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Impact of folic acid fortification on global DNA methylation and one-carbon biomarkers in the Women's Health Initiative Observational Study cohort

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Abbreviations: ANOVA, Analysis of variance; BMI, body mass index; DFE, dietary folate equivalent; DMG, dimethylglycine; FA, folic acid; HPLC, high-pressure liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MMA, methylmalonic acid; MTHFR, methylenetetrahydrofolate reductase; PLP, pyridoxal-5′-phosphate; RBC, red blood cell; TMAO, trimethylamine *N*-oxide; WHI-OS, Women's Health Initiative Observational Study; 5-methyl-THF, 5-methyltetrahydrofolate

DNA methylation is an epigenetic mechanism that regulates gene expression and can be modified by one-carbon nutrients. The objective of this study was to investigate the impact of folic acid (FA) fortification of the US food supply on leukocyte global DNA methylation and the relationship between DNA methylation, red blood cell (RBC) folate, and other one-carbon biomarkers among postmenopausal women enrolled in the Women's Health Initiative Observational Study. We selected 408 women from the highest and lowest tertiles of RBC folate distribution matching on age and timing of the baseline blood draw, which spanned the pre- (1994–1995), peri- (1996–1997), or post-fortification (1998) periods. Global DNA methylation was assessed by liquid chromatography-tandem mass spectrometry and expressed as a percentage of total cytosine. We observed an interaction ($P = 0.02$) between fortification period and RBC folate in relation to DNA methylation. Women with higher (vs. lower) RBC folate had higher mean DNA methylation (5.12 vs. 4.99%; $P = 0.05$) in the pre-fortification period, but lower (4.95 vs. 5.16%; $P = 0.03$) DNA methylation in the post-fortification period. We also observed significant correlations between one-carbon biomarkers and DNA methylation in the pre-fortification period, but not in the peri- or post-fortification period. The correlation between plasma homocysteine and DNA methylation was reversed from an inverse relationship during the pre-fortification period to a positive relationship during the post-fortification period. Our data suggest that (1) during FA fortification, higher RBC folate status is associated with a reduction in leukocyte global DNA methylation among postmenopausal women and; (2) the relationship between one-carbon biomarkers and global DNA methylation is dependent on folate availability.

Introduction

DNA methylation is an epigenetic modification of the genome, which influences gene expression and genome integrity.[1] DNA methylation can be modified by nutrients involved in one-carbon metabolism (e.g., folate, choline, vitamin B12, and vitamin B6), and disturbances in methylation reactions caused by abnormal status of these nutrients have been implicated in a number of human diseases including cancer.[2-6]

Folate, in the form of 5-methyltetrahydrofolate (5-methyl-THF), participates in cellular methylation reactions (including DNA methylation) by donating a methyl group for the vitamin B12-dependent re-methylation of homocysteine to methionine (**Fig. S1**). Folic acid (FA), the synthetic form of folate, can also

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participate in DNA methylation after its reduction to THF and conversion to 5-methyl-THF.[7] Homocysteine re-methylation to methionine can also proceed via a folate and B12-independent route in which betaine (a derivative of choline) serves as the methyl donor.[8]

In January of 1998, the US Food and Drug Administration mandated FA fortification of enriched cereal-grain products (i.e., addition of 140 μg of FA/100 g of grain) in an effort to reduce the occurrence of neural tube defects, a mandate that some food companies initiated in 1996 and 1997.[9] FA fortification of the US food supply has led to significant increases in serum and red blood cell (RBC) folate concentrations as well as decreases in plasma total homocysteine;[10,11] however, less is known about the impact of FA fortification on DNA methylation.

In this report, we investigated the association between mandatory FA fortification and leukocyte global DNA methylation, as well as the relationship between global DNA methylation, RBC folate, plasma choline, and other biomarkers of one-carbon metabolism among postmenopausal women enrolled in the Women's Health Initiative Observational Study (WHI-OS).

Results

Characteristics of the study population

The participants of this study were a subset of the control group from a nested case-control study investigating colorectal cancer risk in the WHI-OS.[12,13] Baseline demographic and biochemical characteristics of the participants, which corresponded to FA fortification periods [pre (1994–1995), peri (1996–1997), or post (1998)], are shown in **Table 1**. BMI differed among FA fortification periods ($P = 0.01$) with a higher mean BMI (28.3 kg/m^2) in the post-fortification period than in the pre- (26.5 kg/m^2; $P = 0.02$) and peri-fortification (26.4 kg/m^2; $P = 0.01$) periods. The ethnic distribution also differed among fortification periods ($P = 0.03$).

Plasma folate differed among FA fortification periods ($P = 0.002$) with higher median plasma folate (20 ng/mL) in the post-fortification period than in the pre- (14 ng/mL; $P = 0.001$) and peri-fortification (16 ng/mL; $P = 0.002$) periods. Similarly, RBC folate differed among FA fortification periods ($P = 0.002$) with higher median RBC folate in the peri- (572 ng/mL; $P = 0.02$) and post-fortification (726 ng/mL; $P < 0.001$) periods compared with the pre-fortification (424 ng/mL) period.

Effect of FA fortification period on baseline leukocyte global DNA methylation

Leukocyte global DNA methylation did not differ ($P = 0.86$, unadjusted; $P = 0.38$, multivariate-adjusted) among FA fortification periods (**Table 2**). However, we observed an interaction ($P = 0.02$) between fortification period and RBC folate status in relation to DNA methylation. Specifically, the highest (vs. lowest) RBC folate group had higher marginal mean DNA methylation (5.12 vs. 4.99%; $P = 0.05$) in the pre-fortification period, but lower DNA methylation (4.95 vs. 5.16%; $P = 0.03$) in the post-fortification period (**Table 3**). In addition, leukocyte global DNA methylation tended to differ ($P = 0.08$; multivariate-adjusted) among fortification periods (post < peri < pre) within the highest RBC folate group, but not within the lowest RBC folate group ($P = 0.20$; multivariate-adjusted) (**Table 3**).

Correlations between baseline leukocyte global DNA methylation and one-carbon biomarkers according to FA fortification period

The univariate Spearman correlations between one-carbon biomarkers and leukocyte global DNA methylation are shown in **Table 4**. Prior to fortification, there were significant, but modest, positive associations of global DNA methylation with plasma folate (r = 0.20, $P = 0.04$) and RBC folate (r = 0.24, $P = 0.01$) as well as a borderline significant positive association with plasma vitamin B12 (r = 0.18, $P = 0.06$). Global DNA methylation was also inversely correlated with plasma methylmalonic acid (MMA; r = -0.26, $P = 0.03$), choline (r = -0.31, $P = 0.002$) and homocysteine (r = -0.26, $P = 0.007$). In the peri-fortification period, no significant relationships were observed between one-carbon biomarkers and global DNA methylation. Finally, in the post-fortification period, global DNA methylation was positively correlated with plasma homocysteine (r = 0.28, $P = 0.02$).

Main predictors of baseline leukocyte global DNA methylation according to FA fortification period

One-carbon biomarkers that predicted global DNA methylation were identified according to FA fortification period, testing them individually in multivariate-adjusted models (**Table 5**). Prior to fortification, RBC folate positively predicted global DNA methylation (β = 0.25, $P = 0.02$) explaining 5% of the residual variation (partial $R^2 = 0.05$). Plasma vitamin B12 also tended to positively predict DNA methylation (β = 0.20, $P = 0.08$) explaining 3% of the residual variation (partial $R^2 = 0.03$); however, plasma homocysteine (β = -21.23, $P = 0.03$), MMA (β = -1.18, $P = 0.05$), and choline (β = -57.82, $P = 0.002$) negatively predicted global DNA methylation explaining 4% (partial $R^2 = 0.04$), 6% (partial $R^2 = 0.06$) and 10% (partial $R^2 = 0.10$) of the residual variation, respectively. In the peri-fortification period, no significant predictors of DNA methylation were detected. Finally, in the post-fortification period, plasma homocysteine tended to positively predict global DNA methylation (β = 29.37, $P = 0.07$) explaining 4% of the residual variation (partial $R^2 = 0.04$). The overall R^2 explained by one-carbon biomarkers, tested in an unadjusted linear regression model in which all variables were included simultaneously, was 0.12 in the pre- and peri-fortification periods and 0.19 in the post-fortification period (**Table S1**) with plasma choline, plasma dimethylglycine (DMG), and plasma homocysteine being the strongest predictors in each period, respectively (**Table S2**).

Discussion

The present study investigated the association between mandatory FA fortification and leukocyte global DNA methylation, as well as the relationship between global DNA methylation, RBC folate, and other biomarkers of one-carbon metabolism in postmenopausal women. The following two main findings emerged: (1) FA fortification period and RBC folate status interacted to influence global DNA methylation and; (2) associations

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Table 1. Baseline characteristics of the study participants (n = 408) according to folic acid (FA) fortification period[1-3]

Characteristic	FA fortification period			P value
	Pre (1994–1995)	Peri (1996–1997)	Post (1998)	
Age (years)[4]	67 ± 7	67 ± 7	67 ± 6	0.87
BMI (kg/m²)[4]	26.5 ± 5[a]	26.4 ± 5[a]	28.3 ± 6[b]	0.01
Race/ethnicity[5]				0.03
Non-Hispanic White	86	88	76	
Other[7]	14	12	24	
Education[5]				0.69
≤ High school	21	24	20	
≥ College	79	76	80	
Pack-years of smoking[6]	0 (0–10)	0 (0–15)	0.3 (0–12.5)	0.49
Leisure physical activity[6] (minutes/week)	60 (0–210)	40 (0–180)	30 (0–150)	0.70
Plasma folate (ng/mL)[6]	14 (8–27)[a]	16 (8–25)[a]	20 (14–31)[b]	0.002
RBC folate (ng/mL)[6]	424 (321–771)[a]	572 (361–852)[b]	726 (431–863)[b]	0.002
Plasma Hcy (µmol/L)[6]	8.6 (6.9–10.8)	8.5 (6.8–9.7)	7.9 (6.8–9.7)	0.13
Plasma MMA (nmol/L)[6]	158 (113–204)	146 (120–179)	168 (136–211)	0.17
Plasma vitamin B12 (pg/mL)[6]	523 (354–703)	481 (352–650)	489 (371–685)	0.43
Plasma PLP (nmol/L)[6]	62 (43–109)	70 (46–114)	68 (41–132)	0.83
Plasma choline (µmol/L)[4]	9.2 ± 2.0	9.3 ± 2.2	9.4 ± 1.7	0.79
Plasma betaine (µmol/L)[4]	28 ± 10	28 ± 10	25 ± 10	0.16
Plasma DMG (µmol/L)[6]	2.5 (2.1–2.9)	2.3 (1.9–2.9)	2.4 (1.9–2.7)	0.07
Plasma TMAO (µmol/L)[6]	3.8 (2.5–6.1)	3.7 (2.6–5.4)	4.1 (2.9–6.1)	0.33
Plasma creatinine (mg/dL)[4]	0.72 ± 0.13	0.70 ± 0.11	0.73 ± 0.11	0.12
DFE (µg/d)[6]	409 (304–537)	411 (319–537)	441 (306–595)	0.51
Dietary vitamin B6 intake (mg/d)[6]	1.4 (1.1–1.9)	1.4 (1.0–1.8)	1.5 (1.0–1.9)	0.43
Dietary vitamin B12 intake (µg/d)[6]	5.1 (3.3–7.3)[a]	4.1 (2.7–6.5)[b]	5.3 (3.5–7.4)[a]	0.01
Supplemental vitamin B2 intake (mg/d)[6]	0.0 (0–1.7)	0.1 (0–1.7)	0.0 (0–1.7)	0.84
Supplemental vitamin B6 intake (mg/d)[6]	0.0 (0–2.0)	1.0 (0–2.0)	0.0 (0–2.0)	0.32
Supplemental vitamin B12 intake (µg/d)[6]	0.0 (0–6.0)	2.3 (0–6.0)	0.0 (0–6.0)	0.89

[1]The study participants were a subset of the control group from a nested case-control study investigating colorectal cancer risk in the Women's Health Initiative-Observational Study. [2]Differences between FA fortification periods were analyzed by chi-square tests (categorical variables), one-way ANOVA (normally distributed continuous variables), or non-parametric Kruskal-Wallis tests (non-normally distributed continuous variables); different superscript letters within a row indicate a difference between FA fortification periods at $P < 0.05$.[3]n = 122 in the pre-fortification period; n = 204 in the peri-fortification period; n = 82 in the post-fortification period. [4]Values are mean ± SD for normally distributed continuous variables. [5]Values are percentage for categorical variables. [6]Values are median (interquartile range) for non-normally distributed continuous variables. [7]African American, Hispanic, Asian or Pacific Islander, American Indian or Alaskan Native. Abbreviations used: BMI, body mass index; RBC, red blood cell; Hcy, homocysteine; MMA, methylmalonic acid; PLP, pyridoxal-5′-phosphate; DMG, dimethylglycine; TMAO, trimethylamine *N*-oxide; DFE, dietary folate equivalent.

between one-carbon biomarkers and global DNA methylation differed between FA fortification periods.

FA fortification period interacted with RBC folate status to influence global DNA methylation

Previous studies have found that global DNA methylation can be altered by folate depletion or repletion in healthy adults.[14-17] In postmenopausal women, global DNA methylation significantly decreased under folate depletion[14,15] and increased upon folate repletion.[14] Based on these findings and the role of folate as a methyl donor, we anticipated that global DNA methylation would be higher among postmenopausal women with higher (vs. lower) RBC folate status. This expected result was observed in the pre-fortification period, but not in the post-fortification period during which women with higher (vs. lower) RBC folate status had lower DNA methylation.

Excess FA intake through fortified foods and supplements can lead to the accumulation of unmetabolized FA,[18] which may interfere with normal folate metabolism[19-22] and lower global DNA methylation.[23] Although supraphysiologic folate status (i.e., total plasma folate concentrations > 19.8 ng/mL)[24] was observed

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Table 2. Baseline leukocyte global DNA methylation levels (%) according to folic acid (FA) fortification period[1]

	FA fortification period						*P* value
	Pre (1994–1995)		Peri (1996–1997)		Post (1998)		
Global DNA methylation (%)	n	Value	n	Value	n	Value	
Unadjusted[2]	122	5.04 ± 0.35	204	5.05 ± 0.37	82	5.03 ± 0.38	0.86
Multivariate-adjusted[3]	118	5.04 ± 0.03	199	5.06 ± 0.03	77	5.00 ± 0.04	0.38

[1]Linear regression models were used to compare mean DNA methylation across fortification periods. [2]Values are mean ± SD for unadjusted analyses. [3]Multivariate analyses were adjusted for age, BMI, ethnicity, creatinine, and *MTHFR* C677T genotype; values are marginal mean ± SE.

Table 3. Baseline leukocyte global DNA methylation levels (%) within the lowest and highest RBC folate groups according to folic acid (FA) fortification period[1,2]

	Lowest RBC Folate group		Highest RBC Folate group		*P* value
	n	Value	n	Value	
Pre-fortification (1994–1995)					
Unadjusted[3]	71	5.01 ± 0.37	51	5.08 ± 0.32	0.29
Multivariate-adjusted[4]	69	4.99 ± 0.04	49	5.12 ± 0.05	0.05
Peri-fortification (1996–1997)					
Unadjusted[3]	102	5.09 ± 0.38	102	5.02 ± 0.36	0.21
Multivariate-adjusted[4]	101	5.08 ± 0.04	98	5.03 ± 0.04	0.37
Post-fortification (1998)					
Unadjusted[3]	29	5.18 ± 0.39	53	4.94 ± 0.34	0.01
Multivariate-adjusted[4]	25	5.16 ± 0.07	52	4.95 ± 0.05	0.03

[1]Participants were divided into tertiles of RBC folate, and lowest (<471 ng/mL) and highest (>672 ng/mL) RBC folate groups were further stratified by FA fortification period. [2]Linear regression models were used to compare differences between RBC folate groups. [3]Values are mean ± SD for unadjusted analyses. [4]Multivariate analyses were adjusted for age, BMI, ethnicity, creatinine, and *MTHFR* C677T genotype; values are marginal mean ± SE.

among postmenopausal women with higher RBC folate in the post-fortification period (25.3 ng/mL; **Table S3**), it was similarly observed among women with higher RBC folate in the pre-fortification period (25.1 ng/mL) and appeared to be mostly attributable to FA supplement use in both periods. Specifically, a higher percentage of FA supplement users was observed among participants with higher (vs. lower) RBC concentrations (76% vs. 20%; **Table S4**), and higher RBC folate concentrations were observed among FA supplement users (vs. non-users) across fortification periods (**Table S5**). Nonetheless, a previous study conducted in the US reported the highest concentration of plasma unmetabolized FA in subjects exposed to both FA fortified foods and supplements as compared to those exposed only to FA fortified foods, or only to supplements.[25] Thus, it is possible that unmetabolized FA was elevated to a greater extent in the post- (vs. pre-) fortification period among women with higher RBC folate status. Measurements of unmetabolized FA in our cohort are needed to further explore this possibility, and additional studies are required to clarify the health outcomes, if any, of the inverse relationship between leukocyte global DNA methylation and high RBC folate in the era of FA fortification.

Associations between one-carbon biomarkers and global DNA methylation differed among FA fortification periods

Previous human studies have reported conflicting results with positive[14,16,26] or no[27,28] relationships between circulating folate (i.e., plasma and RBC folate) and global DNA methylation.[29] In the present study, plasma and RBC folate were positively correlated with DNA methylation in the pre-fortification period, but not in the peri- or post-fortification period. These findings suggest that the relationship between folate status and global DNA methylation is nonlinear and that folate status is likely to be a stronger predictor of global DNA methylation when folate availability is lower (i.e., prior to FA fortification). However, as alluded to above, it is possible that the differences in the relationship between circulating folate and DNA methylation across fortification periods arose from differences in the amounts of metabolized and unmetabolized folate. For example, metabolized folate present in the pre-fortification period may positively associate with global DNA methylation, while unmetabolized FA more likely to be present in the peri- and post-fortification periods[24] may attenuate the positive relationship between folate status and global DNA methylation. Taken together, when total folate status (metabolized plus unmetabolized) is considered across the full spectrum from deficiency to very high, the overall association between folate and global DNA methylation may approximate a reverse U-shaped curve rather than a linear relationship.

Folate intake/status may also modify the relationship between DNA methylation and other nutrients involved in one-carbon metabolism. Indeed, biomarkers of vitamin B12 status (i.e., plasma vitamin B12 and MMA) were associated with leukocyte global DNA methylation in the pre-fortification period, but not in the peri- or post-fortification period. Both folate and vitamin B12 are required for the provision of methyl groups

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Table 4. Spearman rank correlation coefficients (r) between baseline leukocyte global DNA methylation and one-carbon biomarkers according to folic acid (FA) fortification period[1]

	FA fortification period								
	Pre (1994–1995)			Peri (1996–1997)			Post (1998)		
	n	r	*P* value	n	r	*P* value	n	r	*P* value
Plasma folate (ng/mL)	115	0.20	0.04	195	0.05	0.52	75	–0.07	0.56
RBC folate (ng/mL)	118	0.24	0.01	199	–0.01	0.88	77	–0.09	0.47
Plasma vitamin B12 (pg/mL)	115	0.18	0.06	195	–0.08	0.26	75	–0.17	0.16
Plasma MMA (nmol/L)	74	–0.26	0.03	130	0.12	0.19	46	–0.02	0.90
Plasma choline (μmol/L)	102	–0.31	0.002	181	–0.07	0.37	73	0.001	0.99
Plasma betaine (μmol/L)	102	–0.09	0.39	181	–0.06	0.46	73	0.05	0.69
Plasma DMG (μmol/L)	102	–0.15	0.14	181	–0.007	0.92	73	0.05	0.67
Plasma TMAO (μmol/L)	102	0.001	0.99	181	–0.01	0.90	73	0.18	0.13
Plasma Hcy (μmol/L)	115	–0.26	0.007	197	–0.03	0.71	77	0.28	0.02
Plasma cysteine (μmol/L)	115	–0.10	0.28	197	0.04	0.54	77	0.01	0.90

[1]Analyses were adjusted for age, BMI, ethnicity, creatinine, and *MTHFR* C677T genotype. RBC, red blood cell; MMA, methylmalonic acid; DMG, dimethylglycine; TMAO, trimethylamine *N*-oxide; Hcy, homocysteine.

through the methionine synthase reaction (**Fig. S1**). However, folate is suggested to be a stronger determinant of biomarkers of the methylation cycle (e.g., plasma homocysteine) than vitamin B12,[30,31] which may explain the lack of association between vitamin B12 status and global DNA methylation in the peri- and post-fortification periods.

The relationship between plasma choline and global DNA methylation was also modified by FA exposure with an inverse relationship observed in the pre-fortification period, but not in the peri- or post-fortification period. The inverse relationship between choline (a methyl donor) and DNA methylation in the pre-fortification period is unexpected and requires confirmation in other studies. However, when folate is less abundant (i.e., prior to FA fortification), supply of S-adenosylmethionine (SAM) for methylation reactions may be reduced thereby creating a competition among the various methyltransferases. As the affinity of DNA methyltransferase for SAM is ~18 times higher than phosphatidylethanolamine *N*-methyltransferase,[32] the enzyme that produces choline endogenously, SAM may be preferentially partitioned toward DNA methylation thus reducing endogenous choline production. In turn, this could lead to the inverse relationship observed in the pre-fortification period between DNA methylation and plasma choline.

Prior to FA fortification, we observed an inverse relationship between plasma homocysteine and global DNA methylation, which is consistent with previous reports.[26,33] Interestingly, however, plasma homocysteine was positively correlated with DNA methylation in the post-fortification period. The divergent relationships between homocysteine and DNA methylation across fortification periods may arise from the fact that homocysteine is both a precursor and product of cellular methylation reactions. These data collectively suggest that the relationship between homocysteine and DNA methylation is dynamic and likely to be dependent on folate availability.

Strengths and limitations

The present study had several strengths including: (1) a unique opportunity to investigate the impact of mandatory FA fortification on global DNA methylation by stratifying into three fortification periods (pre-, peri-, and post-) and; (2) examination of a wide range of biomarkers involved in one-carbon metabolism as potential predictors of global DNA methylation according to FA fortification period. Several limitations should also be noted: (1) relatively small sample size; (2) potential for residual confounding by factors that were either not collected in the WHI-OS or not measured with sufficient precision and; (3) single measures of one-carbon biomarkers and global DNA methylation within each FA fortification period, which may not fully reflect the true complexity of DNA methylation reactions.

Conclusion

These data suggest that during FA fortification, higher RBC folate status is associated with a reduction in leukocyte global DNA methylation among postmenopausal women. If reductions in leukocyte global DNA methylation are shown to have adverse health outcomes in future studies, FA supplement use may not be advisable among postmenopausal women residing in the US or other countries with mandated FA fortification programs. The present study also suggests that FA intake via fortification modifies the relationship between one-carbon biomarkers and global DNA methylation, but potential biologic mechanisms need discerning.

Materials and Methods

Subjects and study design

The WHI-OS is a prospective cohort study that was established to investigate the predictors and causes of morbidity and mortality in postmenopausal women.[34,35] The study enrolled 93 676 postmenopausal women, aged 50–79 y, at 40 clinical centers throughout the US between 1993 and 1998. These years of enrollment spanned the pre- (prior to January 1, 1996),

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Table 5. Predictors of baseline leukocyte global DNA methylation according to folic acid (FA) fortification period[1,2]

	FA fortification period								
	Pre (1994–1995)			Peri (1996–1997)			Post (1998)		
	n	β Coefficient	*P* value	n	β Coefficient	*P* value	n	β Coefficient	*P* value
Plasma folate (ng/mL)	115	3.98	0.31	195	–0.17	0.92	75	–2.33	0.54
RBC folate (ng/mL)	118	0.25	0.02	199	–0.03	0.73	77	–0.24	0.14
Plasma vitamin B12 (pg/mL)	115	0.20	0.08	195	–0.14	0.20	75	–0.25	0.18
Plasma MMA (nmol/L)	74	–1.18	0.05	130	0.67	0.11	46	0.34	0.56
Plasma choline (μmol/L)	102	–57.82	0.002	181	–7.10	0.57	73	7.65	0.79
Plasma betaine (μmol/L)	102	–2.91	0.41	181	–1.21	0.64	73	1.58	0.74
Plasma DMG (μmol/L)	102	–31.11	0.43	181	6.28	0.79	73	–3.25	0.96
Plasma TMAO (μmol/L)	102	6.10	0.12	181	0.17	0.97	73	1.45	0.83
Plasma Hcy (μmol/L)	115	–21.23	0.03	197	1.38	0.91	77	29.37	0.07
Plasma cysteine (μmol/L)	115	–1.23	0.19	197	0.48	0.53	77	–0.26	0.86

[1]Linear regression models were used, adjusting for age, BMI, ethnicity, creatinine, and *MTHFR* C677T genotype. [2]Beta (β) coefficient indicates mean increase in DNA methylation per 1000-unit increase in one-carbon biomarker. RBC, red blood cell; MMA, methylmalonic acid; DMG, dimethylglycine; TMAO, trimethylamine *N*-oxide; Hcy, homocysteine.

peri- (1996–1997), and post- (after January 1, 1998) FA fortification periods in the US.[9] Women were excluded from the study if they had medical conditions with a predicted survival of less than 3 y; if they had adherence/retention issues (alcoholism, drug dependency, mental illness, or dementia); or if they were participating in another clinical trial. The study was approved by the human subject review boards at the Fred Hutchinson Cancer Research Center where the WHI Clinical Coordinating Center is located and at all 40 clinical centers. Written informed consent was obtained from all participants.[34,35]

In the present study, participants were a subset of those from a nested case-control study investigating colorectal cancer risk in the WHI-OS.[12,13] From the controls of the study, we selected 408 women from the lowest (n = 202) and highest (n = 206) tertiles of baseline RBC folate concentrations, matching on age and timing of the baseline blood draw, which spanned the following FA fortification periods: pre-fortification (1994–1995; n = 71 low tertile, 51 high tertile), peri-fortification (1996–1997; n = 102 low tertile, 102 high tertile), and post-fortification (1998; n = 29 low tertile, 53 high tertile). The proportions in the fortification periods correspond approximately to the recruitment of the WHI-OS. However, because the nested case-control study from which we were sampling did not contain at least 50 participants in the post-fortification period, low RBC folate group, we selected additional participants from the pre-fortification, low RBC folate group in order to maintain approximately the same number of participants in each of the low and high RBC folate tertiles.

Data collection

Baseline demographic and health-related characteristics (i.e., age, race/ethnicity, education, smoking status, and physical activity) were collected using standardized questionnaires.[34] Height and weight were measured using a standardized protocol, and BMI was calculated as weight (kg)/height (m^2). Dietary intake of folate, vitamin B6 and vitamin B12 was based on data derived from the WHI food-frequency questionnaire as previously described.[36] Supplemental vitamin intakes of B2, B6, and B12 were assessed by an inventory in which nutrients were recorded based on participants' current dietary supplement bottles, which they brought to the clinic visits. To account for differences in bioavailability between synthetic FA and natural food folate, dietary folate equivalent (DFE) was used as the unit for total folate intake.[37]

Analytic measurements

Blood samples were drawn at baseline after at least 12 h of fasting. Samples were kept at 4 °C for up to 1 h prior to centrifugation. Plasma and serum were collected and stored at -70 °C until analysis.[13] Leukocyte global DNA methylation was measured in de-identified samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described by Song et al.[38] with modifications based on our instrumentation.[39] A total of 11 batches (40 samples per batch) were run in duplicate. For each batch, all samples from both comparisons (i.e., high and low RBC folate) were randomly ordered and equally represented and matched on period of blood draw by pre-, peri- and post-fortification and on age. Both internal laboratory controls and 10% blind duplicate samples were used to determine assay precision and monitor assay performance. Internal laboratory controls included: (1) unmethylated lambda DNA (Promega); (2) methylated lambda DNA (~30% of DNA methylated); (3) four in-house human biological control samples and; (4) a negative control (water). All quality control samples were prepared in duplicate and interspersed among the samples. DNA methylation is expressed as a percentage of total cytosine: [methylated cytosine / (methylated + unmethylated cytosine)] × 100%.

Plasma concentrations of choline and its metabolites (i.e., betaine, DMG, trimethylamine *N*-oxide [TMAO]) were measured using stable isotope dilution LC-MS/MS methodology.[40] Plasma total homocysteine and cysteine were determined by high-pressure liquid chromatography (HPLC) with post-column

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fluorescence detection[41]; plasma and RBC folate as well as plasma vitamin B12 were measured by radioassay (SimulTRAC; MP Biomedicals); plasma pyridoxal-5′-phosphate (PLP) was analyzed by HPLC with fluorescence detection[42]; plasma MMA was measured by LC-MS/MS[43]; plasma creatinine was quantified by the Jaffe rate reaction method (DxC Instrument; Beckman Coulter); and methylenetetrahydrofolate reductase (*MTHFR*) C677T genotype (rs 1801133) was determined by the Illumina 384-plex BeadXpress genotyping platform (Illumina Inc.).

Inter-assay coefficients of variance of the blind duplicate control samples for each of the assays were as follows: global DNA methylation, 5.5%; choline, 5.6%; betaine, 4.6%; DMG, 11.9%; TMAO, 5.8%; homocysteine, 6.5%; cysteine, 7.1%; RBC folate, 10.2%; plasma folate, 4.8%; vitamin B12, 6.2%; PLP, 5.9%; MMA, 15.0%; and creatinine, 4.1%.

Statistical analysis

Differences in baseline characteristics of the study population between FA fortification periods were analyzed by: (1) one-way analysis of variance (ANOVA) for normally distributed continuous variables; (2) non-parametric Kruskal-Wallis tests for non-normally distributed continuous variables or; (3) chi-square tests for categorical variables. Linear regression models were used to: (1) examine the influence of FA fortification and RBC folate (and their interaction term) on baseline leukcoyte global DNA methylation and; (2) identify the one-carbon biomarkers that predicted leukocyte global DNA methylation. The partial R^2 for each predictor variable was determined to estimate the contribution of each predictor to the total variability in DNA methylation. Spearman rank correlation coefficients (r) were also computed to examine associations between leukocyte global DNA methylation and one-carbon biomarkers. In the multivariate-adjusted analyses, we controlled for age and BMI along with plasma creatinine, ethnicity (white/not white) and *MTHFR* C677T genotype as these three variables were shown to be influential in a univariate model assessing possible confounders on DNA methylation. *MTHFR* C677T genotype was treated as an additive variable (i.e., minor allele count) in our statistical models because of reduced variation; parameter estimates were not changed substantially when *MTHFR* C677T genotype was treated as a categorical variable. Because approximately 25% of plasma creatinine values were missing among the sample due to insufficient sample availability, simple mean imputation was used for the missing creatinine values. Model results using multiple imputation and simple mean imputation were similar. Significance was defined as $P < 0.05$, and all statistical tests were 2-sided. The data were analyzed by SAS version 9.3 (SAS Institute Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
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