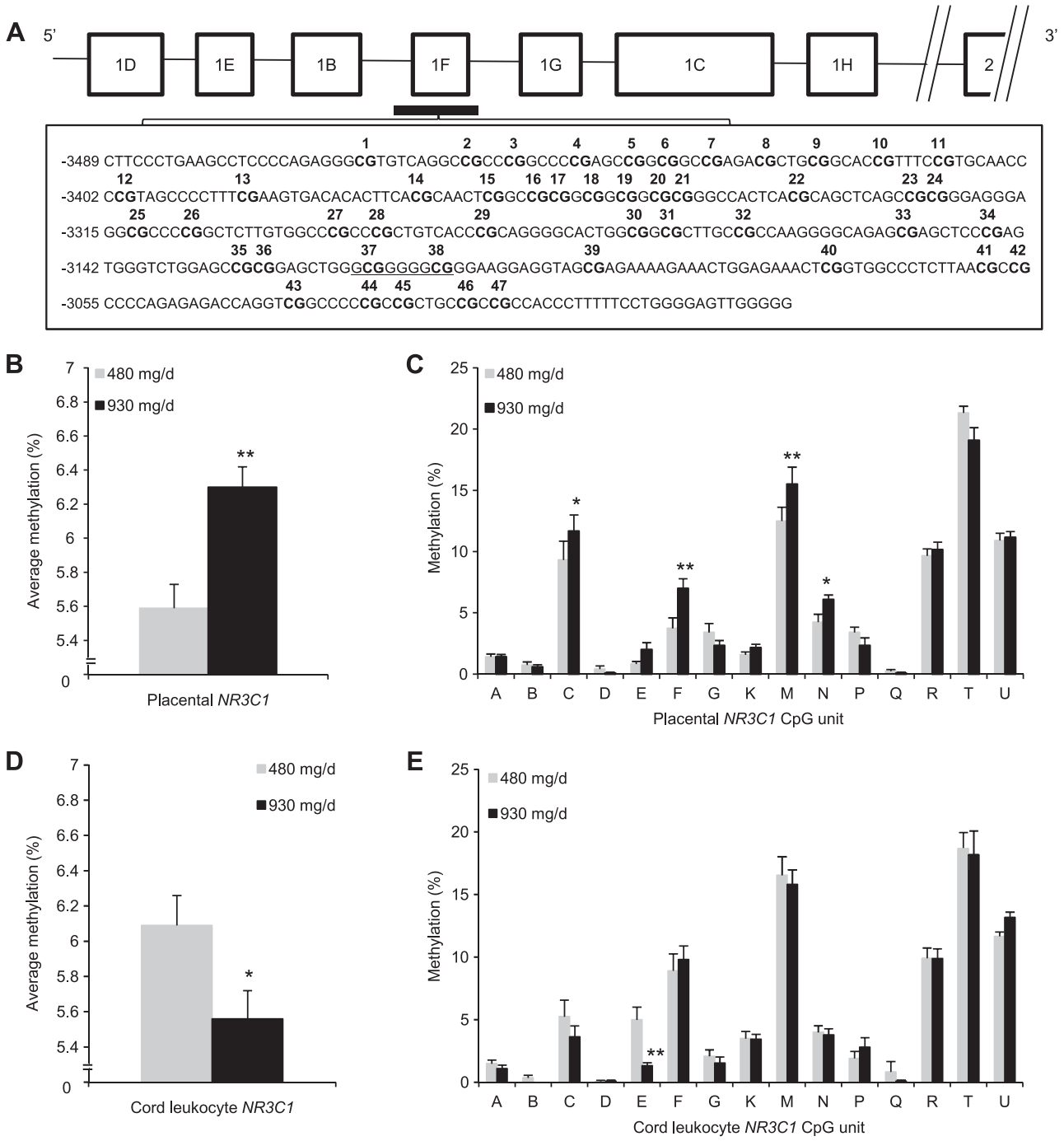

Figure 3. Effect of maternal choline intake (930 *vs.* 480 mg/d) on *NR3C1* promoter region CpG methylation. *A*) Sequence map of individual CpGs analyzed by EpiTyper in *NR3C1* exon 1F (8). *NR3C1* contains multiple nontranslated exon 1s. Translation starts from exon 2. Exon 1F contains a predicted NGFI-A binding site (underlined). *B*) Average percentage of CpG methylation across the placental *NR3C1* target promoter region. *C*) Percentage of methylation of each CpG site within the target promoter region of placental *NR3C1*. CpG unit R represents the NGFI-A binding site. *D*) Average percentage of CpG methylation across the cord leukocyte *NR3C1* target promoter region. *E*) Percentage of methylation of each CpG site within the target promoter region of cord leukocyte *NR3C1*. A CpG unit may contain 1 or multiple CpG sites (Supplemental Table S2). Only CpG units with measurement success rates > 75% were included in the analyses. Shaded bars, 480 mg/d group; solid bars, 930 mg/d group. Values represent means ± SE; n = 8–12/treatment group. $^{**}P < 0.01$ or $^{*}P \leq 0.05$ between choline intake groups.

Fig. 3*C*). In cord leukocytes, the higher maternal choline intake yielded lower (P=0.04) average *NR3C1* promoter methylation (Fig. 3*D*), as well as lower (P=0.003) methylation of CpG unit E (containing CpG sites 10, 11; Fig. 3*E*). However, maternal choline intake did not alter methylation of placental or cord leukocyte CpG unit R (CpG sites 37, 38; P=0.55 and 0.99, respectively), where the predicted NGFI-A binding site resides, nor did it alter placental *NR3C1* transcript abundance (P = 0.44). Maternal choline intake did not

TABLE 3. *Average percentage of CpG methylation across the target regions of select genes in the placental and cord leukocyte samples obtained from the 480 and 930 mg/d maternal choline intake groups*

Gene	Function[a]	Placenta			Cord leukocyte		
		480 mg/d	930 mg/d	*P*	480 mg/d	930 mg/d	*P*
GNAS-AS1	Growth regulation	50.5 ± 1.0	48.0 ± 1.4	0.17	32.2 ± 1.0	32.5 ± 1.0	0.88
IGF2	Growth regulation	36.5 ± 1.5	33.5 ± 1.6	0.21[b]	35.4 ± 0.8	36.4 ± 1.4	0.87
IL10	Anti-inflammation	79.0 ± 0.5	77.6 ± 0.6	0.20	22.6 ± 1.2	21.1 ± 1.1	0.38
LEP	Energy metabolism	24.5 ± 1.6	23.9 ± 1.7	0.78	14.9 ± 0.7	14.0 ± 0.8	0.34[c]

Data without notations are presented as means ± SE. [a]Modified from Talens *et al.* (16).[b]Statistical analysis controlled for parity; values presented are predicted means.[c]Statistical analysis controlled for infant sex; values presented are predicted means.

affect maternal blood leukocyte average CpG methylation of *NR3C1* (480 mg/d group: 5.0±0.2%; 930 mg/d group: 5.5±0.3%; *P*=0.11).

GNAS-AS1, IGF2, IL10, and LEP

To further investigate the influence of maternal choline intake on site-specific DNA methylation, the CpG methylation of 4 additional genes involved in different physiological pathways was assessed. For *GNAS-AS1*, average placental and cord blood leukocyte CpG methylation were not altered by maternal choline intake (*P*=0.17 and 0.88 respectively); however, CpG unit C (containing CpG site 5) of *GNAS-AS1* displayed lower (*P*=0.02) placental methylation in the 930 *vs.* the 480 mg/d intake group (Supplemental Fig. S1). The average and individual CpG methylation of *IGF2*, *IL10*, and *LEP* were not altered (*P*=0.20–0.87) by maternal choline intake in either placental tissue or cord blood leukocytes (**Table 3** and Supplemental Fig. S1). However, it is worth noting that our ability to detect subtle alterations in DNA methylation within these genes was precluded by the study's small sample size.

Global DNA methylation

Placental global DNA methylation was 22% higher (*P*=0.02) in the 930 mg/d maternal choline intake group (4.4±0.2%) compared with the 480 mg/d group (3.6±0.2%) (**Fig. 4*A***). Because the study participants consumed 20% of their total choline intake as trimethyl-d9 choline chloride (*i.e.*, methyl groups of the choline molecule are labeled with deuterium) throughout the last half of the study, we were able to examine placental isotopic enrichment percentage of 5mdC, *i.e.*, d3-5mdC/total 5mdC × 100. Remarkably, placental enrichment of 5mdC was detected in both choline intake groups (480 mg/d: 0.14±0.02 *vs.* 930 mg/d: 0.21±0.03%) and tended to be higher (*P*=0.09) in the 930 mg/d group (Fig. 4*B*). This finding demonstrates unequivocally that dietary choline derived methyl groups are used for placental DNA methylation and support the putative role of choline as an epigenetic regulator of gene expression.

Leukocyte global DNA methylation in maternal (480 mg/d: 5.2±0.3 *vs.* 930 mg/d: 5.0±0.2%; *P*=0.50) and cord (480 mg/d: 5.0±0.1 *vs.* 930 mg/d: 4.9±0.1%; *P*=0.60) blood was not altered by maternal choline intake.

As previously reported by others (28), global DNA methylation percent was lower (*P*<0.001) in placental tissue than cord and maternal leukocytes. No correlations in global DNA methylation were observed among maternal leukocytes, cord leukocytes, and placental tissue.

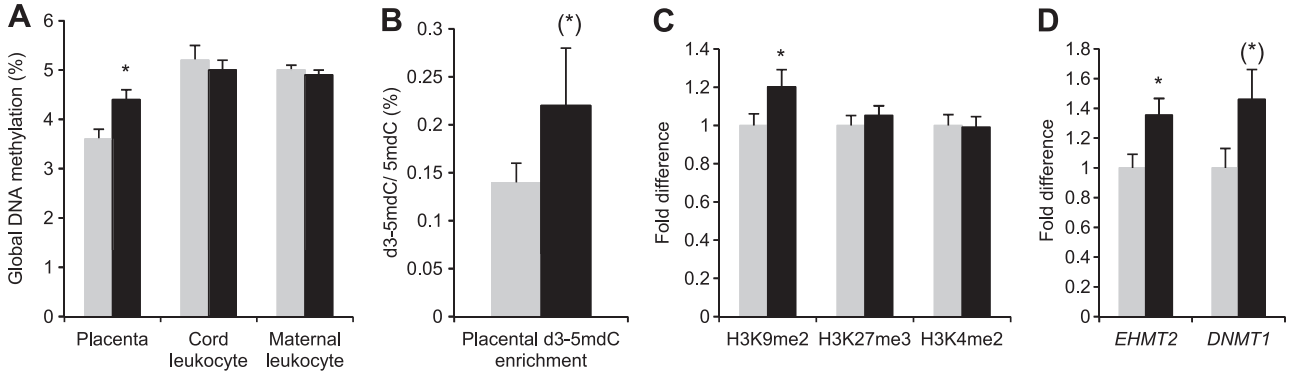

Figure 4. Effect of maternal choline intake (930 *vs.* 480 mg/d) on epigenomic markers and methyltransferase gene transcript abundance. *A*) Percentage of global DNA methylation in placenta, cord leukocytes, and maternal leukocytes. *B*) Deuterium-labeled methyl cytosine enrichment (percentage d3-5mdC to 5mdC) in placenta. *C*) Fold difference of placental global H3K9me2, H3K4me2, and H3K27me3. *D*) Fold difference of placental gene transcript abundance of *DNMT1* and *EHMT2*. Shaded bars, 480 mg/d group; solid bars, 930 mg/d group. Values represent means ± SE; $n = 12$/group. $^{*}P \leq 0.05$, $^{(*)}P < 0.1$ between choline intake groups.

Global histone methylation

Placental H3K9me2, a transcription repression and heterochromatin marker, was 20% higher (P=0.02) among women consuming 930 *vs.* 480 mg choline/d (Fig. 4*C*) and was positively correlated with placental global DNA methylation (Pearson's correlation r=0.40; P=0.05). Maternal (P=0.48) and cord leukocyte (P=0.58) H3K9me2 did not differ between the choline intake groups, nor was placental H3K4me2 (P=0.91), a transcription activation marker or H3K27me3 (P=0.48), a transcription repression marker altered by maternal choline intake (Fig. 4*B*).

Gene expression of DNA and histone methyltransferases

To further clarify the mechanisms by which varied maternal choline intake may modulate epigenetic marks, the expression of several methyltransferases was examined. In placenta, the higher maternal choline intake (930 *vs.* 480 mg/d) yielded higher (36%, P=0.02) gene transcript abundance of *EHMT2*, the histone methyltransferase that mediates H3K9 dimethylation (Fig. 4*D*). In addition, the gene transcript abundance of *DNMT1*, the DNA methyltransferase that functions in DNA methylation maintenance, tended to be higher (33%; P=0.09) in the 930 mg/d group (Fig. 4*D*). The expression levels of DNA methyltransferases that mediate *de novo* DNA methylation establishment (*e.g.*, *DNMT3A* and *DNMT3B*) and histone *SUV39H1* that mediates H3K9 methylation were not altered (P=0.45–0.61) by maternal choline intake (data not shown).

Placental SAM and SAH

SAM is the direct methyl donor for methylation reactions. SAH is formed when the methyl group of SAM is transferred to a methyl acceptor. Despite the changes in global DNA and histone methylation, placental concentrations of SAM and SAH and the SAM/SAH ratio did not differ (P=0.38-0.97) between the maternal choline intake groups (**Table 4**). We suggest that these static measures may not adequately capture the enhanced flow of SAM through the methionine cycle (*i.e.*, homeostatic mechanisms are engaged to prevent alterations in metabolite concentrations).

TABLE 4. *Placental SAM and SAH concentrations in the 930* vs. *480 mg/d maternal choline intake groups*

Metabolite	480 mg/d	930 mg/d	P
SAM (nmol/g)	5.92 ± 0.32	5.90 ± 0.38	0.97
SAH (nmol/g)	1.82 ± 0.11	1.92 ± 0.08	0.53
SAM/SAH	3.31 ± 0.16	3.10 ± 0.17	0.38

DISCUSSION

This study provides compelling evidence that maternal choline intake during the third trimester of human pregnancy can modify global and site-specific epigenetic marks in fetal-derived tissues. Alterations in fetal epigenetic marks by maternal choline intake may have long-lasting functional effects as demonstrated in animal studies (5, 6). The modified epigenetic state of cortisol-regulating genes in response to varied prenatal choline exposure is particularly noteworthy as it implies an effect of maternal choline intake on programming of the HPA axis.

Maternal choline intake alters the epigenetic profile of cortisol-regulating genes

CRH is a main regulator of HPA axis reactivity and cortisol production. During pregnancy, large amounts of CRH are produced by placenta (expressed in high primate species only), which stimulate the HPA axis in both the maternal and fetal compartments to produce cortisol (29, 30). In the present study, promoter methylation of placental *CRH* was higher, and its transcript abundance lower, in women consuming 930 *vs.* 480 mg choline/d. The observed alteration in CpG methylation may have modified the expression of *CRH* by changing accessibility of transcription factors to their regulatory elements in this region (27). The decrease in placental *CRH* expression in the higher maternal choline intake group may attenuate fetal HPA axis reactivity and is consistent with the ~33% lower cord plasma cortisol concentrations in babies of mothers consuming 930 *vs.* 480 mg/d.

NR3C1 encodes the glucocorticoid receptors and is regulated by promoter region methylation (1, 8, 9). In the placenta, glucocorticoids bind to glucocorticoid receptors that stimulate *CRH* expression *via* a feed-forward mechanism (unlike the central HPA axis, where glucocorticoids inhibit *CRH* by a negative feedback mechanism; ref 27). The increased methylation of the placental glucocorticoid receptor observed in the present study may decrease its expression and attenuate placental expression of *CRH*. Nonetheless, we were unable to detect a change in the transcript abundance of placental *NR3C1*.

The change in the epigenetic state of the cortisol-regulating genes was not limited to placenta. However, in contrast to placenta, cord leukocyte *CRH* and *NR3C1* methylation was lower in the 930 *vs.* 480 mg/d intake group. The lower CpG methylation of cord leukocyte *CRH* and *NR3C1* in the 930 mg/d group may represent a secondary response to the altered epigenetic state of the placental HPA axis genes and to the lower circulating cortisol in the fetal compartment. Decreased promoter methylation of *NR3C1* in the central HPA axis is associated with increased sensitivity to cortisol stimuli and improved feedback inhibition (9). Although the value of leukocyte *NR3C1* promoter methylation as a proxy of hypothalamic *NR3C1* and/or HPA reactivity

has yet to be ascertained, a cross-sectional study reported a direct relationship between cord leukocyte *NR3C1* CpG methylation in the NGFI-A binding site and salivary cortisol concentrations in babies at 3 mo of age (8).

These data collectively suggest that a higher maternal choline (*i.e.*, 930 *vs.* 480 mg choline) may lower fetal/neonatal circulating cortisol by altering the methylation state of cortisol-regulating genes in both the placental and fetal compartments. Notably, abnormal increases in placental CRH are associated with obstetric complications such as preeclampsia and intrauterine growth restriction (IUGR; ref. 30), and IUGR neonates exhibit elevated cord blood cortisol (31). Furthermore, a heightened fetal HPA reactivity as a result of early life exposures (*e.g.*, prenatal glucocorticoid overexposure and maternal anxiety) has been shown to enhance lifelong vulnerability to stress-induced illness and chronic conditions such as hypertension and insulin resistance (12, 14, 15). As such, the study findings raise the exciting possibility that prenatal choline supplementation may be employed therapeutically in cases where excess maternal stress (*i.e.*, anxiety, depression) and/or other prenatal conditions might adversely affect fetal HPA axis reactivity and increase vulnerability to stress-related diseases.

Maternal choline intake affects placental global DNA and histone methylation

The higher maternal choline intake (930 *vs.* 480 mg/d) yielded higher placental genome-wide DNA methylation and H3K9me2, as well as increased expression of *DNMT1* and *EHMT2*. These results are consistent with studies in rodents showing that *in utero* choline exposure altered epigenomic marks and methyltransferase expression in several fetal tissues (2, 4). As maintenance of DNA methylation is an important strategy to ensure genomic integrity and H3K9me2 is a marker of transcription repression and heterochromatin formation, the increase of these 2 epigenetic markers in the placenta by a higher maternal choline intake may systematically affect genome stability and expression. In turn, a wide array of placental functions, including endocrine regulation, may be influenced by maternal choline intake which merits further investigation.

Placental tissue is highly susceptible to epigenetic modification

The role of the placenta as a mediator of fetal programming is increasingly recognized (10, 32). More specifically, the placenta acts on behalf of the fetus as both a sensory and effector organ to facilitate the incorporation of environmental information (*i.e.*, nutrient exposure) into the developmental process (33). In the present study, the epigenetic state of the placenta was highly sensitive to varied maternal choline intake during the third trimester of pregnancy, with nearly all the examined placental epigenetic marks (global and site-specific DNA methylation and global histone methylation) showing some degree of alteration. On the contrary, the leukocyte epigenetic state of the mothers, who received the choline treatment directly, was relatively stable. The plasticity of the placental epigenome in response to maternal nutrition may provide a mechanism through which the maternal environment can alter placental function and ultimately fetal development.

Proposed mechanism by which maternal choline intake modulates epigenetic and epigenomic marks

Maternal choline intake may alter the epigenetic and epigenomic marks of fetal derived tissues by influencing the supply of methyl donors (**Fig. 5**). The use of choline as a methyl donor transpires predominately in the liver where the choline derivative betaine provides a methyl group for the conversion of homocysteine to methionine in a reaction catalyzed by betaine-homocysteine *S*-methyltransferase (*BHMT*; ref. 34). Following the activation of methionine to SAM, the choline-derived methyl group may be used in cellular methylation reactions including the methylation of DNA and histones. As the placenta does not express *BHMT*, the choline-mediated placental epigenetic and epigenomic modifications that were observed in the present study depend on the maternal supply, and placental uptake, of methionine and SAM, both of which can transport choline/betaine-derived methyl groups from the maternal liver to extrahepatic tissues including the placenta and fetus.

As shown in Fig. 5, we propose that the higher maternal choline intake increased the use of choline as a methyl donor for methionine and SAM production in

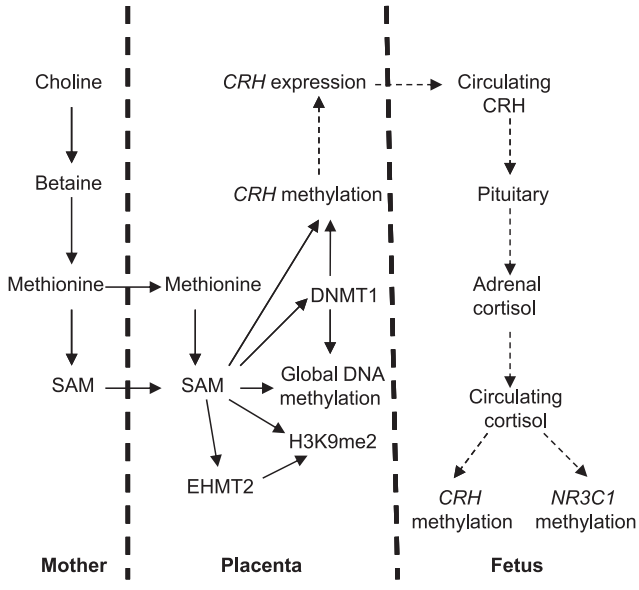

Figure 5. A proposed mechanism by which a higher maternal choline intake may modulate the epigenetic and epigenomic marks of fetal derived tissues. Solid arrows, process enhanced by a higher maternal choline intake; dashed arrows, process attenuated by a higher maternal choline intake.

the maternal compartment. Uptake of methionine and SAM by the placental tissue yielded greater placental global DNA methylation and H3K9me2, as well as greater *CRH* promoter CpG methylation, either directly (increased placental uptake and utilization of methionine and SAM) and/or indirectly *via* up-regulation of DNMT1 and/or EHMT2. A higher *CRH* methylation led to diminished *CRH* transcription and reduced entry of CRH into the fetal compartment. In turn, stimulation of the fetal central HPA axis and adrenal production of cortisol was attenuated thereby lowering circulating concentrations of cortisol. This reduction in circulating fetal cortisol concentrations may have triggered other regulatory events that resulted in a lowering of *CRH* and *NR3C1* CpG methylation.

CONCLUSIONS

The study findings suggest that maternal choline intake throughout the third trimester of pregnancy can alter placental epigenomic marks as well as the epigenetic state of key modulators of placental and fetal HPA axis reactivity. As such, the study findings raise the exciting possibility that a higher maternal choline intake may counter some of the adverse effects of prenatal stress on behavioral, neuroendocrine, and metabolic development in the offspring. Additional studies in larger independent cohorts are needed to replicate these results and explore the long-term functional consequences of these choline-induced epigenetic alterations. FJ

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