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The Protective Association of MTHFR Polymorphism on Cervical Intraepithelial Neoplasia is Modified by Riboflavin Status

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Abstract

Objectives—We previously reported that women polymorphic for the methylenetetrahydrofolate reductase (MTHFR) gene were less likely to have cervical intraepithelial neoplasia (CIN) 2 or 3 (odds ratio [OR] = 0.40, 95 % confidence interval [CI]: 0.21, 0.78, p=0.007). In the current study, we tested whether this protective association is modified by the circulating riboflavin status in the same study population.

Methods—Riboflavin status was assessed by the erythrocyte glutathione reductase (EGR) assay and expressed in terms of an EGR activity coefficient (EGRAC). The status of MTHFR polymorphism, riboflavin, and circulating concentrations of folate, vitamins B-12, A, E, C and total carotene were ascertained in 170 White and 265 African-American women positive for the cervical presence of high-risk human papilloma virus (HR-HPV). Presence/absence of CIN 2 or 3 was determined histologically, and associations with risk factors were examined using multiple logistic regression. Eighty women with CIN 2 or 3 lesions were compared to 355 women without cervical lesions. Based on the median EGRAC of 1.4, women were grouped into low (> 1.4) and high (≤1.4) riboflavin status.

Results—Women with MTHFR polymorphism and low riboflavin status were significantly less likely to have CIN 2 or 3 than the referent group of women without the polymorphism and high riboflavin status (OR = 0.35, 95% CI: 0.13, 0.92, p= 0.034). MTHFR polymorphism was not associated with CIN 2 or 3 in women with high riboflavin status (OR = 0.51, 95% CI: 0.22, 1.19, p = 0.119), nor were any of the associations influenced by folate levels.

Conclusions—A further inactivation of polymorphic MTHFR by low riboflavin status and a resulting shift in the folate metabolic pathway toward DNA synthesis may explain these observations. The practical implications of this complex gene-nutrient-disease interaction will require further investigation.

Keywords

MTHFR; riboflavin; cervical; neoplasia

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Introduction

The apparent role of folate in carcinogenesis in several tissues has stimulated investigations of polymorphisms in folate metabolizing enzymes. Methylenetetrahydrofolate reductase (MTHFR) regulates the metabolism of folate and methionine, both of which are important factors in DNA methylation and synthesis. A base change from C to T at the nucleotide position 677 of the MTHFR gene results in coding for valine (GTC) rather than alanine (GCC). Both heterozygous (Ala/Val) and homozygous (Val/Val) variants, compared to homozygous normal (Ala/Ala), have reduced MTHFR enzyme activity (1). This common polymorphism of the MTHFR gene therefore can lead to abnormal DNA methylation and DNA synthesis, possibly leading to increased risk of cancer. The effect of MTHFR polymorphisms on cancer and pre-cancer susceptibility, however, remains controversial. A protective effect of this polymorphism was shown for colorectal cancer (2,3,4,5), while an increased risk has been reported for endometrial cancer (6), breast/ovarian cancer (7) and cervical intraepithelial neoplasia (8,9). The colorectal cancer studies suggested that the MTHFR polymorphism reduces colon cancer risk, perhaps by increasing 5, 10 methylenetetrahydrofolate levels for DNA synthesis, but that low folate intake or high alcohol intake may negate some of the protective effect. In a study conducted after folate fortification was implemented in the USA, we reported that MTHFR polymorphism is associated with reduced risk of cervical intraepithelial neoplasia (CIN) 2 or 3 (10). This protective effect is also likely to be due to a shift in the folate pathway toward DNA synthesis. Since the activity of MTHFR can also be reduced by a low concentration of its cofactor flavin adenine dinucleotide (FAD) or of riboflavin, the precursor of FAD (11), we tested whether the protective association observed between MTHFR and CIN 2 or 3 is modified by the riboflavin status. In vitro studies have shown that polymorphic MTHFR is ≈ 10 times as likely as the wild-type enzyme to dissociate from its FAD prosthetic group and thus become inactivated, whereas high riboflavin status reduces the degree of this dissociation (12). It is unknown whether riboflavin status affects the activity of the MTHFR in vivo, and consequently, whether it modifies the risk of cancer or pre-cancer conditions.

Materials and Methods

Study Design

A detailed description of the study design has been recently published (10). Briefly, the subjects of this study were a subset of women recruited for the Atypical Squamous Cells of Undetermined Significance-Low-Grade Squamous Intraepithelial Lesion (ASCUS-LSIL) Triage Study (ALTS) at the clinical center in Birmingham, AL, who agreed to participate in an ancillary study of gene-nutrient interactions and risk of developing CIN 2 and 3. The ALTS study was designed to determine the optimal clinical management for low-grade cervical cytological abnormalities. Clinical centers located in Birmingham, AL, Oklahoma City, OK, Pittsburgh, PA, and Seattle, WA were established to enroll and provide follow-up care to women with cytological diagnoses of ASCUS and LSIL from referring community laboratories. Women were randomly assigned to one of three screening arms: immediate colposcopy, HPV triage, and conservative management. Recruitment began in October 1996 and ended in December 1998. Subjects were followed every 6 months for 2 years from the date of recruitment through December 2000. The administration of a risk-factor questionnaire, pelvic examination, collection of specimens for cytology and HR-HPV testing by the Hybrid Capture 2 assay, and cervicography were performed at each visit by the ALTS staff. A more detailed description of the ALTS study is published elsewhere (13).

Seven hundred twenty (n = 720) women were recruited for the ancillary study at the Birmingham ALTS center. These women agreed to provide additional blood samples at baseline and completed a brief questionnaire designed to assess patterns of use of vitamin supplements. The present study is based on analysis of the information collected from

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participants at the baseline of ALTS and the ancillary study. Blood samples were used to determine the status of MTHFR polymorphism and circulating concentrations of folic acid, vitamins B-12, A, E, and C, total carotene and riboflavin status. Ten subjects of races other than White or African American were excluded from the analyses to reduce the potential variation in results by other races and because their numbers were too small to study separately. One woman was excluded because of an incorrect referral diagnosis to ALTS. Thirty-five women without HR-HPV test results at the baseline visit and 232 women who were HR-HPV negative were also excluded. Four additional women who met the above criteria but who were missing riboflavin status were not included in the final analysis population. Lastly, 3 additional women were eliminated from analyses because they were missing MTHFR data. We restricted the analysis to HR-HPV positive women because infection with HR-HPV is a necessary cause in the development of CIN 2 or 3. For this reason, in examining the association of polymorphisms and CIN 2 or 3, we did not control for HPV infection as a confounder because doing so could erroneously nullify the associations of interest (14). The final population consisted of 435 subjects, 170 (39 percent) White and 265 (61 percent) African American, who were HR-HPV positive at the baseline visit. The Institutional Review Board of the University of Alabama at Birmingham (UAB) and the ALTS Ancillary Studies Committee approved our study protocol.

Laboratory Methods

The HC-2 assay (Digene Gaithersburg, MD 20878 USA), selected by the ALTS investigators, detected 13 HR-HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and classified patients as positive or negative for HR-HPV. Detailed descriptions of sample collection and methodologies used to assess MTHFR polymorphism and folate, vitamins B-12, A, E, C and total carotene are published elsewhere (10,15). RBC samples frozen at −80°C within two hours of collection were used to determine riboflavin status using the erythrocyte glutathione reductase activity coefficient (EGRAC), a preferred method for evaluating riboflavin status (16). The principle of this assay is based on the fact that glutathione reductase requires FAD as a coenzyme. If a subject has low riboflavin status, enzyme activity will be reduced and will increase after FAD is added to the reaction mixture. The added FAD has minimal or no effect in subjects with adequate riboflavin status. In order to simplify the procedure and to reduce the time required to perform the determination, we modified it to employ 96-well plates and a plate reader. This assay has been validated in our laboratory by one of the co-authors (PEC). Briefly, each sample was assayed as stimulated (with the addition of in vitro FAD) and un-stimulated (without the addition of in vitro FAD). The following were added to each tube: 2mL phosphate buffer solution (0.1M, pH 7.4), 100μl red blood cell hemolysate (100μl packed, RBC in 1.9mL dH2O), 50 μl 80mM EDTA, and 100μl of 24.5 mM oxidized glutathione. The un-stimulated tubes received an additional 100μl of dH2O and the stimulated tubes received 100 μl of 26.3 uM FAD. All tubes were mixed thoroughly by vortex and incubated for 30 minutes at 37 ° C, then removed from the incubator. When the tubes were cooled to room temperature, 100μl of NADPH was added to all tubes and tubes were mixed by vortex. Quickly, 300μl was taken from each tube and placed into the well of a 96-well plate and read spectrophotometrically at 340nm. The same plate was then read again nine minutes later. The difference in the stimulated samples was divided by the difference in the un-stimulated samples and expressed as an activity coefficient, as shown below.

$$AC=\frac{\text{Reduction of absorbance with added FAD}}{\text{Reduction of absorbance without added FAD}}$$

All samples were assayed by one of the co-authors (MA). Handling of frozen samples when aliquots are made for this assay (e.g., thawing time prior to aliquoting frozen samples) was kept constant throughout the study. The co-efficient of variation of the assay was less than 2%. Since a marked stimulation occurs with inadequate riboflavin status, a higher AC is indicative

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of suboptimal riboflavin status. There has been no standard AC value to define suboptimal riboflavin status in different populations; reported values vary from 1.2 to 1.4 (17,18,19).

Disease Classification

The disease-positive group for this study consisted of subjects with histologically confirmed CIN 2 or 3 lesions at enrollment. The disease-negative group consisted of subjects with histologically or cytologically confirmed CIN 1, ASCUS, or normal cervical cytology at enrollment.

Exposure Classification

MTHFR genotypes were classified as homozygous polymorphic (Val/Val), heterozygous polymorphic (Ala/Val), any MTHFR polymorphism (Val/Val plus Ala/Val), and homozygous normal (Ala/Ala). Micronutrient concentrations were categorized into high or low groups based on median values for the study population. The median EGRAC for our study population was 1.4; this cut off was used to classify subjects as having low or high riboflavin status. Additionally, 33rd and 66th percentile values for the EGRACs were used to divide study subjects into low (> 1.5 EGRAC), medium (>1.3–1.5 EGRAC) and high (≤1.3 EGRAC) riboflavin tertiles.

Statistical Analysis

Frequency distributions and other bivariable analysis methods were used to compare the groups. Unconditional logistic regression models were run with a binary indicator of outcome (CIN 2 or 3 vs. no CIN 2 or 3) as the dependent variable and presence of MTHFR polymorphism as independent predictors of primary interest. To determine whether adjusting for micronutrients including folate confounds the association between MTHFR and CIN 2 or 3, models were fit adjusting for known demographic and behavioral risk factors of CIN 2 or 3 (age, smoking history, race, hormonal contraceptive use, age at first intercourse) alone and in combination with circulating concentrations of micronutrients. The relationship between MTHFR and CIN 2 or 3 was examined by riboflavin status, defined as low vs. high based on the median EGRAC and as low, medium, and high based on the 33rd and 66th percentile values of EGRAC. Statistical testing of the interaction between MTHFR polymorphism and riboflavin status categorized as above and below the median or in tertiles was performed. The purpose was to further evaluate if there is any effect modification present in the various combinations of these factors on CIN risk. These models were also fit adjusting for known demographic and behavioral risk factors of CIN 2 or 3 alone and in combination with circulating concentrations of micronutrients. Subsequent models were adjusted for a combination of demographic and behavioral factors and micronutrients. To determine whether the association of MTHFR, riboflavin, and CIN 2 or 3 was affected by folate status, we examined the association by it restricting to high and low folate and riboflavin groups. All models were adjusted for the study arm assignment since it determined which screening process was used to detect lesions. All statistical analyses were conducted using the statistical package SAS Version 8.2 (SAS Institute, Cary, NC).

Results

Eighty women (18%) had CIN 2 or 3 lesions, and 355 (82%) had CIN 1, ASCUS, or normal cervical cytology. The women with CIN 2 or 3 lesions were less likely to be African American and more likely to have ever smoked than women in the referent group. The use of vitamin supplements and plasma concentrations of folate, vitamin B-12, vitamin C, vitamin A, vitamin E, total carotene, and riboflavin status (EGRAC) did not differ appreciably between disease groups (Table 1).

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Plasma folate concentrations differed significantly depending on the riboflavin status defined as low, medium and high; the low riboflavin group had the lowest plasma folate (8.1 ng/ml) compared to medium and high riboflavin status (9.5 ng/ml and 9.6 ng/ml of plasma folate respectively) ($p<0.0001$). The lowest plasma folate concentrations were found among women with low riboflavin status and MTHFR polymorphism (7.8 ng/ml folate) while the highest folate concentrations were found among women with high riboflavin and no MTHFR polymorphism (9.9 ng/ml folate). As shown in Table 2, the prevalence of any polymorphism (Ala/Val + Val/Val or Ala/Val alone) was lower in the group of women with CIN 2 or 3 lesions; however, these differences were not statistically significant. The distribution of the MTHFR C677T alleles in the case and referent groups fit the Hardy-Weinberg equilibrium. The presence of any MTHFR polymorphism was inversely associated with CIN 2 or 3 after adjustment for demographic and sexual behavioral factors alone (OR = 0.43, 95 % confidence interval [CI]: 0.24, 0.78, $p = 0.005$) or in combination with nutritional factors (OR = 0.40, 95 % CI: 0.21, 0.78, $p = 0.007$). All remaining analyses were conducted with any MTHFR polymorphism, combining Ala/Val and Val/Val genotypes.

As shown in Table 3, women with any MTHFR polymorphism and low riboflavin status were significantly less likely to have CIN 2 or 3 after adjusting for demographic and behavioral factors alone or in combination with plasma micronutrients as compared to women without MTHFR polymorphism and with high riboflavin status (referent group) (OR = 0.31, 95% CI: 0.12, 0.80, $p= 0.016$ and OR = 0.35, 95% CI: 0.13, 0.92, $p= 0.034$ respectively). The presence of any MTHFR polymorphism and high riboflavin status also reduce the odds of having CIN 2 or 3 relative to women without MTHFR polymorphism and high riboflavin status; however, this association does not achieve statistical significance. The results are similar when adjusting for demographic and behavioral factors alone (OR = 0.54, 95% CI: 0.24–1.20, p= 0.129) or in combination with plasma micronutrients (OR = 0.51, 95% CI: 0.22–1.19, p= 0.119). Women without MTHFR polymorphism and low riboflavin status have a non-significant increased risk of CIN 2 or 3 relative to the same referent group as above (adjusting for demographic and behavioral factors alone: OR = 1.46, 95% CI: 0.81–2.63, p= 0.211; in combination with plasma micronutrients: OR=1.62, 95% CI: 0.89–2.97, p=0.117). The association among any MTHFR polymorphism, riboflavin status and CIN 2–3 was also evaluated using tertiles of the distribution of riboflavin levels, yielding similar results whether the models were adjusted for demographic and behavioral factors alone or in combination with plasma micronutrients. The association between any MTHFR polymorphism and low riboflavin status and CIN 2 or 3 was significantly protective (OR = 0.20, 95 % CI: 0.06, 0.74, $p = 0.015$) whereas a non-significant protective effect is shown among those with medium (OR = 0.45, 95 % CI: 0.17, 1.18, $p=0.106$) and high riboflavin status (OR = 0.45, 95 % CI: 0.17, 1.21, $p=0.114$, Table 3) relative to the previously defined referent group. In contrast, women without MTHFR polymorphism and low riboflavin status (OR = 1.07, 95% CI: 0.51, 2.26, p=0.856) or medium riboflavin status (OR = 1.02, 95% CI: 0.51, 2.05, p=0.943) had similar odds of having CIN 2 or 3 as the referent group. The association of low riboflavin status and MTHFR polymorphism with CIN 2 or 3 was similar in women with low plasma folate (OR: 0.26, 95% CI: 0.05–1.33, p=0.10) and high plasma folate (OR: 0.24, 95% CI: 0.06, 1.01, p=0.05). Similarly, the magnitude of the OR was similar when women had high riboflavin status and low folate status (OR: 0.82, 95% CI: 0.16, 4.32, p=0.81) and high riboflavin status and high folate status (OR: 0.83, 95% CI: 0.20, 3.46, p=0.79). Despite this pattern of results, which clearly suggests that riboflavin status modifies the effect of MTHFR polymorphism on CIN risk, formal statistical testing of the interaction between MTHFR polymorphism and riboflavin status did not yield significant results either with riboflavin status categorized as above and below the median (p=0.11) or in tertiles (p=0.29). Thus, the data are statistically compatible with models in which MTHFR polymorphism reduces CIN risk independently from riboflavin.

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Discussion

Interactions between MTHFR and riboflavin status are beginning to be appreciated. Typically, effect modification (interaction) is difficult to demonstrate even in large studies, and the present investigation is no exception. Most reports have focused on the effect of riboflavin on homocysteine concentrations in individuals with MTHFR polymorphism. Three studies conducted in Irish (20), Norwegian (21) and American (22) populations concluded that low riboflavin status may increase homocysteine concentrations in MTHFR polymorphic subjects. A plausible mechanism behind these observations is that FAD is 10 times more likely to dissociate from the less-active polymorphic enzyme than from the normal enzyme, and the resulting reduced activity of MTHFR decreases the availability of 5-methyltetrahydrofolate for homocysteine re-methylation. However, an important difference among these studies was in the influence of folate status on the association between MTHFR polymorphism and homocysteine concentrations. In the Norwegian study, a riboflavin-homocysteine association was present regardless of folate status. In the American study, conducted prior to the implementation of FDA-mandated folic acid fortification of grain products (22), the association was observed only when folate concentrations were low. Although higher riboflavin requirements in relation to folate status have been discussed (23), this relationship may not be relevant in the US, since the proportion of Americans with lower folate status has decreased dramatically after folic acid fortification (24). In fact, our results suggest that higher riboflavin status in a population exposed to folic acid fortified grains negates the protective effect of MTHFR polymorphism on CIN 2 or 3, which are precursor lesions for cervical cancer. In an in vitro system, high riboflavin has been shown to increase chromosomal instability, a risk factor for cancer, in cultured human lymphocytes from MTHFR polymorphic subjects (25). This may be due to increased uracil incorporation into DNA as a result of increased MTHFR activity, which could cause folate to provide methyl groups for methionine synthesis rather than for thymidylate synthesis (26). Our study does not suggest that the higher riboflavin status observed in our study population is likely to be associated with increased chromosomal instability. However, our results suggest that a common genetic polymorphism in the MTHFR gene is protective against CIN 2 or 3 only when riboflavin status is low. This association was independent of folate status. A further inactivation of polymorphic MTHFR in the presence of low riboflavin and a resulting shift in the folate pathway towards DNA synthesis may explain our results. Failure to account for riboflavin status may help to explain varying estimates of the influence of MTHFR polymorphisms on cancer risk. On the other hand, we recognize that the present study, despite its relatively large size and the pattern of results highly suggestive of the effect modification discussed above, does not provide sufficient evidence to reject the null hypothesis that MTHFR polymorphism reduces CIN risk independently from riboflavin. It is common for even large studies to have insufficient power to detect interactions. The practical implications of this complex gene-nutrient-disease interaction will require further investigation in studies sufficiently sized for detecting interaction.

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TABLE 1

Characteristics of the 435 HR-HPV positive women according to presence of CIN 2 or 3 or CIN 1, ASCUS or normal cervical cytology

Risk factors	CIN 2 or 3 *n* (%) or median	CIN 1, ASCUS or normal cervical cytology *n* (%) or median	p^*
Total	80 (18.4)	355 (81.6)	
African American	36 (45.0)	229 (64.5)	0.001
Median age at enrollment	21.5	23.0	0.09
Less than high school education	32 (40.0)	109 (30.7)	0.11
Ever smoked	43 (53.8)	133 (37.5)	0.01
Nulliparous	20 (25.0)	73 (20.6)	0.50
Ever used hormone contraceptives	63 (78.8)	258 (72.7)	0.26
Ever used barrier contraception	56 (70.0)	267 (75.2)	0.34
Ever had a sexually transmitted disease	36 (45.0)	166 (47.7)	0.66
Ever treated for an abnormal pap smear	14 (18.0)	63 (18.0)	0.98
Median number of lifetime sexual partners	4.0	5.0	0.90
Median age at first intercourse	16.0	16.0	0.23
Used vitamin supplements	30 (37.5)	102 (28.7)	0.12
Median micronutrient concentrations			
Plasma folate (ng/ml)	9.1	9.0	0.42
Plasma B_{12} (pg/ml)	452.6	464.7	0.87
Vitamin C (mg%)	0.8	0.9	0.15
Vitamin A (μg%)	39.0	37.0	0.36
Vitamin E (mg%)	0.7	0.7	0.90
Total carotene (μg%)	65.0	68.0	0.74
Riboflavin status (EGRAC)	1.4	1.4	0.90
Study arm			
Immediate colposcopy	29 (36.25)	128 (36.06)	
HPV triage	29 (36.25)	90 (25.35)	
Conservative management	22 (27.50)	137 (38.59)	0.08

* Two-sided *p* for Kruskal-Wallis test when comparing medians and 2-sided *p* for χ^2 when comparing proportions. Statistically significant at $\alpha = 0.05$.

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TABLE 2

Relationship of MTHFR with CIN 2 or 3 among 435 HR-HPV positive women

	Genotype frequencies		CIN[1] 2 or 3			
	CIN 2 or 3 *n* (%)	**CIN 1, ASCUS or normal cervical cytology *n* (%)**	**OR adjusted for demographic and behavioral factors[1]**	**95% CI**	**OR adjusted for demographic, behavioral and snutritional factors[2]**	**95% CI**
MTHFR Ala/Ala	59 (74)	223 (63)	Ref		Ref	
MTHFR Ala/Val	16 (20)	116 (33)	0.40	0.21–0.74	0.40	0.20–.80
MTHFR Val/Val	5 (6)	16 (4)	0.68	0.22–2.09	0.40	0.08–2.04
MTHFR Ala/Val or Val/Val	21 (26)	132 (37)	0.43	0.24–0.78	0.40	0.21–0.78

[1]Multivariable adjustment for age, race, smoking (ever/never), age at first intercourse, use of hormonal contraception (yes/no), and study arm.-

[2]Multivariable adjustment for age, race, smoking (ever/never), age at first intercourse, use of hormonal contraception (yes/no), study arm, and micronutrient concentrations of plasma folate, vitamins B-12, C, A, E, total carotene and riboflavin status.

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TABLE 3

Joint effects of MTHFR polymorphism and riboflavin status on CIN 2 or 3 among 435 women with high-risk human papilloma virus.

MTHFR / RIBOFLAVIN STATUS	Odds Ratio[1], 95% CI, p value	Odds Ratio[2], 95% CI, p value
Any polymorphism /Low: > Median EGRAC (> 1.4)	0.31, 0.12–0.80, 0.016	0.35, 0.13–0.92, 0.034
Any polymorphism /High: ≤ Median EGRAC (≤1.4)	0.54, 0.24–1.20, 0.129	0.51, 0.22–1.19, 0.119
No polymorphism /Low: > Median EGRAC (> 1.4)	1.46, 0.81–2.63, 0.211	1.62, 0.89–2.97, 0.117
No polymorphism /High: ≤ Median EGRAC (≤ 1.4)	1 (Reference)	1 (Reference)
Any polymorphism /Low (> 1.5 EGRAC)	0.20, 0.06–0.74, 0.015	0.22, 0.06–0.82, 0.024
Any polymorphism / Medium (>1.3–1.5 EGRAC)	0.45, 0.17–1.18, 0.106	0.47, 0.18–1.25, 0.132
Any polymorphism / High (≤ 1.3 EGRAC)	0.45, 0.17–1.21, 0.114	0.37, 0.12–1.12, 0.078
No polymorphism / Low (> 1.5 EGRAC)	1.07, 0.51–2.26, 0.856	1.16, 0.54–2.46, 0.708
No polymorphism /Medium (>1.3–1.5 EGRAC)	1.02, 0.51–2.05, 0.943	1.00, 0.49–2.02, 0.998
No polymorphism /High (≤ 1.3 EGRAC)	1 (Reference)	1 (Reference)

[1]OR adjusted for age, race, smoking (ever/never), age at first intercourse, use of hormonal contraception (yes/no), and study arm

[2]OR adjusted for age, race, smoking (ever/never), age at first intercourse, use of hormonal contraception (yes/no), study arm, and micronutrient concentrations of plasma folate, vitamins B-12, C, A, E and total carotene.