DNA methylation profiling at imprinted loci after periconceptional micronutrient supplementation in humans: results of a pilot randomized controlled trial

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ABSTRACT **Intrauterine exposures mediated by maternal diet may affect risk of cardiovascular disease, obesity, and type 2 diabetes. Recent evidence, primarily from animal studies and observational data in humans, suggests that the epigenome can be altered by maternal diet during the periconceptional period and that these programming events may underlie later disease risk. A randomized controlled trial of periconceptional micronutrient supplementation in The Gambia, where seasonal nutritional variations affect fetal growth and postnatal outcomes, provided a unique opportunity to test this hypothesis. Specifically, we targeted imprinted genes, which play important roles in allocation of maternal resources while being epigenetically regulated. DNA methylation at 12 differentially methylated regions (DMRs) was analyzed in cord blood samples from 58 offspring of women participating in a double-blind randomized-controlled trial of pre- and periconceptional micronutrient supplementation (including folate, zinc, and vitamins A, B, C, and D). We observed sex-specific effects of micronutrient supplementation, reducing methylation levels at two of the DMRs analyzed, *IGF2R* in girls and *GTL2*-2 in boys. This pilot study is the first to analyze DNA methylation in the context of a randomized controlled trial, and it provides suggestive evidence that periconceptional maternal nutrition alters offspring methylation at imprinted loci.—Cooper, W. N., Khulan, B., Owens, S., Elks, C. E., Seidel, V., Prentice, A. M., Belteki, G., Ong, K. K., Affara, N. A., Constância, M., Dunger, D. B. DNA methylation profiling at imprinted loci after periconceptional micronutrient supplementation in humans: results of a pilot randomized controlled trial. *FASEB J.* 26, 1782–1790 (2012). www.fasebj.org**

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The hypothesis that adverse early life exposures might increase susceptibility to diseases of affluence in later life was initially proposed by Barker and Osmond (1). Subsequent work confirmed and extended this hypothesis by demonstrating striking inverse correlations between birth weight and adult diabetes and cardiovascular disease risk (2–4). Analysis of adults who had been exposed to undernutrition *in utero* during the Dutch Hunger Winter showed that increased disease risk can occur even in individuals who do not exhibit profound prenatal growth failure (5, 6), suggesting that size at birth may be only a surrogate of more complex effects on fetal development.

The link between early nutritional exposures and later disease risk could be mediated by environmentally induced alterations to the epigenome (which include DNA methylation and histone modifications). Although usually stably maintained during mitosis, epigenetic marks are more labile than nucleotide sequence and thus are potentially susceptible to modulation in response to environmental influences. One class of genes regulated by epigenetic mechanisms is imprinted genes, whose expression is dependent on their parent of origin. Imprinted genes are important regulators of fetal outcomes, as they control fetal and placental growth and are involved in the adaptive response to the

Abbreviations: DMR, differentially methylated region; ISRCTN, International Standard Randomized Controlled Trial Number; IUGR, intrauterine growth retardation; LMP, last menstrual period; SNP, single-nucleotide polymorphism; UNIMMAP, United Nations international multiple micronutrient preparation.

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uterine environment (7). Epimutations (alterations to the epigenetic marks on DNA) at imprinted loci have been associated with human syndromes of fetal overgrowth (8, 9), intrauterine growth retardation (IUGR; ref. 10), and disregulated neonatal glucose homeostasis (11). Imprinted genes have 1 or more transcripts that are preferentially or exclusively expressed from 1 parental allele in ≥1 tissue or developmental stage. This monoallelic expression is likely to be initiated by differential methylation between the oocyte and sperm at gametic differentially methylated regions (DMRs). In the periconceptional period following fertilization, this differential methylation induces additional allele specific methylation at somatic DMRs and thus monoallelic expression. The methylation imprints at paternally methylated gametic DMRs are thought to be initiated while the father was himself a fetus, whereas those at maternally methylated gametic DMRs are thought to be set up postnatally during the final stages of oocyte maturation (12). Therefore, maternally methylated DMRs may be particularly susceptible to nutritional insufficiencies in the pre- and periconceptional period. After fertilization, methylation at gametic DMRs resist genome-wide demethylation events that occur in the early embryo (zygote to preimplantation).

The evidence in the literature that human DNA methylation may be affected by periconceptional nutrition is scarce and does not control for postnatal effects on the epigenome. Accordingly, individuals conceived during the Dutch Hunger Winter demonstrated decreased methylation at *IGF2* DMR0 and *INSIGF* and increased methylation at *IL10*, *LEP*, and *GNASAS*-DMR in adult blood (13, 14). In addition, increased methylation at *IGF2* DMR0 was detected in blood from 17-mo-old children whose mothers had taken periconceptional folic acid (15). However, these differences could have been mediated through the postnatal consequences of earlier exposures (studies of monogenic twins illustrate the influence of the postnatal environment on methylation; ref. 16). Direct evidence of the effects of periconceptional maternal nutrition on the epigenome can only be confirmed by controlled studies where the end point is methylation changes in fetal tissue such as cord blood.

The recently completed randomized controlled trial of United Nations international multiple micronutrient preparation (UNIMMAP) periconceptional micronutrient supplementation in rural Gambia [International Standard Randomized Controlled Trial Number (ISRCTN) 13687662] provided a unique opportunity to demonstrate that periconceptional nutritional exposures affect DNA methylation at imprinted loci. There is evidence that this population has a high incidence of micronutrient deficiencies (17–19). Nutritional factors involved in 1-carbon metabolism (*e.g.*, folic acid and vitamin B12) may be particularly important for the supply of methyl donors and thus normal DNA methylation. Hematological analysis of women in this trial who did not become pregnant demonstrated a marked effect of the UNIMMAP intervention on increasing hemoglobin concentrations and reducing the risk of anemia (20). Males and females have been shown to differently respond to periconceptional famine in their DNA methylation levels at some loci (13) and in other studies to generate sex-specific changes in cord blood hormone (IGF1 and leptin) levels following antenatal UNIMMAP supplementation (21). We therefore hypothesized that periconceptional micronutrient supplementation in this cohort might have sex-specific effects on methylation of critical imprinted DMRs in the offspring.

Using MassArray (Sequenom, San Diego, CA, USA), we analyzed patterns of DNA methylation at imprinted loci in children whose mothers had or had not taken periconceptional UNIMMAP supplementation. In this pilot study of 58 individuals where statistical power was further limited by confounding effects of gender, we identified effects of periconceptional multiple micronutrient supplementation on DNA methylation at 2 of 13 regions analyzed, and these changes were sex specific.

MATERIALS AND METHODS

Subjects

DNA was extracted from residual volumes of umbilical cord blood samples collected at birth from the live-born offspring of women successfully completing a randomized placebo-controlled trial of multiple micronutrient supplementation in rural Gambia (ISRCTN 13687662; ref. 20). Briefly, consenting, nonpregnant women aged 17–45 were randomized to receive daily supplementation with either UNIMMAP (for composition see **Table 1**) or placebo. Once pregnancy was confirmed by positive pregnancy test at mean gestational age 9.5 wk (interquartile range: 7.4–13.2 wk), women ceased supplementation, and all women received a standard antenatal preparation of iron (60 mg) and folate (250 μg). The trial was approved by the Medical Research Council (MRC)/Gambian Government Ethics Committee, and the current study was also approved by the MRC Laboratories Fajara Scientific Co-ordinating Committee (L2007.77, September 21, 2007) and the Gambian Government Ethics Committee (October 25, 2007). Following unblinding of the study and initial *post hoc* analysis of compliance, sufficient cord blood was available to extract DNA from 22 newborns of compliant mothers in the intervention group and 36 newborns from the

TABLE 1. *Composition of the UNIMMAP micronutrient supplementation*

Micronutrient	Daily dose
Vitamin A	800 RE
Vitamin D	200 IU
Vitamin E	10 mg
Vitamin C	70 mg
Vitamin B1	1.4 mg
Vitamin B2	1.4 mg
Niacin	18 mg
Vitamin B6	1.9 mg
Vitamin B12	2.6 mg
Folic acid	400 μg
Iron	30 mg
Zinc	15 mg
Copper	2 mg
Selenium	65 μg
Iodine	150 μg

placebo group. Children were described as wet-season conceptions if the mother's last menstrual period (LMP) occurred during the 6 mo June to November, and dry-season conceptions when the mother's LMP occurred in the period December to May. Gestational age was determined by ultrasound scan performed between wk 7 and 24 of pregnancy in >90% cases, and for those presenting later than 24 wk, we used the woman's recollection. Women were excluded from this analysis if they were enrolled for <14 d before LMP (mean duration of supplementation was 155 d, range of 20–441 d) or if they apparently consumed <70% of allocated tablets. Tablets were allocated at weekly clinic visits, and clinic workers counted the remaining pills to determine the number apparently consumed. The median compliance for this subsample was 94% (interquartile range 84–100%), which compares favorably with that described by Gulati *et al.* (20). In a secondary analysis, the persistence of treatment effects was further investigated in 57 infancy blood samples collected at 9 mo of age (27 UNIMMAP and 30 placebo infants, of which 19 could be matched to a cord blood sample from the same child; **Fig. 1**).

Molecular analysis

The loci chosen for quantification of methylation using mass spectrometry included 2 paternally methylated germline DMRs [*H19*-DMR and the intragenic (IG) DMR at the *DLK1/GTL2* (also known as *MEG3* locus)], 3 paternally methylated somatic DMRs [*GTL2*-DMR (2 regions), *IGF2* DMR0, and *GNAS*-DMR] and 7 maternally methylated germline DMRs [*PLAGL1*-DMR (also known as *ZAC*-DMR), *IGF2R*-DMR, *PEG1*-DMR (also known as *MEST*-DMR), KvDMR, *SNRPN*-DMR, *PEG3*-DMR, and *GNASAS*-DMR]. The DMRs, their allelic methylation, the expressed transcripts, their putative roles, and disease associations are summarized in **Table 2**. IGF2R is polymorphically imprinted in humans; however, the methylation status of the DMR is conserved even in the absence of imprinted expression.

DNA was extracted at the MRC Fajara Laboratories using the Gentra Puregene reagents (Qiagen, Hilden, Germany). Methylation analysis was performed at the Institute of Metabolic Science; unmethylated cytosines were converted to uracil by treatment with sodium bisulfite (EZ DNA Methylation Kit; Zymo Research, Irvine, CA, USA). Experiments were performed in triplicate (from 3 independent bisulfite conversions). DNA was then diluted to 2 ng/μl (based on the input amount) and used for PCR amplification with HotStarTaq (Qiagen) according to the protocol recommended by Sequenom. The annealing temperature was 56°C for all primers except *PEG3*-DMR and *IGF2R*-DMR, where 60°C was required. Primers (listed in **Table 3**) were designed using EpiDesigner (Sequenom), screened to avoid known single-nucleotide polymorphisms (SNPs), and synthesized (Sigma, St. Louis, MO, USA) with a 5′-AGGAAGAGAG added to the forward primer and a 5′-CAGTAATACGACTCACTATA GGGAGAAGGCT added to the reverse primer. The PCR tags are required for the MassCleave chemistry (Sequenom), which was performed exactly according to the manufacturer's instructions. Samples were then spotted onto SpectroCHIPs (Sequenom) for analysis by mass spectrometry (MassArray Compact Analyzer; Bruker; Sequenom). Complete conversion was verified using the R-package MassArray (22). EpiTyper analysis interrogated 245 CpG units (7–27 CpG units/locus). CpGs were excluded from further analysis where there were SNPs reported in dbSNP (13 CpG units), where <90% of samples generated data at that CpG (67 CpG units), or where a titration curve indicated a nonlinear relationship between input and output (8 CpG units). Where the molecular mass of a CpG unit rendered it a duplicate with other CpG units, the second and subsequent appearances of that CpG unit were excluded (13 CpG units). The primers used for analysis of IGF2-DMR0 are those from Heijmans *et al.* (14); however, there is no overlap with the CpGs analyzed by Talens *et al.* (23) or Waterland *et al.* (24).

Statistical analysis

To identify differences by treatment group, sex, or season of conception, continuously distributed anthropometric variables were compared by Student's t test, and categorical measures were compared by χ^2 test.

Quantification of methylation was performed in triplicate; therefore, for each sample at each CpG unit, the average of

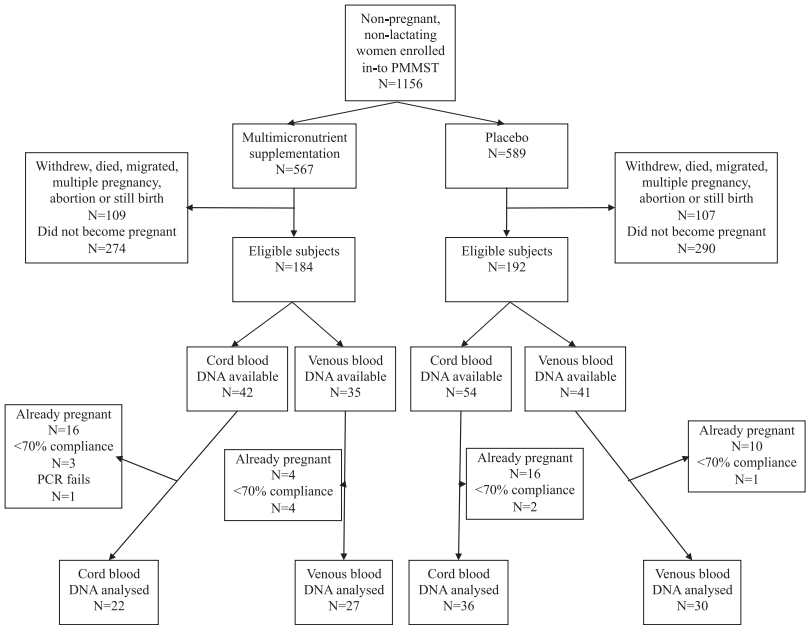

Figure 1. Trial profile.

TABLE 2. *Summary of DMRs analyzed, their allelic methylation, allele from which transcripts are expressed, and disease associations*

Gametic DMR (methylation allele)	Somatic DMR (methylation allele)	Coding transcripts (expressed allele)	Noncoding transcripts (expressed allele)	Associated disease
H19-DMR/IC1(P)	*IGF2* DMR0(P)	*IGF2*(P), *IGF2AS*(P), *INS*(P)	*H19*(M)	SRS (LOM) BWS (GOM)
IG-DMR(P)	GTL2-DMR(P)	*DLK1*(P), *DIO3*(P), *RTL1*(P)	*MEG3/GTL2*(M), *RTL1AS*-mirs(M), snoRNAs(M), *MEG8*(M)	Maternal UPD14 syndrome (LOM at IG DMR)
PLAGL1-DMR(M)		*PLAGL1/ZAC*(P)	*HYMAI*(P)	TNDM (LOM)
IGF2R-DMR(M)		*IGF2R*(M), *SLC22A2*(M), *SLC22A3*(M)	*AIRN*(P)	LOS (LOM)
PEG1-DMR(M)		*KLF14*(M), *CPA4*(M), *MEST/PEG1*(P)	*MESTIT1*(P), *COPG2IT1*(P)	
KvDMR/IC2(M)		*ASCL2*(M), *KCNQ1*(M), *KCNQ1DN*(M), *CDKN1C*(M), *SLC22A18AS*(M), *SLC22A18*(M), *PHLDA2*(M), *OSBPL5*(M)	*KCNQ1OT1*(P)	BWS (LOM)
SNRPN-DMR(M)		*UBE3A*(M), *ATP10A*(M), *SNRPN*(P), *MKRN3*(P), *MAGEL2*(P), *NDN*(P), *SNURF*(P), *UBE3A-AS*(P), *C15ORF2*(P), *PWRN1*(P)	*SNORD* snoRNA cluster(P)	AS (LOM) PWS (GOM)
PEG3-DMR(M)		*PEG3*(P), *ZNF331*(P), *ZIM2*(P)	*MIMT1*(P), *PEG3AS*(P)	
GNASAS-DMR(M)	*GNAS*-DMR(P)	*GNAS*(M), *GNASXL*(P)	*Exon-1A*(P), *GNASAS*(P)	PHP-1b (LOM at *GNASAS*-DMR, GOM at *GNAS*-DMR)

AS, Angelman syndrome; BWS, Beckwith-Wiedemann syndrome; GOM, gain of methylation; LOM, loss of methylation; LOS, large offspring syndrome (in cloned cattle); M, maternal; P, paternal; PHP, pseudohypoparathyroidism; PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome; TNDM, transient neonatal diabetes mellitus; UPD, uniparental disomy.

the 2 most concordant replicates was taken. To calculate the group mean ± SD methylation level at each locus, the mean percentage or level of methylation across the locus was calculated for each sample, and the mean of these values was calculated for the group of individuals.

To estimate the effects of intervention on methylation at each locus, multilevel mixed-effects linear regression analyses were performed, allowing data across multiple CpG units to be modeled simultaneously while accounting for correlation between methylation of adjacent CpG units. In separate models for each locus, the *xtmixed* command in Stata 11 (StataCorp LP, College Station, TX, USA) was used with the sample ID entered as a random effect and the sex, season of conception, and treatment group entered as fixed effects.

Due to reported sex-specific effects of antenatal UNIMMAP supplementation on cord blood hormone levels (21), and also sex-specific effects of periconceptional famine on DNA methylation levels (13), we repeated these analyses including a sex × intervention group term to test for sex-specific effects and also performed stratified analyses to estimate effect sizes in male and female babies separately. Individual *P* values for each CpG site were obtained by linear regression of treatment group on methylation status, adjusting for season, separately for boys and girls. For all analyses, 2-sided *P* values are reported.

RESULTS

Of the 1156 women enrolled in the trial, 376 delivered a live-born singleton offspring. Comparison of mother and child anthropometric variables between those individuals initially enrolled in the clinical trial and the subset analyzed in this study indicated that they were broadly comparable. However, parity and newborn head circumference were slightly greater among the current study members (parity: mean 5, interquartile range 3–7; head circumference: mean±SD 34.6±1.1 cm) compared with the trial as a whole (parity: mean 4, interquartile range 0–7, $P=0.003$; head circumference: mean±SD 34.2±1.4, $P=0.01$). In the subsample available for methylation analysis, there were no detectable

TABLE 3. *Sequences of unique regions of primers*

Primer and location of amplicon (build GRCh37)	Sequence	
	Forward (has additional 5′-AGGAAGAGAG)	Reverse (has additional 5′-CAGTAATACGACTCACTATAGGGAGAAGGCT)
H19-DMR chr11: 2020904–2021331	GTTGTGTTTTGGGATAGATG	CTCTAACAAACACAAAACCC
IG-DMR chr14: 101277148–101277684	GGGAATTGGGGTATTGTTTATATTT	ACAATTTAACAACAACTTTCCTCCA
IGF2 DMR0 (from ref. 14) chr11: 2169459–2169796	TGGATAGGAGATTGAGGAGAAA	AAACCCCAACAAAAACCACT
GTL2-DMR_1 chr14: 101291608–101291975	GGTTTTTTTAAAGGGTATGTGTGTG	ATATAAACCAAAAACTATCACCCCC
GTL2-DMR_2 chr14: 101292283–101292796	TTTTTTTTGTTGTAATTTGGGTG	CCTTAAAAACTAAACCCTCCAAAAA
GNAS-DMR chr20: 57415713–57416152	GTTTTAGAGTTTTAGGGAAGGGGAG	CAACAAACCTTTAACCACCAAAAC
PLAGL1-DMR chr6: 144329448–144329951	GGGTTGAATGATAAATGGTAGATGT	CAAAATACTTAAAACAATACCTAACTCAC
IGF2R-DMR chr6: 160426403–160426850	GAGGTGTAGGGGATTTAGGGAG	CAATCCCTACCTAACCCTTATCTAAA
PEG1-DMR chr7: 130131325–130131792	GTGGTGGGTTTAATAGAGTTTGTTG	AACCACAAAAATAAAATACCCCTCT
KvDMR chr11: 2721164–2721465	GGTAGGATTTTGTTGAGGAGTTTTT	CTCACACCCAACCAATACCTCATA
SNRPN-DMR chr15: 25200224–25200805	TGGTTTTTTTTAAGAGATAGTTTGGG	CCTAATCCACTACCATAACCTCCTC
PEG3-DMR chr19: 57351734–57352308	TAGATTTGGTTTTGGGGTTTTTTA	ACACCAACCATCCACAACCTAAC
GNASAS-DMR chr20: 57429802–57430242	AGGGGTATTTTTTTGATTTTGAGAG	AAAAACTCCCACTACCCCAAC

differences between intervention and placebo groups in maternal age, parity, height, weight, and upper arm circumference (a measure of maternal nutritional status; at recruitment and at delivery); season of conception; offspring's sex, birth weight, length, or head circumference; placental weight; or gestational age (**Table 4**).

Table 5 shows the mean methylation levels at each locus in cord blood DNA samples. When analyzed in males and females combined, no differences by treatment group were observed at any locus.

However, the sex × intervention interaction term was statistically significant for 2 of the 13 regions, *IGF2R*-DMR and *GTL2*-DMR_2 (**Table 6**). In stratified

TABLE 4. *Characteristics of mothers and infants in UNIMMAP treated and placebo groups*

Characteristic	Cord blood DNA			Infancy DNA		
	Placebo	UNIMMAP	*P*	Placebo	UNIMMAP	*P*
n	36	22		30	27	
Mother						
Age at delivery (yr)	31.1 ± 7.2	29.2 ± 8.0	0.38	33.0 ± 7.3	32.4 ± 8.0	0.75
Parity	5.1 ± 2.8	4.6 ± 3.5	0.64	5.5 ± 2.9	5.1 ± 3.2	0.61
Height (cm)	161.9 ± 5.5	160.7 ± 4.2	0.34	160.1 ± 5.6	162.2 ± 5.3	0.15
Recruitment weight (kg)	57.0 ± 8.7	54.8 ± 9.4	0.39	54.7 ± 9.4	57.7 ± 12.7	0.33
Recruitment MUAC (mm)	266 ± 28	264 ± 30	0.80	265 ± 33	269 ± 36	0.62
Delivery MUAC (mm)	257 ± 26	256 ± 29	0.91	257 ± 26	259 ± 29	0.76
Infant						
Male (*n*)	18	10	0.74	15	11	0.48
Wet season conception (*n*)	16	13	0.28	22	20	0.95
Birth weight (kg)	3.1 ± 0.4	3.0 ± 0.4	0.95	3.0 ± 0.4	2.9 ± 0.4	0.19
Birth length (cm)	49.5 ± 3.3	49.7 ± 2.3	0.80	49.8 ± 2.0	49.2 ± 2.5	0.35
Head circumference (cm)	35.0 ± 2.7	34.7 ± 1.3	0.58	34.5 ± 0.9	34.6 ± 1.2	0.71
Placental weight (g)	498.3 ± 78.1	479.0 ± 72.7	0.35	469.4 ± 96.0	462.3 ± 94.6	0.79
Gestational age (wk)	40.3 ± 1.7	40.2 ± 1.3	0.95	40.2 ± 1.5	40.0 ± 0.9	0.57

Data are means ± SD. MUAC, mid-upper arm circumference.

TABLE 5. *Methylation levels by treatment group in cord blood DNA samples collected at birth*

Locus	Methylation level (%)[a]		Difference in methylation, treatment *vs.* placebo (%)		Effect of treatment adjusted for sex	
	Placebo, $n = 36$	UNIMMAP, $n = 22$	Absolute	Relative	Absolute difference[b]	P
H19-DMR	41.9 ± 6.3	40.7 ± 4.9	−1.2	−2.9	−2.3 ± 1.5	0.13
IG-DMR	50.2 ± 5.0	49.4 ± 4.9	−0.8	−1.7	−0.4 ± 1.5	0.77
IGF2 DMR0	40.5 ± 5.2	42.0 ± 4.9	1.5	3.7	1.6 ± 1.8	0.37
GTL2-DMR_1	30.7 ± 3.7	30.4 ± 3.2	−0.3	−1.0	−0.6 ± 1.1	0.56
GTL2-DMR_2	25.9 ± 10.7	26.8 ± 10.8	1.0	3.7	−0.7 ± 2.7	0.80
GNAS-DMR	33.5 ± 4.3	33.1 ± 5.5	−0.4	−1.2	−1.0 ± 1.4	0.47
PLAGL1-DMR	28.1 ± 3.3	28.8 ± 4.5	0.7	2.5	1.1 ± 1.3	0.40
IGF2R-DMR	53.6 ± 10.6	51.7 ± 8.4	−1.8	−3.4	−3.1 ± 2.8	0.27
PEG1-DMR	34.6 ± 3.9	35.1 ± 4.2	0.6	1.6	−0.8 ± 1.5	0.59
KvDMR	34.4 ± 11.6	31.9 ± 4.6	−2.5	−7.3	−3.7 ± 3.1	0.24
SNRPN-DMR	30.4 ± 6.8	32.3 ± 6.1	1.9	6.1	2.5 ± 1.9	0.19
PEG3-DMR	27.9 ± 7.2	27.4 ± 6.8	−0.5	−1.7	−1.3 ± 2.0	0.51
GNASAS-DMR	22.5 ± 3.5	22.9 ± 4.0	0.4	1.8	0.1 ± 1.1	0.90

Difference in methylation levels between treated and untreated samples was calculated for all individuals together (columns 2–5) and additionally using a multilevel mixed-effects linear regression analysis to account for the sex of the child (columns 6, 7) ($n = 58$). [a]Data are means ± SD. [b]Data are means ± SE.

analyses, UNIMMAP intervention significantly reduced methylation levels at *IGF2R*-DMR in girls, and *GTL2*-DMR_2 in boys (Table 6 and **Fig. 2*A, B***). Within these loci, the sex-specific effects of UNIMMAP intervention appeared to be consistent across individual CpG sites at each locus (**Fig. 3**).

To determine whether these methylation changes persisted during postnatal life, we analyzed further DNA samples extracted from infant blood samples. Estimation of the sex-specific effects of treatment on methylation levels (**Table 7** and Fig. 2) indicated that neither of the significant treatment effects observed in cord blood DNA was replicated in infant DNA. However, significant reductions in methylation levels following treatment were observed in female infants at *PEG1*-DMR and *GNASAS*-DMR.

DISCUSSION

Observational data in humans and animal experimental models (13–15, 24–25, 26) have already indicated that maternal periconceptional nutrition might be an important determinant of subsequent imprinted gene methylation. However, species differences in animal studies and delayed effects in adult human studies raise doubts as to the strength of these observations. Here we report the first analysis of methylation levels in human DNA samples from a randomized placebo-controlled trial of periconceptional nutrient supplementation in human newborns. Despite its strengths as a randomized trial, this study is limited by its small sample size and hence limited power to detect small differences, especially in the presence of potential confounding factors, such as the heterogeneous

TABLE 6. *Effect of UNIMMAP intervention on methylation levels in cord blood DNA samples collected at birth in the 2 genders separately*

Locus	Effect in girls		Effect in boys		Sex × intervention interaction (P)
	EAD (%)	P	EAD (%)	P	
H19-DMR	−2.6 ± 2.3	0.26	−1.8 ± 2.2	0.40	0.78
IG-DMR	−2.8 ± 2.2	0.20	2.8 ± 2.1	0.18	0.08
IGF2 DMR0	1.9 ± 2.4	0.44	2.4 ± 2.9	0.41	0.89
GTL2-DMR_1	−1.0 ± 1.6	0.54	0.7 ± 1.5	0.63	0.80
GTL2-DMR_2	4.2 ± 4.2	0.32	−6.5 ± 3.3	0.044*	0.036*
GNAS-DMR	0.0 ± 1.8	1.00	−1.6 ± 2.2	0.48	0.54
PLAGL1-DMR	2.6 ± 1.9	0.18	0.1 ± 1.9	0.97	0.22
IGF2R-DMR	−8.6 ± 3.8	0.023*	2.6 ± 4.4	0.57	0.038*
PEG1-DMR	−1.5 ± 2.4	0.52	−0.7 ± 1.9	0.70	0.99
KvDMR	−6.8 ± 5.4	0.21	−2.4 ± 3.0	0.42	0.47
SNRPN-DMR	1.5 ± 2.3	0.52	2.6 ± 3.3	0.43	0.56
PEG3-DMR	−0.3 ± 2.8	0.91	−1.6 ± 2.8	0.56	0.52
GNASAS-DMR	−0.4 ± 1.3	0.78	1.9 ± 1.6	0.24	0.48

Data are means ± SE. EAD, estimated absolute difference. *$P < 0.05$, adjusted for season of conception; $n = 58$.

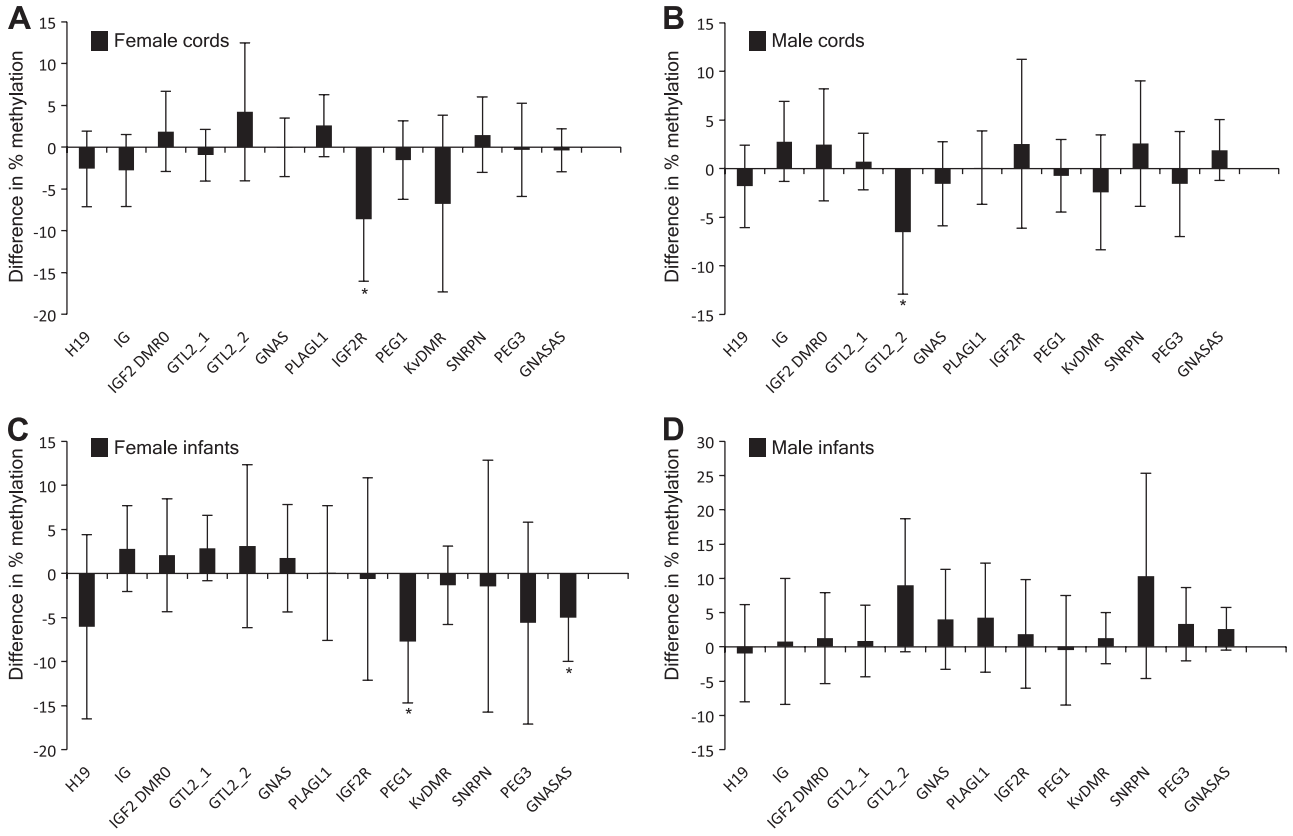

Figure 2. Effects of the UNIMMAP supplementation on methylation levels at imprinted loci in girls (*A*, *C*) and boys (*B*, *D*) at birth (*A*, *B*) and during infancy (*C*, *D*). Bars represent effect of treatment ± 95% confidence intervals. Treatment effects at birth and in infancy adjusted for season were generated in boys and girls separately. $*P \leq 0.05$.

cell populations found in blood. The power of the study was further limited by gender-specific methylation changes in both the degree and direction of change, necessitating the analysis of males and females separately. Our pilot observations indicate that, independent of seasonal effects, periconceptional nutrition could also be an important determinant of the methylation of critical fetal imprinted genes in both genders.

We observed gender-specific differences in methylation following treatment at 2 loci (*IGF2R*-DMR in girls and *GTL2*-DMR_2 in boys). The effects that we observed were consistent across all the CpGs within the locus analyzed (Fig. 3) and were analyzed using multilevel mixed-effects linear models. However, we did not make formal Bonferroni adjustments for the 13 loci studied. Thus, our data must be considered as pilot, requiring confirmation using

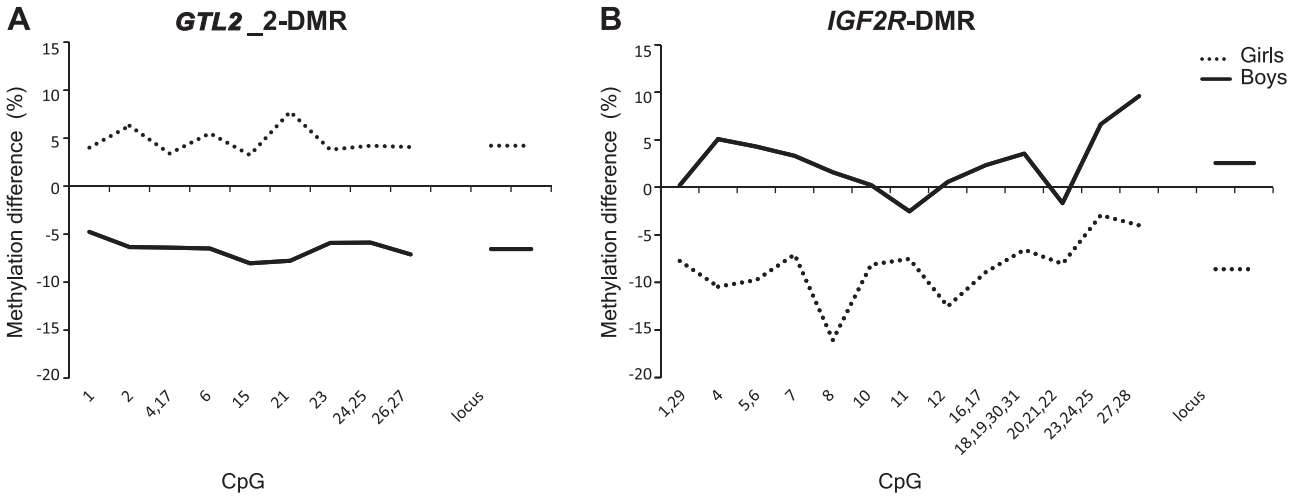

Figure 3. Effect of UNIMMAP treatment on methylation levels in girls (dotted trace) and boys (solid trace). Difference in level of methylation at each CpG within the locus between treated and untreated groups, adjusted for season. *A*) *GTL2*_2-DMR. *B*) *IGF2R*-DMR.

TABLE 7. *Effect of UNIMMAP treatment adjusted for season of conception on methylation levels by sex in venous blood DNA samples collected at 9 mo*

	Methylation level (%)[a]		Effect in girls		Effect in boys		
Locus	Placebo, $n = 30$	UNIMMAP, $n = 27$	Value[b]	P	Value[b]	P	Sex × treatment interaction (P)
H19-DMR	48.9 ± 11.2	45.9 ± 12.0	−6.1 ± 5.3	0.26	−0.9 ± 3.6	0.80	0.57
IG-DMR	53.6 ± 7.0	53.5 ± 9.8	2.8 ± 2.5	0.26	0.8 ± 4.7	0.87	0.57
IGF2 DMR0	42.9 ± 6.8	44.9 ± 9.4	2.1 ± 3.3	0.53	1.3 ± 3.4	0.71	0.90
GTL2-DMR_1	33.8 ± 5.1	36.6 ± 6.5	2.9 ± 1.9	0.13	0.9 ± 2.7	0.75	0.45
GTL2-DMR_2	24.7 ± 12.0	33.2 ± 19.2	3.1 ± 4.7	0.51	9.0 ± 4.9	0.07	0.44
GNAS-DMR	37.3 ± 7.7	40.2 ± 8.0	1.7 ± 3.1	0.58	4.0 ± 3.7	0.28	0.64
PLAGL1-DMR	29.6 ± 8.1	31.9 ± 9.9	0.0 ± 3.9	0.99	4.3 ± 4.1	0.29	0.43
IGF2R-DMR	57.1 ± 14.2	55.7 ± 10.6	−0.6 ± 5.9	0.91	1.9 ± 4.0	0.65	0.72
PEG1-DMR	38.5 ± 7.3	34.6 ± 9.6	−7.7 ± 3.6	0.030*	−0.5 ± 4.1	0.90	0.26
KvDMR	34.4 ± 3.6	33.9 ± 5.8	−1.3 ± 2.3	0.56	1.2 ± 1.9	0.51	0.37
SNRPN-DMR	40.6 ± 15.4	46.3 ± 18.1	−1.4 ± 7.3	0.84	10.3 ± 7.7	0.18	0.25
PEG3-DMR	25.4 ± 14.5	26.3 ± 15.6	−5.6 ± 5.9	0.34	3.3 ± 2.7	0.23	0.31
GNASAS-DMR	23.8 ± 6.2	25.0 ± 6.7	−5.0 ± 2.5	0.047*	2.6 ± 1.6	0.10	0.019*

*$P < 0.05$; $n = 57$. [a]Data are means ± SD. [b]Data are means ± SE.

a similar trial design in a larger population. Gender-specific differences in DNA methylation have also been observed following periconceptional famine exposure (13). Methylation marks at imprinted loci are instigated before sexual hormones are produced, but they may subsequently become modified by them. In addition, there are known differences in transcription (27), antigenicity (28), metabolic rate (29), and developmental rate (30, 31) between male and female blastocysts. The faster development of male embryos has been postulated to render them more susceptible to a suboptimal environment (32). Our observation of sex differences on methylation of genes in relation to micronutrient supplementation has parallels in sex-specific differences observed in newborn IGF1 and leptin concentrations following antenatal UNIMMAP supplementation (21).

Mechanisms to sense and respond to nutrient availability may be able to exploit the periconceptional windows of opportunity that potentially exist, while the oocyte is acquiring its imprints and in the early embryo when somatic DMRs become methylated. As different classes of imprinted loci acquire their methylation at different times, they may be differentially susceptible to nutritional programming. We found methylation levels at the *H19* and IG loci were refractory to nutritional status; these are 2 of only 3 known human paternally methylated gametic DMRs (the other being GPR1/ZDBF2), and as such one would expect that their imprints would be initiated during the fathers' embryonic period (and maintained in offspring). Consistent with our data, a preimplantation low-protein diet did not alter *H19* methylation (despite reducing expression) in male rats (25).

The hypothesis that periconceptional micronutrient supplementation may be useful clinically as well as affecting future postnatal outcomes is based on previous studies of the Gambian population, where seasonal alterations in nutrition are reflected in alterations in infant and childhood morbidity and mortality (33) and young adult mortality (34, 35), although a recent analysis of methylation at the *IGF2* locus and *GNASAS*-DMR in children's blood did not detect any seasonal effects (24). In this pilot study, we report effects of micronutrient supplementation at *IGF2R*-DMR and *GTL2*-DMR_2. In addition, at *IGF2* DMR0 we observed a trend toward increased methylation following periconceptional micronutrient supplementation, consistent with data described in an observational study of periconceptional folic acid use (2.1% absolute difference or 4.5% relative difference; ref. 15).

To determine whether the micronutrient induced alterations in DNA methylation identified in cord blood DNA samples persisted over a period of months, we were able to analyze infant venous blood collected at 9 mo. Neither of the significant alterations observed in cord blood DNA were observed in infant blood DNA; however, this may be due to the small sample size limiting the power to detect these alterations. Permanent methylation changes may be required to continually modify the physiology. Alternatively, a methylation change may not be required to persist if its effects have been exerted by altering the child's histology and morphology during embryonic and fetal life. These effects may differ by tissue, and while we were able to observe small alterations in this analysis of nucleated cord blood cells, if it were possible to analyze a tissue with more relevance to physiological adaptations to nutrient stress, larger changes or changes in a larger proportion of the imprinted gene set may be revealed.

Our study of cord blood DNA provides preliminary evidence from a randomized controlled trial that variation in periconceptional micronutrients can affect methylation of imprinted genes. These changes may be sex specific, and the subsequent postnatal nutritional conditions may also be important in longer term effects on metabolism and disease outcomes, which are ultimately determined by postnatal genetic/epigenetic environmental interactions. FJ

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