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Maternal Periconceptional Folic Acid Supplementation and DNA Methylation Patterns in Adolescent Offspring

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Abstract

Background: Folate, including the folic acid form, is a key component of the one-carbon metabolic pathway used for DNA methylation. Changes in DNA methylation patterns during critical development periods are associated with disease outcomes and are associated with changes in nutritional status in pregnancy. The long-term impact of periconceptional folic acid supplementation on DNA methylation patterns is unknown.

Objectives: To determine the long-term impact of periconceptional folic acid supplementation on DNA methylation patterns, we examined the association of the recommended dosage (400 μg/d) and time period (periconceptional before pregnancy through first trimester) of folic acid

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supplementation with the DNA methylation patterns in the offspring at age 14–17 y compared with offspring with no supplementation.

Methods: Two geographic sites in China from the 1993–1995 Community Intervention Program of folic acid supplementation were selected for the follow-up study. DNA methylation at 402,730 CpG sites was assessed using saliva samples from 89 mothers and 179 adolescents (89 male). The mean age at saliva collection was 40 y among mothers (range: 35–54 y) and 15 y among adolescents (range: 14–17 y). Epigenome-wide analyses were conducted to assess the interactions of periconceptional folic acid exposure, the 5,10-methylenetetrahydrofolate reductase (*MTHFR*)-*C677T* genotype, and epigenome-wide DNA methylation controlling for offspring sex, geographic region, and background cell composition in the saliva.

Results: In the primary outcome, no significant differences were observed in epigenome-wide methylation patterns between adolescents exposed and those non-exposed to maternal periconceptional folic acid supplementation after adjustment for potential confounders [false discovery rate (FDR) P values < 0.05]. The *MTHFR-C677T* genotype did not modify this lack of association (FDR P values < 0.05).

Conclusions: Overall, there were no differences in DNA methylation between adolescents who were exposed during the critical developmental window and those not exposed to the recommended periconceptional/first-trimester dosage of folic acid. *J Nutr* 2022;00:1–8.

Keywords

folic acid; DNA methylation; epigenetic; periconceptional; adolescents; pregnancy; dietary supplements

Introduction

Folic acid is a form of folate (vitamin B-9), which is a key source of the one-carbon group that methylates DNA, RNA, and proteins. DNA methylation regulates gene expression, imprinting, and developmental gene regulation (1–4). The availability of methyl groups to methylate DNA during early embryogenesis can be critical for fetal programming and long-term disease susceptibility (4). In the early embryo, DNA methylation patterns are erased and reset across most of the epigenome (4–7). Methylation of genomic DNA modifies gene expression and provides a mechanism for transmitting and perpetuating epigenetic information through DNA replication and cell division across the life span and even transgenerationally (8, 9). A change to this patterning during early development would be propagated to all of the cell's descendants and may affect long-termhealth and development becauseaberrant DNA methylation is a hallmark of cancer and other disease processes (3, 4, 10, 11).

DNA methylation patterns vary between sexes and change across the life course (12–17). In addition, environmental exposure to air pollution in general, particulates in air, and benzene have also been linked to changes in DNA methylation and adverse outcomes such as cancer initiation or progression (12–17). Studies have shown that maternal dietary changes in one-carbon availability (as a cocktail of one-carbon sources) can affect the DNA methylation patterns and phenotype of the offspring as best described in the agouti mouse model (18,

19). A growing and substantial body of data supports the Developmental Origins of Health and Disease (DOHaD) hypothesis, which postulates that early embryonic exposures program lifelong health and disease risks in part through changes in DNA methylation (17, 20–22). Extreme exposures, such as starvation during pregnancy, have been demonstrated to change DNA methylation at loci that are associated with adverse health outcomes such as diabetes (23–25). Secondtrimester folic acid supplementation has been associated with a change in a single locus in female offspring 47 y of age (26). Two other studies have examined maternal folate concentrations near delivery and associated cord/infant DNA methylation (20,27).Another study examined periconceptional through breastfeeding intakes of one-carbon methyl donors (choline, betaine, folate, methionine) and found changes in DNA methylation dependent on exposure and timing (28). Overall, replication of findings across studies is needed to confirm results. In addition, studies are lacking in humans of the impact of periconceptional exposure of folic acid only (without use of multivitamins),at the recommended dosage and on methylation patterns in adolescent offspring of both sexes.

The recommendation to consume folic acid before and during early pregnancy for the prevention of neural tube defects (NTDs) (29) is based on strong evidence from 2 randomized controlled trials published in the early 1990s (30). This led to effective fortification programs in several countries (31–34). The US CDC collaborated with Chinese investigators in a public health Community Intervention Program (CIP) during 1993–1995 in 21 counties in China using a recommended dosage of 400 μg/d of folic acid alone (29). The CIP population had low dietary intake of folate and no use of folic acid supplements nor exposure to folic acid fortification. The CIP was a public health campaign in which all women planning their first pregnancy were asked to take folic acid supplements starting at their premarital examination and extending to the end of the first-trimester time of pregnancy. Prospective pill taking was collected monthly for each woman. Comparison of birth defects in the offspring of “exposed” (400 μg/d) as opposed to “unexposed”(no folic acid supplements) mothers demonstrated prevention of ≤85% of NTDs among ~250,000 pregnancies (35). Unexposed women were generally those who registered their pregnancy after the first trimester and had no opportunity to take folic acid. The unexposed women were ~2 y older than the exposed women. A comparison of the characteristics of the exposed and unexposed women is described in detail elsewhere (36, 37). The current analysis was based on a sample of mothers and adolescent offspring from the CIP population (38). The 5,10-methylenetetrahydrofolate reductase (*MTHFR*)-*C677T* variant moderately decreases blood folate concentrations across folic acid intakes (4, 31, 39) and likely increases the risk of NTDs dependent on background folate concentrations (lower concentrations resulting in more risk above baseline) (40). The *MTHFR* genotype affects the relation of folate concentration with both DNA replication (cell division/survival) and DNA methylation (cell regulation) (4).

The primary objective of the current study was to evaluate whether there were differences in epigenome-wide methylation patterns between adolescent offspring “exposed” and “unexposed” to maternal periconceptional folic acid supplementation. A secondary goal was to assess whether there were changes in folate metabolism genotype frequencies between mothers and adolescents, because it had been hypothesized that folic acid supplementation

may result in increased frequency of detrimental genetic variants—specifically *MTHFR 677 TT* (rs1801133) (41).

Methods

Cohort and follow-up

The current investigation was part of 2 interrelated studies conducted during 2011–2013. The overarching goal of the studies was to test the feasibility of carrying out a large-scale follow-up observational cohort study of late health effects of periconceptional folic acid (38, 42). The planned cohort to be followed up was based on the historical CIP population participating in the public health program of periconceptional folic acid supplementation and initially followed up to ascertain neural tube and other birth defects during 1993–1995.

During 2011–2013 Study #1 tested the feasibility of recontacting and interviewing a targeted sample of 500 families from an urban and a rural community that had participated in the historical CIP program. Stratified (on geographic region and on the pregnant mothers' status of being "exposed" or "unexposed" to folic acid supplements) random sampling was used to identify families in the historical CIP population to approach among CIP members who were still residing 15–18 y later in the study communities. Participating in the feasibility study were 460 families who responded to an in-person interview including questions about sociodemographic, lifestyle, school/work, and health factors (38). Participants in Study #1 were informed that they might be asked to participate in a second study (Study #2) focusing on nutrition, physical activity, and UV radiation exposure (42).

A total of 120 mother–child pairs included in Study #1 were targeted for Study #2. A stratified sample targeting 60 from the urban and 60 from the rural community were selected, with half of each group of 60 from families in which the mothers took and half from families in which the mothers did not take periconceptional folic acid supplements. Agreeing to participate in Study #2 were 92 mothers and 92 offspring among the 120 pairs identified from Study #1. All saliva samples came from Study #2 with the exception of the DNA methylation study component, for which we sought an increased sample size by approaching adolescents who had participated in Study #1 but declined participation in the entirety of Study #2. Participation in the entirety of Study #2 was declined because of the extensive time commitment required by the questionnaires and measurements for Study #2 (see Genetic and epigenetic samples: Selection of study subjects) (Supplemental Figure 1).

The institutional review boards and ethics committees of the collaborating institutions [Chinese Center for Disease Control and Prevention (China CDC), the US CDC, and the US National Cancer Institute (NCI)] approved the current project before data collection began. A 3-d training was conducted at each study center with staff from Maternal and Child Health Hospitals, including interviewers, phlebotomists, and hospital laboratory staff. Team leads from the China CDC, the US CDC, and the US NCI led the training and practice sessions with interviewers. Approximately 1 mo after training, supervisory staff from Beijing returned to each center to oversee data collection, monitor, and retrain staff as needed. Two additional supervisory visits occurred in the field at 2- to 3-wk intervals to address additional questions and concerns from each center.

Genetic and epigenetic samples

Selection of study subjects.—Among the 120 mother–adolescent dyads approached for the DNA methylation component of Study #2, a total of 92 mothers and 92 adolescents completed saliva collection. An additional 104 adolescents were approached for saliva samples to increase the sample size, with 92 of them completing saliva collection, thus providing a total of 276 samples (including 92 mothers and 184 adolescents). Seven of the collected samples were missing data (3 mothers and 4 adolescents) and thus excluded, resulting in a total of 269 viable saliva samples available for analysis (180 adolescents) (Supplemental Figure 1). Details regarding participation rate in the follow-up study have been reported previously (42).

Collection of saliva samples.—Saliva samples were collected and extracted using noninvasive Oragene® (OG-500) DNA self-collection kits and DNA Genotek's prepIt.L2P DNA Extraction Kit according to the manufacturer's instructions. Genomic DNA was evaluated and quantified using a Nanodrop UV spectrometer (Thermo Fisher Scientific). Respondents were instructed not to eat, drink, or smoke for 30 min before their appointment and then instructed to accumulate saliva in their mouths (~2–5 min), and spit into the opened vial until the saliva reached a designated line on the vial. Study staff then screwed on the cap of the vial and shook it gently. Samples were stored in local freezers (−80°C) until the end of the study.

After the collection of all samples, they were first transported to a central repository in Beijing without being thawed and similarly transported to the NIH repository in Maryland. DNA methylation was assessed using the Infinium Human Methylation® 450k array. One adolescent offspring sample was removed during the quality control step of analysis, leaving a total of 268 samples (179 adolescents) available for analysis (see Supplemental Figure 1).

Genotyping methods, frequencies, and quality control.—DNA was genotyped for a panel of folate metabolic genes, including *MTHFR* variants, by Sequenom MassARRAY iPlex®. The single nucleotide polymorphisms (SNPs) included in the plex are identified by Reference SNP cluster ID (rs number) in Supplemental Table 1. Duplicates were used to determine concordance rates. Supplemental Table 1 shows a summary of available genotyping data for each gene for mother and adolescent populations.

Statistical analysis

The primary analysis was to determine whether maternal folic acid supplementation in the periconceptional/first-trimester period was associated with changes to DNA methylation patterns in adolescent offspring of the mothers who took the supplements compared with those in adolescent offspring of mothers who did not take the supplements. To this end, raw data from the Infinium HM 450k bead array were imported into R statistical software, version 3.6.1 (R Core Team,2018) and quality control was completed using the minfi package, version 1.32.0 (43). Data were normalized using a single-sample normal-exponential convolution with out-of-band probes as controls, ssNoob (44), which reduces technical variations due to array batch or bisulfite plate, providing probes for 485,512 CpG methylation sites. After normalization, probes were masked using an updated manifest from

Zhou et al. (45). CpG sites were removed if >10% of samples had missing probes for the specified site, leaving a total of 402,730 CpG sites available for epigenome-wide association analyses. One adolescent sample was removed for having >5% missing CpG probes. No data were imputed.

Epigenome-wide association analyses were conducted with R software, version 3.6.1 (R Core Team,2018).We examined associations between maternal periconceptional folic acid supplementation and DNA methylation intensity of the 402,730 CpG sites of adolescent offspring remaining after quality control in an epigenome-wide association study. Logit-transformed β-values (i.e.,M-values) were used as the outcome variable for each CpG probe (46). Sex and geographic region (rural and urban) were determined to be potential confounders based on principal component analyses. Surrogate variable analysis (SVA) was conducted as a reference-free technique to estimate residual cell-mixture effect (47), after controlling for sex and geographic region. Using a permutation procedure by Buja and Eyuboglu (48), the number of surrogate variables was estimated to be 15. These 15 continuous surrogate variables were then calculated using the sva package, version 3.35.0 (49).

The primary analysis fit linear models to the M-values at each probe to assess periconceptional folic acid exposure and epigenome-wide methylation (46). The analysis controlled for calculated surrogate variables (using SVA to estimate background cell composition), sex, and geographic region (i.e., rural and urban). Models were fit using the limma package, version 3.42.2 (50). From the linear models, moderated F statistics and P values were calculated; P values were then adjusted for multiple comparisons using the Holm–Bonferroni method (51). Supplemental Figure 2 shows a Q-Q plot. Gene Set Enrichment Analysis (GSEA) and region-based analysis were conducted using the missMethyl, version 1.28, and DMRcate packages (52, 53). Additional models were fit to assess the interaction between periconceptional folic acid exposure and *MTHFR-C677T* genotype, owing to its established role in modulating folate status (39) genotype in the adolescent offspring. Epigenome-wide methylation models controlled for sex, geographic region, and background cell composition. As a positive control, models were fit comparing mothers with adolescent daughters to assess background methylation changes due to the mother–daughter age differences controlling for geographic region and cell composition (Supplemental Figure 3). We evaluated potential interaction by comparing differential methylation patterns due to the age differences in mothers and daughters according to folic acid exposure status (Supplemental Figure 4). Significant CpG sites were analyzed for significant overlap with previous studies on the effects of aging and methylation, and mother–daughter pairs and mothers and unrelated adolescents were analyzed independently (Supplemental Figures 5 and 6).

A secondary analysis was conducted to examine potential differences in allelic frequencies at selected SNPs implicated in folate metabolism. ANOVAs were performed to see if there were any main generational effects (comparing mothers and children) on allelic frequencies of *MTHFR-C677T* (rs1801133). These were defined as the number of recessive alleles among folic acid–consuming mothers and adolescents exposed to periconceptional folic acid compared with the distribution among non–folic acid consuming mothers and adolescents

not exposed to periconceptional folic acid. An additional ANOVA was conducted to examine the interaction of periconceptional folic acid exposure and generational changes on allelic frequencies. Analyses on all SNPs selected are available in Supplemental Table 1. Linkage disequilibrium (LD) was calculated for available genotype data using the Population Genetics R Package, version 1.3.8.1.2 (54). A threshold of $R^2 > 0.5$ was used to determine SNPs that were in LD with one another (Supplemental Table 2, Supplemental Figure 7).

Results

Adolescents were between 14 and 17 y old and mothers were between 35 and 54 y old at the time of participation (Table 1). After qualitycontroltherewere179adolescentsand 89mothers included in the analysis (Table 1). Among the 89 mothers, 44 received periconceptional folic acid supplementation and 45 were unexposed to folic acid by design. Among adolescents, there were 77 (43%) exposed and 102 (57%) unexposed to maternal periconceptional folic acid supplement use. The mean age was 15.2 y for adolescents and 40.0 y for mothers. The participants were from both rural (46.1% among mothers, 52.5% among adolescents) and urban centers (53.9% among mothers, 47.5% among adolescents). Table 1 presents the demographics and periconceptional folic acid exposure of the final analytic set. Genotypes were available for 29 polymorphisms involved in the folate metabolism pathway (Supplemental Table 1, Table 2).

For the primary analysis, the association of folic acid supplementation in the periconceptional period with DNA methylation level across the epigenome in the adolescent offspring is shown in the Manhattan plot in Figure 1. No loci reached statistical significance with a false discovery rate (FDR) <0.05. The top 100 loci with the highest FDR values are shown in Supplemental Table 3, each with an FDR consistent with the null hypothesis. The association of folic acid exposure compared with no exposure was adjusted for *MTHFR* genotype (rs1801133) and no changes were found (Supplemental Figure 3); the top 100 loci with the highest FDR values are shown in Supplemental Table 4, each with an FDR consistent with the null hypothesis. No enrichment was found in analyzing the top 1000 or top 5000 CpG sites, and region-based analysis yielded no differentially methylated regions (DMRs).

As a positive control, DNA methylation in mothers was compared with that in daughters for established epigenetic differences due to aging and developmental stage. From a total of 23,994 probes, 5.96% of all loci were significantly different between mothers and daughters (Supplemental Figure 3). The CpG sites that were significantly different between mothers and daughters were compared with previously reported CpG sites in the literature. Overlap with Clock CpGs: 116 of 353 clock CpGs overlapped (55). This overlap was significant using *GeneOverlap* to test for enrichment: $P < 0.01$. Supplemental Table 5 reports genes that overlapped with Horvath Clock CpGs. There was overlap with methylated probes associated with childhood aging: 771 of 2078 CpGs overlapped (56). This overlap was significant using *GeneOverlap* to test for enrichment: $P < 0.0001$. Overlap with Thompson et al. (57), methylated probes associated with puberty in females: 181 of 347 CpGs overlapped. This overlap was significant using *GeneOverlap* to test for enrichment: $P < 0.0001$. In the comparison of the differential methylation patterns between mothers and daughters in

the folic acid–exposed group and those in the unexposed group we found no evidence of interaction (Supplemental Figure 4). An additional analysis was conducted comparing only related mother–daughter pairs with unrelated mothers and adolescent females and found similarly significant CpG overlap (Supplemental Figures 5 and 6).

In the secondary analysis, no significant differences in genotype frequencies were found between mothers and adolescents. Some of these polymorphisms were in LD with each other; as such, each test is not independent and may reflect the close neighbors (SupplementalTable 2, SupplementalFigure 7).There was no significant change of allelic frequency at *MTHFR-C677T* between mother and daughter cohorts due to folic acid exposure ($P = 0.17$) (Table 2).

Discussion

In this first investigation (to our knowledge) comparing long-term DNA methylation patterns in adolescent offspring of both sexes in mothers who consumed folic acid supplements (400 μg/d) during the periconceptional period with those in offspring of mothers who did not take folic acid supplements, we found no differences in DNA methylation, regardless of *MTHFR-C677T* genotype. Overall, these results suggest that there are no widespread persistent changes in DNA methylation or folate metabolism genotypes in adolescent offspring in response to their mothers consuming the recommended dosage of 400 μg folic acid/d during this critical periconceptional period.

We are aware of 1 other longitudinal investigation that has examined the long-term impact of folic acid intake using an epigenome-wide approach; however, these participants consumed folic acid starting at a mean gestation of 17 wk (26). In a 47-y follow-up of 170 women whose mothers were enrolled in the Aberdeen Folic Acid Supplementation Trial the investigators found an association at cg09112514 ($P = 4.03 \times 10^{-9}$), a CpG located in the 5’ untranslated region of platelet-derived growth factor receptor (*PDGFRA*), and 8% reduction in methylation at this site (26). The investigators reported a dose-response reduction in methylation at this site in relation to the intervention. Richmond et al. (26) identified 46 regions of the genome that were differentially methylated in response to the intervention including major histocompatibility complex, class II, DP beta 2 (*HLA-DPB2*), major histocompatibility complex, class II, DP beta 1 (*HLADPB1*),paired box 8 (*PAX8*),and vault RNA 2–1 (*VTRNA2–1*). The investigators concluded that the findings from their study suggest that use of folic acid supplements during pregnancy was linked with persisting changes in the DNA methylation of offspring that were in genes implicated in embryonic development, immune response, and cellular proliferation.

A randomized controlled trial that evaluated continued folic acid intake into the second and third trimesters compared with stopping at the end of the first trimester (58) revealed significant changes in DNA methylation in cord blood of genes related to brain development; however, the changes were small and of uncertain clinical relevance (58,59).Thus, the findings from the randomized trial taken together with our findings may suggest that continuation of folic acid supplementation beyond the first trimester may be needed to observe long-standing changes in methylation patterns. Indeed, in a recent report

from the same trial, the adolescents exposed to continued supplementation with folic acid experienced significantly improved cognitive function at age 7 y compared with those exposed to folic acid supplementation through the first trimester (60).

Additional studies are needed because findings of the impact of prenatal exposures and cord blood/infant outcomes are subject to a lack of replication and small effect sizes as demonstrated in recent meta-analyses (61). Other studies of DNA methylation changes in offspring associated with pregnancy exposures (e.g., starvation in pregnancy compared with no starvation) discovered methylation changes at a handful of candidate genes (25, 62–64), demonstrating that extreme deprivation in nutrition during pregnancy can change DNA methylation in a very targeted fashion. In contrast, DNA methylation changes in cancer (in the tumor) are generally widespread because tumors are clonal and DNA methylation changes happen early in tumorigenesis (65, 66, 67, 68). Likewise, studies of pregnancy exposures such as the known teratogen smoking have found substantial changes in DNA methylation of the offspring at several loci in large meta-analyses (66, 69). Most DNA methylation changes in healthy individuals due to nutritional changes (such as folic acid and vitamin B-12) are extremely small (expressed as % methylation changes) and are of unclear or confirmed clinical significance (27, 61, 67, 70, 71).

To address the hypothesis that folic acid supplementation might increase the frequency of detrimental genetic variants— specifically *MTHFR 677 TT* (rs1801133) (41)—we examined the frequencies of *MTHFR 677* alleles in both mothers and adolescents in addition to other folate-specific genotypes. We lacked information about paternal genotypes to enable traditional transmission disequilibrium testing. However, the frequencies are consistent with previous studies in this population where the *MTHFR-C677T* variant is more common than the ancestral form (39). Reassuringly, across >24 genotypes assessed in the folate pathway there were no systematic increases in possible deleterious variants, including *MTHFR 677 TT*, among the adolescents compared with their mothers in our study. However, these results should be interpreted with caution owing to lack of paternal genotypes and the small sample size.

The current study has a number of strengths. There was no other source of folic acid (such as food fortification) or supplements used or widely available during these pregnancies in the geographic regions in China where the study was carried out (35).Mothers who participated in the original CIP had their folic acid intake recorded monthly (monthly pill counts) before and during early pregnancy(35).Women consumed 400 μg folic acid/d and were not provided with a multivitamin, permitting us to examine the independent effect of folic acid. Even in well-defined animal systems such as the agouti mouse where maternal diet increases DNA methylation at specific loci in the offspring and there have been associated changes in the phenotype of offspring, folic acid was not the only vitamin ingested in the classic experiments because the dams were fed a multicomponent cocktail of folic acid, choline, betaine, and other one-carbon sources (17, 22). Moreover, prospective assessment of the use of folic acid supplements before and during the first trimester as compared with continued use for the duration of the study allows for comparisons with other trials and adds a unique contribution to the literature, because many countries recommend discontinuation of folic acid supplements after the first trimester. These characteristics (prepregnancy,

prospective, single nutrient, recommended dosage) are major strengths for interpreting the findings of this study. In addition, this study was the first to show an ≤85% reduction in NTD prevalence with periconceptional high compliance with use of a 400 μg/d folic acid supplement alone (35). The high participation rates in the DNA methylation component of our feasibility work are another important strength that reduces the possibility of selection bias.

Our study is limited by sample size. Although the methylation status of any single CpG in a cell is obligatorily 0%, 50%, or 100% methylated, assays measure groups of cells that are often of different origins. The current analysis was conducted from saliva samples rather than blood, which may affect the type of changes seen (72). Power calculations using the 450K BeadChip suggested that in a case–control study design changes of 10%–15% would be detectable with sample sizes of 50– 100 individuals with $P < 10^{-6}$ (73). A methylation change that happens in the very early embryo would be passed to all the descendants of that early cell (74,75) and would therefore likely involve entire tissues. The occurrence of this kind of event was not supported by the current analysis, although rarer events cannot be excluded (such as CpG sites that did not reach the significance threshold that corrects for multiple comparison but may be of interest). Small although statistically significant changes in DNA methylation levels have been reported in response to nutritional exposures by others although they are generally un-replicated and of unknown clinical impact (24, 64, 67, 70, 71, 76). We confirmed the expected differences in DNA methylation between mothers and adolescent daughters due to aging and puberty (55–57), showing that the sample has sufficient statistical power to detect DNA methylation differences when they are present.

In addition, we are limited in that the assay used does not detect other changes such as 5-hydroxymethylcytosine, which affects global gene expression. The effects at CpGs not covered on this platform of large although still limited size are also clearly unknown. The use of saliva samples as opposed to the more commonly used blood is a limitation, but findings from other studies support the value of saliva as a noninvasive type of sample for DNA methylation characterization, although clearly DNA methylation changes in other tissues are known (26, 77, 78). This is a limitation of all studies using living persons where noninvasive samples are a necessity. Although it is possible that in the case of an exposure limited to the early embryonic period the effect may be present in more tissues, this is not certain. It is also not known if the adolescents born with birth defects had aberrant DNA methylation patterns (with or without folic acid supplement intake) or if any of the adolescents selected for the current study would have had a defect if their mothers had not chosen to consume the folic acid supplement. The study participants were all healthy adolescents (no external birth defects) regardless of their mother’s folic acid supplementation status in the CIP public health program.

In conclusion, our findings suggest that among the women in this study, folic acid intake at the recommended dosage did not result in widespread long-term changes in their offspring’s folate metabolism, genetic variant frequencies, or DNA methylation patterns epigenome-wide at an average follow-up of 15 y past birth. If these findings are confirmed in large studies using other populations and using a more expansive array and, ideally, blood samples, the results may provide reassurance of the benefits of the long-standing

recommendation of daily periconceptional use of 400 μg folic acid supplements to prevent NTDs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used:

CIP	Community Intervention Program
FDR	false discovery rate
LD	linkage disequilibrium
MTHFR	methylenetetrahydrofolate reductase
NTD	neural tube defect
SNP	single nucleotide polymorphism
SVA	surrogate variable analysis

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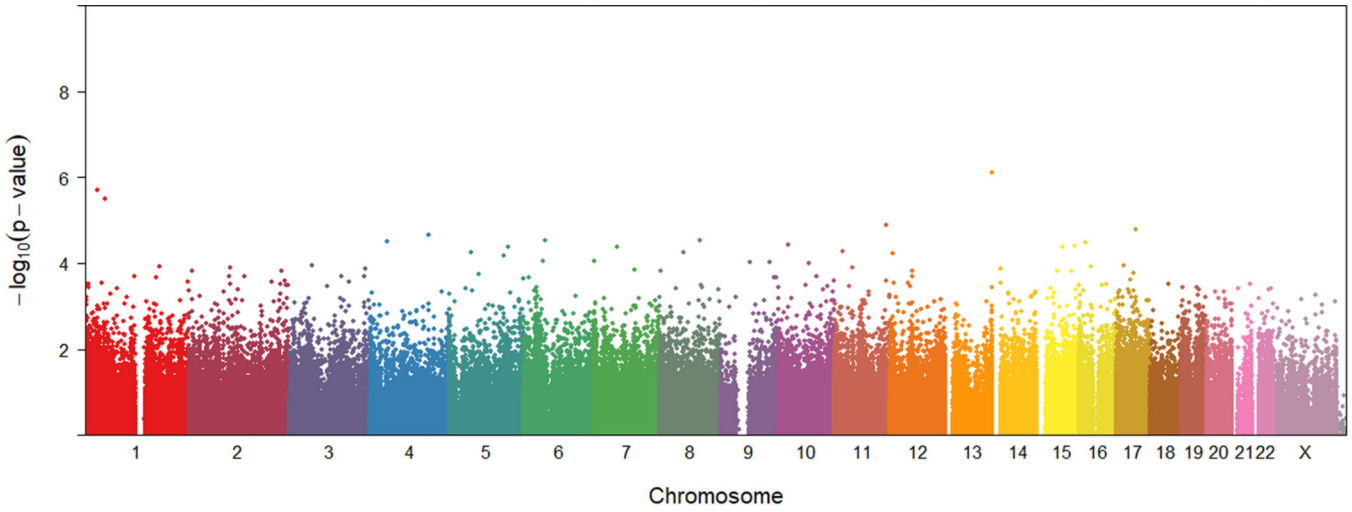

FIGURE 1.

Differential methylation between prenatal periconceptional folic acid exposure (n = 77) and no exposure (n = 102) in adolescents at 402,730 CpG sites. Adjusted for sex (90 female), region (94 rural), and background cell composition (surrogate variables modeled using surrogate variable analysis). Each point represents the negative log-transformed P value comparing methylation of folic acid–exposed with non-exposed adolescents at 1 CpG site grouped by chromosome (color). No site reached the statistical significance threshold comparing folic acid exposed with unexposed.

TABLE 1

Demographics and folic acid supplementation status[1]

	Mothers	Adolescents
Sample size, *n*	89	179
Folic acid supplementation		
Periconceptional	44 (49.4)	77 (43.0)
None	45 (50.6)	102 (57.0)
Age at time of study follow-up, y		
Mean (95% CI)	40.0 (39.2, 40.8)	15.2 (15.1, 15.3)
Range	35–54	14–17
Median	39	15
Gender		
Male	0 (0.0)	89 (49.7)
Female	89 (100.0)	90 (50.3)
Region		
Rural (Laoting)	41 (46.1)	94 (52.5)
Urban (Taicang)	48 (53.9)	85 (47.5)

[1]Values are *n* (%) unless indicated otherwise.

TABLE 2

Genotype frequencies for *MTHFR-C677T* (rs1801133), stratified by mothers and adolescents[1]

	Folic acid supplementation				No folic acid supplementation				
	n	**Mothers**	**Adolescents[2]**	***P* value[3]**	***n***	**Mothers**	**Adolescents[2]**	***P* value[3]**	***P* value[4]**
MTHFR-C677T (rs1801133)	115	44	71		138	43	95		
Homozygote major		10 (22.7%)	11 (15.5%)	0.07		11 (25.6%)	28 (29.5%)	0.88	0.17
Heterozygote		26 (59.1%)	36 (50.7%)			22 (51.2%)	45 (47.4%)		
Homozygote minor		8 (18.2%)	24 (33.8%)			10 (23.3%)	22 (23.2%)		

[1] *MTHFR*, 5,10-methylenetetrahydrofolate reductase.

[2] The genotype frequencies shown for adolescents were <179 (see Table 1) owing to some alleles not passing quality assurance/quality control criteria.

[3] *P* value from an ANOVA examining the main effect of generation (mother compared with child) on allele frequency.

[4] *P* value from an ANOVA examining the interactive effect of generation (mother compared with child) and folic acid supplementation on allele frequency.