Clinical Chemistry 49:2
295–302 (2003)

Nutrition

Effect of Riboflavin Status on the Homocysteine-lowering Effect of Folate in Relation to the *MTHFR* (C677T) Genotype

Stuart J. Moat,[1*] Pauline A.L. Ashfield-Watt,[1] Hilary J. Powers,[2] Robert G. Newcombe,[3] and Ian F.W. McDowell[1]

Background: **Riboflavin (vitamin B_2) is the precursor for FAD, the cofactor for methylenetetrahydrofolate reductase (MTHFR). MTHFR catalyzes the formation of 5-methyltetrahydrofolate, which acts as a methyl donor for homocysteine remethylation. Individuals with the *MTHFR* 677C→T mutation have increased plasma total homocysteine (tHcy) concentrations, particularly in association with low folate status. It has been proposed that riboflavin may act together with folate to lower plasma tHcy, particularly in individuals with the thermolabile *MTHFR* T variant.**

Methods: **We measured B-vitamin status and plasma tHcy in 126 healthy individuals 20–63 years of age (42 CC, 42 CT, and 42 TT *MTHFR* genotypes) at baseline and after three interventions (4 months): placebo plus natural diet; daily 400 μg folic acid supplement plus natural diet; and increased dietary folate to 400 μg/day.**

Results: **At baseline and after nutritional intervention, lower riboflavin status was associated with increased plasma tHcy concentrations. Plasma tHcy was 2.6 μmol/L higher in the lowest plasma riboflavin quartile compared with the highest ($P < 0.02$) and was 4.2 μmol/L higher in the highest erythrocyte glutathione reductase activation coefficient (EGRAC) quartile compared with the lowest ($P < 0.001$). This effect was not restricted to those with the T allele. Folic acid given as a 400 μg/day supplement appeared to exacerbate a tendency toward riboflavin deficiency, as suggested by an increase in the proportion of individuals with EGRAC ≥1.4 from 52% to 65% after supplementation ($P < 0.05$).**

Conclusions: **Folate and riboflavin interact to lower plasma tHcy, possibly by maximizing the catalytic activity of MTHFR. The effect may be unrelated to *MTHFR* genotype.**

Current evidence suggests that increased plasma homocysteine (hyperhomocysteinemia) is a risk factor for cardiovascular disease in the general population *(1, 2)*, although it is unclear whether homocysteine itself is the causal factor. Homocysteine is a metabolite of methionine, and once formed, it can be metabolized by transsulfuration through a vitamin B_6-dependent pathway or remethylated to methionine in a folate- and vitamin B_{12}-dependent reaction. Genetic defects or inadequate provision of these vitamin cofactors can increase plasma total homocysteine (tHcy)[4] concentrations.

Methylenetetrahydrofolate reductase (MTHFR) is a FAD-dependent enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5MeTHF), which acts as a methyl donor for homocysteine remethylation. A common mutation in the *MTHFR* gene (677C→T) has been identified with a frequency of 0.32 in Caucasian populations. Individuals homozygous (TT) for this mutation have ~50% of the normal MTHFR activity *(3)*. This reduced activity is associated with a reduced ability to catalyze the reduction of 5,10-methylenetetrahydrofolate to 5MeTHF and predisposes homozygous individuals to hyperhomocysteinemia. It has been proposed that individuals with the TT

[1] Cardiovascular Sciences Research Group, Wales Heart Research Institute, and [3] Department of Epidemiology, Statistics and Public Health, University of Wales College of Medicine, Cardiff, Wales CF14 4XN, United Kingdom.
[2] Centre for Human Nutrition, Division of Clinical Sciences, Northern General Hospital, University of Sheffield, Sheffield S5 7AU, United Kingdom.
*Author for correspondence. Fax 44-29-2074-3500; e-mail moatsj@cardiff.ac.uk.
Received August 20, 2002; accepted October 29, 2002.

[4] Nonstandard abbreviations: tHcy, total homocysteine; MTHFR, methylenetetrahydrofolate reductase; 5MeTHF, 5-methyltetrahydrofolate; EGRAC, erythrocyte glutathione reductase activation coefficient; RNI, reference nutrient intake; and CI, confidence interval.

genotype are more likely to have vascular disease because, in combination with a low folate status, these TT individuals had higher plasma concentrations of homocysteine than do those with the CT and CC genotypes. However, a recent metaanalysis has failed to confirm this *(4)*.

Plasma homocysteine is inversely associated with plasma folate even within the range that is considered to be normal. However, the associations between homocysteine and plasma concentrations of vitamin B_{12} and B_6 are weak. Folate, vitamin B_{12}, and vitamin B_6 all have homocysteine-lowering activity, but folate is the most effective *(5)*.

Dietary riboflavin (vitamin B_2) is the precursor for FAD, the cofactor for MTHFR. MTHFR activity and hepatic 5MeTHF concentrations are lower in riboflavin-deficient rats than in controls *(6)*. Increased homocysteine in the skin of riboflavin-deficient rats has also been reported *(7)*. Recently, plasma riboflavin has been reported to be an independent predictor of tHcy in a group of healthy blood donors. This relationship was confined to individuals with the CT and TT genotypes for the *MTHFR* 677C→T mutation *(8)*. Separate in vitro studies suggest that the *MTHFR* mutation may restrict FAD binding, leading to its dissociation from the MTHFR enzyme. Both riboflavin and folate can protect against loss of function of MTHFR *(9, 10)*. Riboflavin intake was a determinant of plasma homocysteine in men and women in the Framingham Offspring Cohort *(11)*. In individuals with the TT genotype and a low folate status from the same population, plasma homocysteine was inversely associated with plasma riboflavin concentration *(12)*.

It thus has been postulated that riboflavin status may affect the metabolism of reduced folates, particularly in individuals with the *MTHFR* T variant. Individuals with the TT and, possibly, CT genotypes may require a higher intake of both folate and riboflavin to overcome the partial metabolic block and maintain low plasma homocysteine concentrations. To date this proposal has not been investigated in a nutritional intervention study. This has implications for nutritional advice relevant to prevention policies for neural tube defects, cancer, and cardiovascular disease.

The aim of this study was to evaluate the effect of plasma riboflavin status on the homocysteine-lowering response of folate intervention in individuals with different *MTHFR* genotypes. In addition, this study explores the interaction between intracellular riboflavin stores, measured as the erythrocyte glutathione reductase activation coefficient (EGRAC), tHcy, folate and *MTHFR* genotype.

Participants and Methods

For the following study, we used appropriately stored blood samples and dietary questionnaires from a previous study funded by the UK Food Standards Agency (FSA Projects N05002 and N05006). These projects investigated the effects of enhanced folate intake from dietary sources (mainly fortified foods) or low-dose (400 μg) folic acid supplements in healthy individuals recruited according to *MTHFR* genotype (42 TT, 42 CT, and 42 CC). The study protocol is outlined below.

STUDY PARTICIPANTS

Healthy individuals 18–65 years of age were recruited from workplace screening and blood donor sessions in South Wales. Exclusion criteria included smoking, history of cardiovascular disease or epilepsy, use of vitamin supplements, use of drugs known to interfere with folate metabolism, and pregnancy. After completion of an eligibility questionnaire, suitable individuals were screened for the thermolabile *MTHFR* genotype. A total of 634 volunteers were genotyped for *MTHFR* status. All participants provided informed written consent, and ethics approval was obtained from the Local Research Ethics Committee (Bro Taf Health Authority).

STUDY DESIGN

A total of 126 volunteers (42 TT, 42 CT, and 42 CC) were recruited to a cross-over intervention study. Within each genotype group, individuals were allocated equally to all six possible sequences of the three 4-month interventions, thus achieving a Latin square design balanced for carryover from all preceding treatment periods.

INTERVENTIONS

The interventions were are follows:

1. Control diet. Participants were asked to consume their usual diet but to avoid folate-fortified foods and to take a placebo tablet daily.

2. Natural folate-enriched diet. Participants were encouraged to consume their usual diet plus additional folic acid-fortified foods and naturally folate-rich foods to achieve a total of at least 400 μg/day.

3. Supplement. Participants were asked to consume the same diet as in intervention 1 but to take a 400 μg folic acid supplement daily.

Interventions 1 and 3 were carried out double blind such that neither the nutritionist nor the participant knew which treatment (control or supplement) he or she was receiving. It was not possible to blind either party regarding folate intake from dietary sources.

Volunteers attended the Research Institute for assessment on four occasions. Baseline measurements were made at visit 1 and post-intervention measurements at visits 2, 3, and 4. Each visit consisted of venipuncture, dietary interview, and assessment of vascular endothelial function. The results of the vascular endothelial function and dietary aspects have been reported elsewhere *(13, 14)*.

NUTRITIONAL ASSESSMENT

Baseline and follow-up dietary assessments were by semiquantitative food frequency questionnaire. Nutritional analysis was based on McCance and Widdowson's Composition of Foods *(15)* and UK food portion sizes *(16)*. This questionnaire was designed specifically to assess dietary folate intake and included questions on the intake of 32 folate-rich foods (e.g., broccoli, spinach, Brussels sprouts, and oranges) or frequently consumed folate-poor foods (e.g., dairy products, meats, and unfortified breads). Participants were asked to report how often they ate each of these foods (number of days per week, fortnightly, monthly/rarely). Frequency of intake was multiplied by standard average portion sizes and then by the folate content derived from UK nutrient composition tables to provide total folate intake data. Data on fortified foods were obtained directly from manufacturers and supermarkets and updated regularly.

Estimates of riboflavin intake were derived from the original folate questionnaire. This was possible because many of the folate-providing foods included on the questionnaire were also good sources of riboflavin. Data on the riboflavin content of these foods were also derived from UK nutrient composition tables. The amounts of these nutrients used as fortificants were obtained from food manufacturers retrospectively. Some manufacturers were unable to provide this information; therefore, missing values have been entered for those participants who consumed their products. This affected only a small number of participants and is unlikely to bias our results significantly.

LABORATORY MEASUREMENTS

Genotyping. The thermolabile 677CT *MTHFR* genotype was determined by heteroduplex analysis involving extraction of DNA from buccal cells, PCR, and electrophoresis as described previously *(17)*.

Biochemistry. Fasting blood samples for the determination of plasma folate, plasma tHcy, and plasma flavins were collected into Vacutainers containing EDTA. Samples for the measurement of pyridoxal phosphate and vitamin B_{12} were collected into Vacutainers containing lithium heparin. Blood samples were then centrifuged within 10 min at 3000*g* for 10 min at 4 °C, and the plasma was stored at −70 °C until assayed. Erythrocytes from lithium heparin tubes were washed three times with an equal volume of saline (9 g/L NaCl) and resuspended in an equal volume of distilled water and stored at −70 °C until analysis. Samples were analyzed in batches with all four samples from each individual assayed together to reduce any variation.

Plasma tHcy was measured by enzymatic immunoassay, and plasma folate and plasma vitamin B_{12} were measured by competitive protein-binding methods on an Abbott IMx instrument (Abbott Diagnostics Ltd.). The between-batch CVs for these measurements were 5.3%, 9.3%, and 4.0%, respectively. Plasma pyridoxal phosphate (vitamin B_6) was measured by HPLC with fluorescence detection as described previously (between-batch CV, 5.1%) *(18)*.

Plasma riboflavin, FAD, and FMN were measured by HPLC using a modification of the method by Capo-chichi et al. *(19)*. Briefly, 50 μL of isoriboflavin (internal standard) was added to 300 μL of plasma and calibrators. Plasma samples and calibrators were then deproteinized with 150 μL of chilled 100 mL/L trichloroacetic acid and centrifuged at 10 000*g* for 5 min at 4 °C. A 100-μL portion of the resulting supernatant was injected on the HPLC column (PLRP-S; 5 μm bead size; 100Å pore size; 250 × 4.6 mm; Polymer Laboratories). Plasma flavins were eluted isocratically with a 10 mmol/L potassium phosphate buffer containing 15 mmol/L magnesium acetate and 130 mL/L acetonitrile (adjusted to pH 2.0 with orthophosphoric acid) at a flow rate of 1.1 mL/min and detected fluorometrically at excitation and emission wavelengths of 445 and 530 nm, respectively. HPLC analysis was performed using an Agilent 1100 series autoinjector, isocratic pump, and fluorescence detector (Agilent Technologies, UK Limited). The between-batch CV for the plasma flavins was <5.2%.

Evaluation of intracellular riboflavin status was measured by the EGRAC *(20)*. Glutathione reductase is an FAD-dependent enzyme, and the activity of the erythrocyte form of this enzyme is used as the basis of a functional test for riboflavin status. The activity of erythrocyte glutathione reductase (EGR) is measured in the presence and absence of added FAD to an optimum concentration, and results are expressed as an activation coefficient (EGRAC), such that the lower the endogenous saturation of enzyme with FAD the higher the EGRAC. Thus, low riboflavin status is reflected by high EGRAC values. Enzyme activity is measured in hemolysates of red blood cells in the presence of NADPH as reducing agent and oxidized glutathione as substrate. Oxidation of NADPH is monitored at 340 nm in an automated centrifugal analyzer (Cobas Bio automated analyzer; Roche Diagnostica). The assay has high precision (within- and between-batch CVs <3.0%). We adopted a conservative threshold of >1.4 to indicate biochemical ariboflavinosis *(21)*. Plasma flavins and EGRACs were measured blind in separate laboratories. Plasma creatinine was measured by the Jaffe method on the Hitachi 747 analyzer (Roche Diagnostics).

STATISTICAL ANALYSIS

The distribution of each measure at baseline by genotype and for each intervention period is given as the mean and SD. In subsequent analyses, plasma riboflavin, FMN, EGRAC, tHcy, vitamin B_{12}, and vitamin B_6 were log-transformed (base 10) because of skewness. One-way ANOVA was used to compare baseline values among the three genotypes. Gender effects on biochemical variables were assessed by unpaired two-sample *t*-tests. Differences

Table 1. Riboflavin status, homocysteine, and folate in all participants at baseline, stratified by genotype.[a]

		MTHFR genotype			
	All participants	**CC**	**CT**	**TT**	***P***
Plasma riboflavin, nmol/L	10.9 (7.4)	11.4 (7.2)	11.5 (8.5)	9.9 (6.4)	0.80
Plasma FMN, nmol/L	4.9 (3.0)	4.6 (2.4)	5.1 (3.2)	4.9 (3.4)	0.83
Plasma FAD, nmol/L	30.9 (5.3)	31.6 (5.1)	31.4 (4.7)	29.7 (5.9)	0.24
EGRAC[b]	1.44 (0.18)	1.41 (0.17)	1.47 (0.21)	1.43 (0.15)	0.36
Plasma tHcy, μmol/L	10.2 (4.2)	8.8 (2.4)	9.3 (2.5)	12.5 (5.7)	0.001
Plasma folate, nmol/L	17.7 (7.7)	20.6 (8.0)	17.2 (6.9)	15.2 (7.4)	0.004
Plasma B_{12}, nmol/L	266 (104)	281 (113)	262 (100)	256 (101)	0.58
Plasma B_6, nmol/L	40.9 (18.3)	39.7 (17.1)	42.2 (19.0)	40.9 (19.2)	0.88

[a] Data are presented as the mean (SD).
[b] Lower EGRAC values are indicative of better riboflavin status.

among the four visits according to dietary regimen were assessed by two-way ANOVA, modeling on individual and treatment. Pearson correlations expressing the interrelationship between measures of riboflavin status at baseline with one another and with tHcy, folate, age, *MTHFR*, creatinine, and vitamin B_{12} and B_6 concentrations were calculated for all participants and for each genotype. Pearson correlations were also calculated for changes in different variables from baseline and control to diet and supplement regimen. Multiple linear regression was used to assess the dependence of each measure of riboflavin status at baseline on age, gender, and genotype and to model the dependence of tHcy on riboflavin status and other variables. The proportions of participants with EGRAC values ≥1.4 and riboflavin intake below the current reference nutrient intake (RNI) were calculated with 95% confidence intervals (CIs). Summary statistics for baseline tHcy were calculated for each quartile of plasma riboflavin and EGRAC, and means in the upper and lower quartiles were compared by unpaired *t*-test. Joint contributions of folate and riboflavin to tHcy lowering were assessed by fitting regression models to baseline tHcy by plasma folate and EGRAC or plasma riboflavin concentrations, all log-transformed, and using these to calculate the predicted effect on tHcy of moving from the diet to the supplement regimen and from baseline to control.

Results

riboflavin, homocysteine, folate status, and *MTHFR* genotype at baseline

The study population consisted of 53 men and 73 women 20–63 years of age, with a mean age of 40 years. Measures of riboflavin status were not different among genotypes. In contrast, plasma folate and tHcy were significantly influenced by genotype (Table 1). Individuals with the TT genotype had significantly higher tHcy concentrations than did the CT and CC genotypes ($P < 0.001$). Plasma folate was lower in the TT group than either the CT or the CC genotype ($P < 0.004$). CT individuals had intermediate values. Men had a higher median plasma tHcy (9.6 μmol/L) than did women (8.4 μmol/L; $P < 0.002$). The median plasma folate concentration was higher in women (18.6 nmol/L) compared with men (14.0 nmol/L; $P < 0.006$). Men also had a higher median plasma B_6 concentration (44.2 nmol/L) than did women (36.6 nmol/L; $P < 0.004$).

riboflavin status at baseline in the study population

The mean dietary intake of riboflavin in our study population was 1.45 (SD, 0.51) mg/day. Using a RNI of 1.3 mg/day for males and 1.1 mg/day for females, we calculated the proportion of participants not attaining the UK RNI for riboflavin (Table 2). Overall, 30% of the study population at baseline did not achieve the UK RNI for riboflavin. If EGRAC were used as the measurement of riboflavin status, 52% of the study population would be deemed biochemically riboflavin deficient (EGRAC >1.4). Riboflavin intake was negatively correlated with EGRAC ($r = -0.27$; $P < 0.003$).

bivariate correlations

Plasma tHcy was significantly inversely correlated with plasma riboflavin and significantly positively associated with EGRAC (Table 3). Plasma riboflavin was inversely associated with EGRAC for all participants and each genotype group. Plasma riboflavin was also positively associated with FMN, but only in the CT group. EGRAC was inversely associated with plasma folate in the whole group and in all genotypes, in contrast to plasma riboflavin, for which the association with plasma folate was significant only in the CC genotype (Tables 3 and 4). Plasma tHcy and folate were inversely correlated in the whole group ($r = -0.5$; $P = 0.001$). The inverse correlation between tHcy and plasma folate was strongest in the TT individuals ($r = -0.6$; $P = 0.001$). In addition, plasma riboflavin and EGRAC showed a strong relationship with age (Table 3).

When expressed as quartiles, plasma tHcy was 2.6 μmol/L (95% CI, 0.48–4.7 μmol/L) higher in the lowest plasma riboflavin quartile than the highest ($P = 0.016$) and was 4.2 μmol/L (95% CI, 2.0–6.4 μmol/L) higher in the highest EGRAC quartile compared with the lowest (*P*

Table 2. Riboflavin intake and status of the study population.

	Proportion with intake below the UK RNI		Proportion with EGRAC ≥1.4	
	n (%)	95% CI	n (%)	95% CI
Baseline, all	36/120 (30%)	23–39%	64/122 (52%)	44–61%
Genotype				
TT	12/41 (29%)	18–44%	22/42 (52%)	38–67%
CT	14/40 (35%)	22–50%	24/41 (59%)	43–72%
CC	10/39 (26%)	15–41%	18/39 (46%)	32–61%
Age, years				
<30	11/29 (38%)	23–56%	23/31 (74%)	57–86%
30–39	7/29 (24%)	12–42%	18/29 (62%)	44–77%
40–49	10/37 (27%)	15–43%	15/37 (41%)	26–57%
≥50	8/25 (32%)	17–52%	8/25 (32%)	17–52%
Gender				
Male	14/50 (28%)	17–42%	21/50 (42%)	29–56%
Female	22/70 (31%)	22–43%	43/72 (60%)	48–70%

<0.001). The association between tHcy and plasma riboflavin or EGRAC was significant only in the CC and TT groups.

MULTIPLE REGRESSION ANALYSES

We explored the independent predictive effect of riboflavin status on plasma tHcy. When plasma folate was included as a covariate in the analysis of EGRAC or plasma riboflavin and tHcy, the significance of the relationship was reduced ($P = 0.023$ and 0.035, respectively). In a stepwise linear regression analysis with log-transformed baseline tHcy concentration as the dependent variable and selecting from all variables in Table 3 and *MTHFR* genotype, four factors were significant, independent determinants of plasma tHcy: plasma folate ($P = 0.002$), *MTHFR* TT genotype ($P < 0.001$), log EGRAC ($P < 0.001$), and creatinine ($P = 0.004$).

EFFECT OF FORTIFIED FOODS ON RIBOFLAVIN STATUS

Riboflavin intake was increased by 0.4 mg/day in the fortified period, from 1.45 ± 0.51 mg/day at baseline to 1.85 ± 0.65 mg/day ($P < 0.001$). Riboflavin intake in the control (1.24 ± 0.52 mg/day) and supplement (1.19 ± 0.40 mg/day) periods was similar and was significantly lower in both than in the baseline period ($P < 0.001$), which is consistent with the advice given to avoid fortified cereals during these periods.

RIBOFLAVIN STATUS AND VASCULAR ENDOTHELIAL FUNCTION

Riboflavin status, either at baseline or after increased intake from fortified foods, was unrelated to vascular endothelial function as assessed by flow-mediated dilatation (data not shown).

EFFECT OF INCREASED FOLIC ACID INTAKE ON RIBOFLAVIN STATUS

In terms of folate intake relative to baseline (254 ± 83 μg/day), the response to the interventions was greatest for the supplement (561 ± 98 μg/day), moderately effective in the dietary intervention (482 ± 127 μg/day), and minimal during the control phase (216 ± 82 μg/day) when fortified cereals were excluded from the diet *(14)*. The effects of these interventions on measures of riboflavin status are shown in Table 5. There was some evidence for a modest improvement in riboflavin status after the dietary intervention, shown as a significant decrease in EGRAC, compared with other interventions. In contrast and unexpectedly, two measures of riboflavin status, plasma riboflavin and EGRAC, deteriorated after folate

Table 3. Pearson correlation coefficients for measures of riboflavin status at baseline.

	Log riboflavin	Log FMN	FAD	Log EGRAC
Log plasma FMN	0.30[a]			
Plasma FAD	−0.08	0.04		
Log EGRAC	−0.48[a]	−0.14	−0.11	
Log plasma tHcy	−0.21[b]	−0.03	−0.10	0.36[a]
Plasma folate	0.27[c]	0.18	0.06	−0.39[a]
Age	0.31[a]	−0.12	0.00	−0.46[a]
Creatinine	0.07	0.02	−0.08	−0.07
Log vitamin B_{12}	0.18[b]	−0.07	0.13	−0.38[a]
Log vitamin B_6	0.15	0.14	0.08	−0.16

[a–c] Significant: [a] $P < 0.001$; [b] $P < 0.05$; [c] $P < 0.01$.

Table 4. Pearson correlation coefficients for riboflavin, FMN, and EGRAC by genotype.

	Genotype		
	CC	CT	TT
Log riboflavin			
Log plasma FMN	0.14	0.49[a]	0.23
FAD	−0.08	0.10	−0.33[b]
Log EGRAC	−0.52[c]	−0.52[c]	−0.41[b]
Log plasma tHcy	−0.39[b]	0.08	−0.38[b]
Plasma folate	0.43[a]	0.13	0.24
Log FMN			
FAD	0.15	−0.04	0.03
Log EGRAC	0.03	−0.30	−0.16
Log plasma tHcy	−0.02	−0.04	−0.07
Plasma folate	0.26	0.09	0.24
Log EGRAC			
FAD	0.04	−0.24	−0.15
Log plasma tHcy	0.52[c]	0.28	0.48[c]
Plasma folate	−0.32[b]	−0.34[b]	−0.55[c]

[a–c] Significant: [a] $P < 0.01$; [b] $P < 0.05$; [c] $P < 0.001$.

supplementation. Thus, the folic acid supplement regimen increased the proportion of individuals with an EGRAC ≥1.4 from 52% at baseline to 65% ($P < 0.05$). The proportion of individuals with EGRAC values ≥1.4 was 40% after the diet regime and 48% in the control period ($P < 0.001$). This effect of folic acid on reducing riboflavin status was also evident in the ≥50 age group, who had a better EGRAC status than did the ≤30 age group at other measurement points ($P < 0.001$). The proportion of individuals with an EGRAC >1.4 in the ≥50 age group was increased from 32% at baseline to 56% after folic acid supplementation ($P < 0.005$).

RELATIVE CONTRIBUTIONS OF FOLATE AND RIBOFLAVIN TO HOMOCYSTEINE LOWERING

Reductions in plasma tHcy after the folic acid supplements and the fortified diet were not significantly different (8.6 vs 8.7 μmol/L; $P = 0.73$; Table 5), despite higher concentrations of plasma folate being reached in the supplement group compared with the dietary group (31.7 vs 25.8 nmol/L; $P < 0.001$). This may be explained by the concomitant reduction in riboflavin status (EGRAC, 1.46 vs 1.38).

These data are consistent with riboflavin having a homocysteine-lowering effect in addition to folate after the combined intervention (dietary folate phase). A model to combine the effects of folate and riboflavin on plasma homocysteine has been derived from baseline data using plasma folate and either plasma riboflavin or EGRAC. These models have been used to predict the effects of folate and/or riboflavin on plasma tHcy after intervention:

$$\text{Log tHcy} = 1.102 - (0.306 \times \log \text{folate}) + (0.880 \times \log \text{EGRAC})$$

$$\text{Log tHcy} = 1.385 - (0.242 \times \log \text{folate}) - (0.125 \times \log \text{riboflavin})$$

The relationship of tHcy with plasma folate and EGRAC at baseline would predict that the differences in plasma folate and EGRAC between the supplement and diet regimens would produce only a 1.6% difference in tHcy, a small change that is not significantly greater than the 0.7% that occurred. Similarly, the reduction in riboflavin status observed on the control regimen can explain the increase in tHcy relative to baseline. In each model, the effects of folate and riboflavin status are both statistically significant ($P < 0.05$).

Discussion

The observed age and gender differences in riboflavin intake and biochemical markers of status are consistent with national trends. The recent National Diet and Nutrition Survey of young people found that 79% of adolescent girls had EGRAC values >1.4 compared with 59% of adolescent boys *(22)*. Biochemical evidence of poor riboflavin status among adults is sparse *(23)*, but the recent National Diet and Nutrition Survey of the elderly found that 18% of free-living men and women 65 years of age or older had EGRAC values >1.4 *(24)*. Although riboflavin intake estimates are commonly reported to correlate strongly with EGRAC, values of the latter tend to overestimate the prevalence of deficiency, a pattern repeated in the present study.

These studies provide further information on the relative value of different biochemical markers of riboflavin status. Estimates of riboflavin intake showed a significant association with EGRAC values but not with other biochemical measures of riboflavin status. In contrast to

Table 5. Effect of the different treatment periods on measures of riboflavin status.

	Mean (SD)					
Treatment	EGRAC[a]	Riboflavin, nmol/L	FMN, nmol/L	FAD, nmol/L	tHcy, μmol/L	Folate, nmol/L
Baseline	1.44 (0.18)	10.9 (7.4)	4.9 (3.0)	30.9 (5.3)	10.2 (4.2)	17.7 (7.7)
Control	1.43 (0.19)	10.1 (10.2)	5.0 (2.4)	31.7 (5.2)	10.9 (6.9)	17.2 (7.2)
Diet	1.38 (0.18)	9.8 (6.6)	5.3 (2.5)	30.9 (5.7)	8.7 (3.3)	25.8 (8.4)
Supplement	1.46 (0.18)	8.3 (6.0)	5.0 (2.7)	31.0 (6.2)	8.6 (3.1)	31.7 (16.5)

[a] Lower EGRAC values are indicative of a better riboflavin status.

plasma FAD or FMN, plasma riboflavin concentrations were strongly correlated with EGRAC, indicating the relative usefulness of these plasma variables as indicators of riboflavin status.

Our baseline observations showed no relationship between *MTHFR* genotype and riboflavin status and are in line with those of Hustad et al. *(8)* and Jacques et al. *(12)*. The finding that plasma tHcy concentrations are inversely related to plasma riboflavin and positively associated with EGRAC is in agreement with previous studies demonstrating inverse associations between plasma tHcy and plasma riboflavin and riboflavin intake *(8, 11, 12)*. The association between tHcy and EGRAC remained when adjusted for plasma folate. In a stepwise linear regression analysis, plasma folate, *MTHFR* TT genotype, EGRAC, and creatinine were the main predictors of tHcy. These findings are similar to those of Hustad et al. *(8)*, who found significant effects of age, plasma riboflavin, folate, cobalamin, creatinine, and genotype in a cross-sectional analysis of 423 healthy Norwegian blood donors.

We found an inverse association between tHcy and plasma riboflavin and a positive association between tHcy and EGRAC in individuals with the CC and TT *MTHFR* genotypes but not in individuals with the CT genotype. This is in contrast to Hustad et al. *(8)*, who found no relationship with plasma riboflavin in the CC group but an inverse association in a group consisting of CT (83%) and TT (17%) genotypes, suggesting that the effect was largely confined to those with the T allele. Furthermore, Hustad et al. *(8)* showed that this relationship was not influenced by plasma folate, which is in contrast to the recent findings of the cross-sectional study of Jacques et al. *(12)*, who studied 450 participants from the Framingham Offspring Study. In these individuals, the association between plasma tHcy and plasma riboflavin was confined to those with a low plasma folate and TT homozygotes.

These conflicting data with respect to genotype mean that the proposal that riboflavin has an effect only in those individuals carrying the T allele for the *MTHFR* gene remains unproven *(8, 12)*. Further studies will be required to directly investigate the role of *MTHFR* genotype in response to riboflavin interventions.

The fortified diet intervention led to an increase in both folate and riboflavin intake, confirming that fortified cereals can have a significant impact on the intake of these vitamins. An interesting and unexpected observation was that the folate supplementation regimen of 400 μg/day for 3 months produced a significant decrease in riboflavin status, whether assessed as plasma riboflavin or EGRAC. This effect has not been observed previously and requires confirmation in other studies. If confirmed this would imply that folic acid supplements given in isolation may adversely affect riboflavin status. A possible mechanism for this effect could be an increase in the rate of turnover of flavins, presumably through increased MTHFR activity. It is known that riboflavin deficiency can reduce MTHFR activity and lower 5MeTHF concentrations in the livers of riboflavin-deficient rats *(6)*. Alternatively, the folate supplement might increase the binding of FAD to MTHFR *(9, 10)* and thereby reduce the circulating concentrations of flavins.

The baseline data and those obtained after interventions indicate that both folate and riboflavin have a homocysteine-lowering effect. The observation that the degree of homocysteine lowering after intake of fortified foods (which increased both folate and riboflavin intake) is comparable to that from a higher dose of folic acid suggests that these vitamins have an additive or synergistic effect on homocysteine lowering. However, these results could also be partly explained by a plateau effect of increasing folic acid on homocysteine lowering *(5)*.

Our results suggest that an increase in folic acid intake might also increase the requirement for riboflavin to achieve maximal catalytic function of MTHFR. However, the lack of any association between riboflavin status and vascular endothelial function at baseline or after increased intake from fortified foods suggests that increasing riboflavin intake may not have additional benefits to the cardiovascular system.

In conclusion, our results indicate a high prevalence of riboflavin deficiency in our study population and suggest that riboflavin status could be compromised further by folate supplementation in the absence of additional riboflavin.

This study was funded by the UK Food Standards Agency (Project Reference N05002). The Kellogg Company of Great Britain provided funding for reimbursement of participants for the cost of fortified foods. Dr. S.J. Moat is currently funded by the British Heart Foundation. We appreciate the donation of the isoriboflavin internal standard by Dr. S. Hustad and Prof. P. Ueland (University of Bergen, Bergen, Norway) and thank Z.E. Clark for technical assistance.

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