

# A 19-Base Pair Deletion Polymorphism in Dihydrofolate Reductase Is Associated with Increased Unmetabolized Folic Acid in Plasma and Decreased Red Blood Cell Folate<sup>1,2</sup>

Renee D. Kalmbach,<sup>3</sup> Silvina F. Choumenkovitch,<sup>3</sup> Aron P. Troen,<sup>3</sup> Paul F. Jacques,<sup>3</sup> Ralph D'Agostino,<sup>4</sup> and Jacob Selhub<sup>3\*</sup>

<sup>3</sup>Vitamin Metabolism and Aging Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA 02111; and <sup>4</sup>Framingham Heart Study, Boston University School of Medicine, Framingham, MA 01701

## Abstract

Dihydrofolate reductase (DHFR) catalyzes the reduction of folic acid to tetrahydrofolate (THF). A 19-bp noncoding deletion allele maps to intron 1, beginning 60 bases from the splice donor site, and has been implicated in neural tube defects and cancer, presumably by influencing folate metabolism. The functional impact of this polymorphism has not yet been demonstrated. The objective of this research was to determine the effects of the *DHFR* mutation with respect to folate status and assess influence of folic acid intake on these relations. The relationship between *DHFR* genotype and plasma concentrations of circulating folic acid, total folate, total homocysteine, and concentrations of RBC folate was determined in 1215 subjects from the Framingham Offspring Study. There was a significant interaction between *DHFR* genotype and folic acid intake with respect to the prevalence of high circulating unmetabolized folic acid (defined as >85th percentile). Folic acid intake of  $\geq 500$   $\mu\text{g}/\text{d}$  increased the prevalence of high circulating unmetabolized folic acid in subjects with the deletion (del/del genotype (47.0%) compared with the wild type (WT)/del (21.4%) and wild type (WT)/WT genotypes (24.4%) ( $P$  for interaction = 0.03). Interaction between the *DHFR* polymorphism and folic acid intake was also seen with respect to RBC folate ( $P$  for interaction = 0.01). When folic acid intake was  $< 250$   $\mu\text{g}/\text{d}$ , the del/del genotype was associated with significantly lower RBC folate (732.3 nmol/L) compared with the WT/WT genotype (844.4 nmol/L). Our results suggest the del/del polymorphism in *DHFR* is a functional polymorphism, because it limits assimilation of folic acid into cellular folate stores at high and low folic acid intakes. J. Nutr. 138: 2323–2327, 2008.

## Introduction

The enzyme dihydrofolate reductase (DHFR)<sup>5</sup> converts ingested folic acid, the fully oxidized and synthetic form of the vitamin folate found in supplements and fortified foods, to the reduced physiological folates that are utilized by the cells. DHFR's endogenous substrate, dihydrofolate, is formed during thymidylate synthesis and is reduced by DHFR back to tetrahydrofolate (THF), which accepts and donates 1-carbon units that are used to synthesize DNA precursors (1). The importance of this reaction is demonstrated by the effectiveness of antifolate

medications used to treat cancer by inhibiting DHFR, thus depleting THF and slowing DNA synthesis and cell proliferation (2). Optimal synthesis of THF is also necessary for biological methylation activity as the precursor of 5-methylTHF, which is needed for synthesis of *S*-adenosylmethionine, a universal methyl donor (1).

Recently a prevalent polymorphism of the *DHFR* gene has been described involving a 19-bp deletion/insertion in intron-1, 60 bases from the splice donor site (3). The biological consequences of this mutation have been controversial. Originally, Johnson et al. (3) suggested that the deletion (del)/del genotype in mothers is related to an increased risk of having a baby born with spina bifida, whereas another study has suggested the del/del genotype is protective and decreases the risk of having a baby born with spina bifida (4); yet another study reported no effect (5). Inconsistent results have also been reported for the effect on measures of folate status, with one study reporting that those with the del/del genotype had lower plasma homocysteine and another reporting no effect on homocysteine but increased plasma and RBC folate in women (6,7).

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<sup>5</sup> Abbreviations used: del, deletion; DHFR, dihydrofolate reductase; Hb, hemoglobin; THF, tetrahydrofolate; tHcy, total homocysteine; WT, wildtype.

\* To whom correspondence should be addressed. E-mail: jacob.selhub@tufts.edu.

There is other evidence suggesting the deletion is a functional mutation. The deletion is in a noncoding part of the DNA sequence, and in other genes this is an important site for transcription, translation, and other regulatory functions (8–10). The del/del genotype has been associated with up to a 4.8-fold increase in mRNA levels compared with the wildtype (WT)/WT genotype (11), but similar studies have reported no effect (5) or a smaller nonsignificant trend of increased mRNA levels (4).

Given its role in reducing folic acid, we hypothesized the *DHFR* polymorphism alters the capacity to reduce dietary folic acid, thereby limiting the assimilation of folic acid into the endogenous forms of folate. The objective of this study was to: 1) determine the functional impact of the *DHFR* polymorphism with regard to plasma concentrations of circulating unmetabolized folic acid, total folate, and total homocysteine (tHcy) and RBC folate concentrations; and 2) evaluate a possible interaction between folic acid intake and *DHFR* genotype with any of the measures of folate status.

## Materials and Methods

**Study population.** Subjects were participants of the 6th examination of the Framingham Offspring Study. The Framingham Offspring Study started in 1971 and includes the offspring of the original Framingham Heart Study. A total of 3532 individuals participated in the 6th examination of the Framingham Offspring Cohort, which started in January 1995 and was completed by August 1998 and thus includes samples from subjects who were unexposed and exposed to mandatory folic acid fortification, which was phased in between September 1996 and July 1997 (12). Individuals who were examined between October 1996 and August 1997 were excluded from these analyses because of uncertainty of their folic acid intake. Of the 1703 individuals remaining, we had *DHFR* genotype information for 1258 individuals. Of these, 1215 had data for circulating folic acid and total folate concentrations, 1212 had complete data on plasma creatinine and plasma tHcy, and 882 individuals had complete data on RBC folate concentrations. This study was approved by the Human Investigations Review Committee at New England Medical Center and by the Institutional Review Board for Human Research at Boston University Medical Center.

**Dietary assessment.** Dietary intake was assessed by a semiquantitative, self-administered FFQ (13). Subjects were asked to report on frequency of food consumption in the previous year, vitamin and mineral supplements, and type of breakfast cereal most commonly consumed. To estimate intake of specific nutrients, the frequency of consumption was multiplied by the nutrient content of the specified portions. If  $\geq 12$  food items were left blank or reported energy intakes were  $< 2512$  kJ/d or  $> 16,747$  kJ/d, the information was excluded from analysis. To determine total pre- and post-fortification folic acid intake, the FFQ food item “folic acid” was created to reflect fortification as previously described (12). Total folic acid intake included folic acid consumed in fortified foods and in vitamin supplements.

**Biochemical methods.** As part of the 6th examination, blood samples were taken from fasting subjects. Circulating plasma folic acid concentrations were determined using an updated affinity/HPLC method with electrochemical (coulometric) detection (14), plasma tHcy was determined by HPLC with fluorometric detection (15), plasma total folate by a 96-well plate microbial (*Lactobacillus casei*) assay (16,17), plasma creatinine by the Jaffe method (18), and RBC folate by heat extraction and centrifugation followed by *L. casei* microbial assay with chicken pancreas conjugase treatment (19). RBC folate was expressed as nmol folate/g hemoglobin (Hb). Values were converted to nmol folate/L packed cells using the mean cell Hb concentration (20).

***DHFR* genotype determination.** Genotype was determined using a 7300 RT-PCR system from Applied Biosystems. The following PCR primers were used: forward primer (5'-TCGCTGTGTCCAGAACATG-3') and re-

verse primer (5'-AGCGCAGACCGCAAAGTCTG-3'). Two TaqMan TAMRA probes were used to target the 19-bp deletion. The assay identifies the *DHFR* alleles by measuring the change in fluorescence of the dyes associated with the probes. A FAM fluorescent probe was used to identify the insertion allele (ACC TGG GCG GGA CGC G TAMRA) and a VIC fluorescent probe was used for the deleted allele (TGG CCG ACT CCC GGC G TAMRA). The PCR consists of 3 ng template (genomic DNA), 950 nmol/L for the forward and backward primers, 250 nmol/L of each probe (Applied Biosystems), and TaqMan Universal PCR Master mix at  $2\times$  concentration in a total reaction volume of 20  $\mu$ L. The samples were denatured at 95°C for 10 min, followed by 50 cycles of 92°C for 15 s, then 60°C for 1 min.

**Statistical methods.** All analyses were performed with SAS statistical software version 9.1 (SAS Institute). Because the concentrations of tHcy, RBC folate, and total folate were positively skewed, we used natural logarithmic transformation on these variables and reported geometric means with 95% CI. We used ANCOVA (PROC GLM) to calculate the prevalence of high circulating folic acid and mean concentrations for the other measures of folate status for each *DHFR* genotype. The prevalence of high circulating folic acid (defined as  $> 1.35$  nmol/L, the 85th percentile of the cohort) was used instead of actual concentrations, because 32% of the population did not have detectable circulating folic acid and the distribution was skewed toward lower values. All means are adjusted for age and sex. Plasma tHcy concentrations are also adjusted for plasma creatinine. The *P*-values reported represent the overall significance test for differences across *DHFR* genotype after the Tukey adjustment for multiple comparisons.

Interactions between age, sex, folic acid intake, and *DHFR* genotype were assessed by including the interaction terms between these factors and *DHFR* genotype in the model. When significant interactions were observed ( $P < 0.05$ ), we stratified the analyses. Folic acid intake was stratified into 3 folic acid categories to represent different levels of non-saturating and saturating folic acid intake ( $< 250$   $\mu$ g/d, 250–500  $\mu$ g/d, and  $> 500$   $\mu$ g/d). These categories were based on metabolic studies that have indicated folic acid intake between 200–300  $\mu$ g/d is the lower threshold that typically does not saturate DHFR and will not result in folic acid in circulation; thus, 250  $\mu$ g/d was used as a cut off point. The upper threshold of 500  $\mu$ g/d was used, because this level of intake saturates the intestinal conversion of folic acid to 5-methylTHF and result in the appearance of folic acid in circulation (21–25).

## Results

Demographic and biochemical characteristics by *DHFR* genotype are shown in Table 1. Of the total cohort, 216 were homozygous for the deletion allele (del/del), 646 were heterozygous (WT/del), and 396 individuals for homozygous for the wild-type allele (WT/WT). The genotypes did not differ in age, BMI, gender, folic acid intake, total plasma folate, prevalence of high circulating folic acid, RBC folate, or tHcy concentrations.

There was an interaction between *DHFR* genotype and folic acid intake ( $P = 0.03$ ) in relation to the prevalence of high circulating folic acid ( $> 85$ th percentile); thus, we stratified our analyses by folic acid intake (0–250, 250–500, and  $> 500$   $\mu$ g/d) (Fig. 1A). The prevalence of high circulating folic acid was higher in those homozygous for the deletion allele who consumed  $> 500$   $\mu$ g/d of folic acid (47.0%, 95% CI: 31.6–62.5) compared with heterozygotes (21.4%; 95% CI: 13.6–29.3;  $P < 0.05$ ) and WT/WT (24.4%; 95% CI: 13.9–35.0;  $P < 0.05$ ). The prevalence of having high circulating folic acid in those consuming  $< 500$   $\mu$ g/d was unrelated to *DHFR* genotype.

There was also an interaction ( $P = 0.01$ ) between folic acid intake and *DHFR* genotype with RBC folate concentrations (Fig. 1B). Among those consuming  $< 250$   $\mu$ g/d of folic acid, mean RBC folate concentrations were 732.3 nmol/L (95% CI: 669.1–801.4) for those del/del compared with 793.0 nmol/L

**TABLE 1** Characteristics of the Framingham Offspring Study according to *DHFR* genotype

	<i>DHFR</i> genotype <sup>1</sup>			<i>P</i> -value
	WT/WT ( <i>n</i> = 396)	WT/del ( <i>n</i> = 646)	del/del ( <i>n</i> = 216)	
Age, <i>y</i> (range)	58.8 (36–86)	57.8 (29–85)	58.5 (33–80)	0.14
Sex, % <i>males</i>	51.0	48.8	51.9	0.90
BMI, <i>kg/m</i> <sup>2</sup> (range)	28.0 (27.5–28.4)	28.1 (27.7–28.4)	27.6 (27.0–28.1)	0.33
B vitamin supplement use, <i>n</i> (%)	176 (36.7)	315 (38.6)	103 (36.7)	0.70
Total folic acid intake, <sup>2</sup> $\mu\text{g}/\text{d}$ (range)	171.7 (148.8–196.3)	206.5 (186.6–227.5)	177.5 (146.3–211.7)	0.07
Plasma total folate, <sup>3</sup> <i>nmol/L</i> (range)	18.3 (17.2–19.7)	19.3 (18.1–20.4)	17.9 (16.3–19.9)	0.40
Plasma tHcy, <sup>3,4</sup> $\mu\text{mol/L}$ (range)	9.2 (8.9–9.4)	9.1 (8.9–9.3)	9.3 (8.9–9.6)	0.77
Homocysteine >13 $\mu\text{mol/L}$ <sup>4</sup>	11.9 (8.8–14.9)	9.4 (7.0–11.8)	13.9 (9.8–18.0)	0.15
RBC folate, <sup>3</sup> <i>nmol/L</i>	1020.6 (960.4–1084.5)	1017.7 (971.5–1066.1)	982.1 (906.0–1064.8)	0.72
Plasma folic acid >85th percentile	16.3 (12.7–19.9)	15.3 (12.4–18.1)	15.5 (10.5–20.4)	0.90

<sup>1</sup> Values are means and percentages with 95 percent CI adjusted for age and sex unless otherwise indicated; *P* < 0.05 is considered different.

<sup>2</sup> Total folic acid intake from food and supplements square root transformed.

<sup>3</sup> Geometric mean and 95% CI.

<sup>4</sup> Means also adjusted for creatinine.

(95% CI: 750.3–837.4) (WT/del) and 844.4 nmol/L (95% CI: 787.8–905.1) (WT/WT) (*P* < 0.05 for del/del with WT/WT). RBC folate concentrations in those consuming >250  $\mu\text{g}/\text{d}$  was not different between the 3 genotypes.

Interactions between *DHFR* genotype and folic acid intake with plasma tHcy and plasma total folate were not significant,

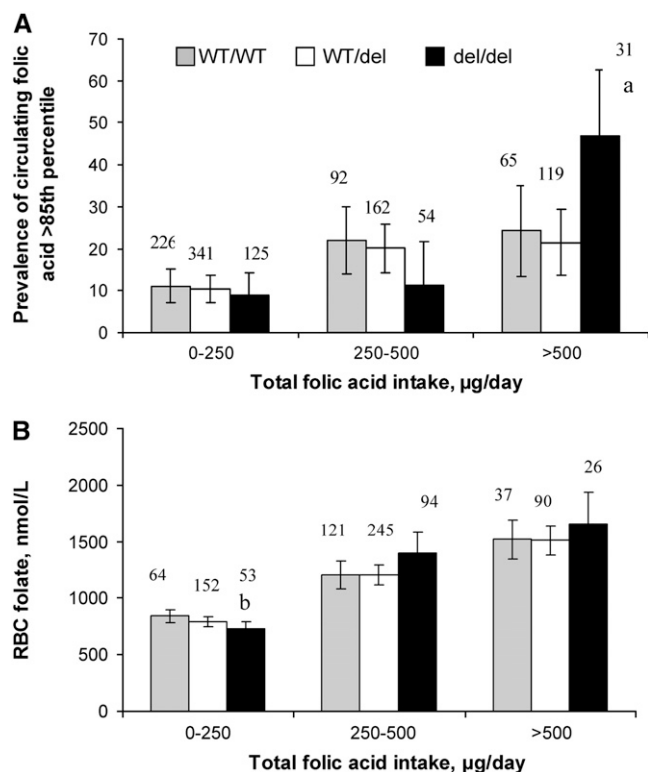
nor were there any interactions between *DHFR* genotype and age or sex for any outcome measures.

## Discussion

This cross-sectional, population-based study describes an effect of a 19-bp deletion allele in the *DHFR* gene on measures of folate status that depends on folic acid intake. Homozygotes for the 19-bp deletion in *DHFR* who consume >500  $\mu\text{g}/\text{d}$  of folic acid had an increased prevalence of high circulating folic acid compared with the WT/WT and WT/del genotypes. Additionally, homozygotes who consume <250  $\mu\text{g}/\text{d}$  of folic acid had lower RBC folate concentrations compared with the WT/WT genotype. Together, these results suggest that the *DHFR* polymorphism directly impairs folic acid metabolism at both low and high folic acid intakes.

During intestinal absorption, folic acid is readily metabolized to 5-methylTHF, the normal transport form of folate, when given in low doses (26). However, there is a threshold level of intake at ~250  $\mu\text{g}$  that saturates the capacity of the enzyme, allowing folic acid to be directly transported into circulation unmetabolized (21,22,24). In the present study, the upper threshold of folic acid intake that revealed a functional effect on the enzyme was ~500  $\mu\text{g}/\text{d}$ . Intakes above this amount in those homozygous for the deletion allele seemed to overwhelm the ability of the enzyme to reduce exogenous folic acid to the physiological folate forms. This functional effect also occurred if folic acid intake was <250  $\mu\text{g}/\text{d}$ . Folic acid can only be incorporated into the cell if it is reduced by DHFR. Our findings suggest the del/del polymorphism results in a diminished capacity of the enzyme to reduce folic acid, thus limiting assimilation of reduced folates into tissue folate stores, although at higher intakes, there appears to be enough folate to overcome any deficiencies of the enzyme associated with the *DHFR* polymorphism.

*DHFR* genotype did not affect total plasma folate and tHcy concentrations, so it seems in this population the del/del genotype may have a greater negative effect on tissue stores of folate rather than plasma stores. Our findings are in contrast to a study in a Dutch population in which the del/del genotype was associated with lower homocysteine but did not affect total plasma folate or RBC folate (6) and an Irish study in which women with the del/del genotype had higher plasma folate and RBC folate. The Irish



**FIGURE 1** Prevalence of having circulating folic acid >85th percentile (A) and geometric mean RBC folate concentration (B) with 95% CI according to folic acid intake by *DHFR* WT/WT, del/WT, and del/del genotypes. The numbers above the bars represent the number of subjects in each category. Prevalence and means were calculated by general linear models adjusted for age and sex. Letters indicate a difference between genotypes at the same level of folic acid intake, *P* < 0.05: <sup>a</sup> del/del vs. WT/WT and WT/del; <sup>b</sup> del/del vs. WT/WT.

study found no relationship in men or in homocysteine concentrations (7). The conflicting results for all these studies could be at least partially explained by differences in populations. For example, the Irish population was generally younger and their outcomes depended on gender, although in our population, genders did not differ.

Another factor could be folic acid intake, which in our population dictated the effect of the polymorphism on RBC folate and high circulating folic acid concentrations. Our population included individuals exposed and unexposed to mandatory folic acid fortification; while there is no folic acid fortification of grains in the Netherlands and fortification is voluntary in Ireland. Evidence of a gene-nutrient interaction from this and other studies highlight the importance of nutritional status when evaluating the effect of the *DHFR* polymorphism on folate-related health outcomes (11,27). For example, in 1 study, women with the del/del genotype had increased risk of breast cancer, but only if they also used multivitamin supplements (11). Folate seems to be protective against some cancers, presumably by its positive effect on genomic stability and DNA methylation. However, there is also increasing, albeit controversial, evidence that folic acid can promote cancer development in those with an existing cancer or who are at high risk for cancer, possibly by increasing DNA synthesis (28–30).

There is very little mechanistic evidence that would help explain the inconsistent findings concerning the *DHFR* polymorphism. An in vitro study reported that removal of intron-1 did not effect transcription, but the protein produced was unstable and subject to lysosomal degradation, suggesting the deletion polymorphism may lower the *DHFR* function or amount at the translational level (31). Bioinformatics reveal that intron-1 in *DHFR* contains strong potential transcription factor binding sites for transcriptional factors Sp1, Sp3, and estrogen receptor 1. The insertion contains Sp1 and Sp3 sites and the deletion contains ESR1 sites (32). ESR1 is a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription. The Sp1 site is reported to be important for growth/cell cycle regulation of *DHFR* transcription (33). Removal of the Sp1 transcription factor binding site could act to increase or decrease transcription, because Sp1 regulates *DHFR* transcription during the cell-cycle through the recruitment and interactions of coactivators and repressors (34). Confirmation in laboratory studies is clearly needed to understand how gene expression and enzyme activity are affected, which would also help clarify the public health importance of the polymorphism.

In conclusion, this study describes an important gene-nutrient interaction where the functional effect of the *DHFR* polymorphism depends on folic acid intake. Future studies relating the *DHFR* polymorphism and health outcomes should include data on folic acid intake in their analyses. The clinical significance of the *DHFR* polymorphism may be most significant in individuals with either very low folic acid intake, which results in diminished tissue folate stores, or very high intake, which results in increased folic acid in circulation. The safety of high synthetic folic acid intake has been questioned due to potential adverse health effects in those with the highest intakes of folic acid or highest plasma folate concentrations (30,36–39). If the potential harm is a consequence of circulating folic acid, then the deletion in *DHFR* that results in the highest circulating folic acid concentrations could represent additional risk.

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