The Relationship between Riboflavin and Plasma Total Homocysteine in the Framingham Offspring Cohort Is Influenced by Folate Status and the C677T Transition in the Methylenetetrahydrofolate Reductase Gene[1]

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ABSTRACT Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate, the methyl donor for remethylation of homocysteine to methionine. The C677T MTHFR polymorphism is associated with mild hyperhomocysteinemia, but only in the presence of low folate status. Because MTHFR contains flavin adenine dinucleotide (FAD) as a prosthetic group, riboflavin status may also influence homocysteine metabolism. The objective of this study was to examine the association between riboflavin status and fasting plasma total homocysteine (tHcy) concentration while also considering MTHFR C677T genotype and folate status. The study was conducted using fasting plasma samples (n = 450) from the fifth examination of the Framingham Offspring Study cohort. All persons with the TT genotype and age- and sex-matched sets of individuals with the CT and CC genotypes were selected for determination of plasma riboflavin and flavin mono- and dinucleotide levels. Plasma riboflavin was associated with tHcy concentrations, but the association was largely confined to persons with plasma folate <12.5 nmol/L and TT genotype. In these persons, the mean tHcy among individuals with riboflavin levels <6.89 nmol/L was 14.5 μmol/L, whereas the mean tHcy for those with riboflavin ≥11 nmol/L was 11.6 μmol/L (P-trend <0.03). Plasma flavin nucleotides were unrelated to tHcy concentrations. Our data suggest that riboflavin status may affect homocysteine metabolism, but only in a small segment of the population who have both low folate status and are homozygotes for the MTHFR C677T mutation. J. Nutr. 132: 283–288, 2002.

KEY WORDS: • *homocysteine* • *riboflavin* • *folate* • *methylenetetrahydrofolate reductase* • *humans*

Methylenetetrahydrofolate reductase (MTHFR,[3] EC 1.7.99.5) is a flavoprotein that catalyzes the reduction of N^5,N^{10}-methylenetetrahydrofolate to N^5-methyltetrahydrofolate, which is used for homocysteine methylation. In 1988, Kang et al. (1) reported the presence of a MTHFR variant, which was thermolabile and had reduced activity (≤50%). This variant was identified in fibroblast and lymphocyte extracts from two unrelated patients with moderate elevations of plasma homocysteine. These characteristics were later found to be due to a cytosine to thymidine transition at nucleotide 677 (C677T) in the MTHFR gene, resulting in the substitution of a valine for an alanine residue in the protein (2,3). The frequency of this mutant allele was shown to differ in various ethnic groups (3–6).

The discovery of this common mutation was of interest because plasma total homocysteine (tHcy) concentrations on average were higher in persons who expressed the thermolabile MTHFR than in those who did not (1,3,7,8) and because elevated tHcy was associated with an increased vascular disease risk (9). Nevertheless, the subsequent studies not only cast doubt on the magnitude of the contribution of this mutation to the risk for vascular disease (10), but it also became clear that a large proportion of individuals with thermolabile MTHFR had normal plasma tHcy levels (7). This latter fact strongly implied that there are other factors that influence the activity of the thermolabile enzyme. Two potential factors that might influence activity of the thermolabile enzyme are folate and riboflavin. As described above, MTHFR converts methylenetetrahydrofolate to methyltetrahydrofolate, which is required for the methylation of homocysteine to methionine; riboflavin, in the form of flavin adenine dinucleotide (FAD), is a cofactor for MTHFR.

A study from our group (11) demonstrated an interaction between folate status and the C677T mutation. When folate

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[3] Abbreviations used: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine.

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status is low, plasma tHcy concentrations are significantly higher in homozygotes for this mutation than in other genotypes. However, when folate status is adequate, plasma tHcy was low and not affected by the MTHFR genotype. The possible role of riboflavin in the activity of thermolabile MTHFR has received less attention. A recent study of Hustad et al. (12) investigated the relationship between this C677T MTHFR mutation, riboflavin status and plasma tHcy. They demonstrated that low riboflavin was associated with higher plasma tHcy levels in people who have at least one MTHFR mutant allele. However, folate status did not alter this relationship between this mutation, riboflavin status and plasma tHcy as might be expected based on the known influence of folate status on the relation between C677T MTHFR mutation and plasma tHcy concentrations.

The present study was undertaken to further explore this hypothesis by studying the relationships between riboflavin, folate status and plasma homocysteine in a cohort that included a large number of individuals with the C677T mutation ($n = 149$).

SUBJECTS AND METHODS

Study subjects. The Framingham Heart Study, an epidemiologic study of heart disease, was established in Framingham, MA between 1948 and 1950 with a cohort of 5209 men and women age 30 to 59 y (13). By 1971, the original cohort included 1644 husband-wife pairs and 378 individuals who had developed cardiovascular disease. The offspring of these subjects and the offspring's spouses were invited to participate, and 5135 of the 6838 eligible individuals participated in the first Framingham Offspring Study examination (14). The offspring cohort has undergone repeat examinations at approximately 3- to 4-y cycles. The fifth examination of the offspring cohort began in January 1991 and was completed in December 1994. 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.

Laboratory measurements. As part of the fifth offspring cohort examination, blood samples from fasting (>10 h) subjects were obtained and stored at −70°C. Plasma tHcy was determined by HPLC with fluorometric detection (15), plasma folate by a 96-well plate microbial (*Lactobacillus casei*) assay (16,17), plasma pyridoxal-5′-phosphate (the active circulating form of vitamin B-6) by the tyrosine decarboxylase apoenzyme method (18), plasma vitamin B-12 by a radioassay (Biorad Quantaphase II, Hercules, CA), and riboflavin and combined flavin nucleotides [flavin mononucleotide (FMN) and FAD] in plasma using HPLC analysis with fluorimetric detection (19). Coefficients of variation for these assays were 8% for tHcy, 13% for folate, 16% for pyridoxal phosphate, 7% for vitamin B-12 and 9% for the B2 vitamers.

MTHFR thermolability is associated with an alanine to valine substitution due to C to T transition in the coding region of the gene. Frosst et al. (3) identified the primers for the identification of this transition. These primers generate a fragment of 198 base pairs (bp). Replacement of C by T creates a HinfI recognition sequence that digests the 198-bp fragment into fragments of 175 and 23 bp. Normal genotypes (CC) have intact 198-bp fragments, whereas the heterozygote (CT) displays both the 198- and 175-bp fragments.

Riboflavin and combined flavin nucleotide (FMN + FAD) concentrations were measured on stored plasma samples from a subset of 457 subjects seen at cycles 5 and 6. The selection of subjects was based on MTHFR C677T genotype. Subjects ($n = 1903$) who had MTHFR C677T genotype and plasma tHcy data also had archived blood samples from both the 5th and 6th examinations. Of these, 609 subjects were excluded for reasons unrelated to the present analyses if they had attended the sixth examination cycle during implementation of folic acid fortification (October 1996 through August 1997) (20). Of the remaining 1295 subjects, all subjects ($n = 158$) who were homozygotes for the MTHFR C677T mutant genotype were selected. Equal numbers of CC and CT subjects were sex and age matched (to within 5 y) to the TT subjects. There was insufficient plasma volume to measure riboflavin and FMN + FAD for 6 CC, 5 CT and 6 TT subjects. Consequently, riboflavin and FMN + FAD were determined for 152 TT, 153 CT and 152 TT subjects. Of these, 7 subjects were missing data on folate concentrations. These 450 subjects comprised the sample for our analyses.

Statistical analyses. Because plasma tHcy concentration was positively skewed, analyses were done using natural logarithm transformations. Inverse transformations were performed to provide geometric mean tHcy concentrations and their 95% confidence limits. The geometric mean tHcy concentrations were adjusted for age, sex, genotype and logarithm of plasma folate concentrations using SAS PROC GLM (21).

To estimate mean tHcy concentration across levels of plasma riboflavin and FMN + FAD, we divided these measures of B2 vitamin status into three categories of approximately equal size using tertile values (i.e., the 33.3rd and 66.7th percentile values) based on all subjects with available values. The lowest tertile category was used as the reference category for statistical comparisons. Tests for linear trend were based on the statistical significance of the linear regression coefficient relating the logarithm of riboflavin and FMN + FAD in their continuous form to the logarithm of plasma tHcy. We examined interactions between the logarithm of riboflavin and both plasma folate and MTHFR C677T genotype. For testing the interaction with riboflavin and for subsequent stratification, we divided plasma folate into two categories using the median folate concentration (12.5 nmol/L or 5.5 ng/mL) for the entire cohort. There was a slight imbalance in the number of subjects in the resulting categories because the median for the subset of subjects used in these analyses was slightly lower than the median for the entire cohort. Unless specified, statistical significance refers to $P < 0.05$.

RESULTS

Women comprised one half of the sample and the mean age was ~57 y (**Table 1**). Neither age nor sex was associated with MTHFR C677T genotype. tHcy was significantly higher in persons with the TT genotype than in those with the CC ($P = 0.01$) or CT ($P = 0.04$) genotypes (Table 1). The B2 vitamers were unrelated to genotype and the overall *F*-statistic for the association between plasma folate concentrations and genotype was not significant ($P = 0.09$), although the comparison of the geometric mean plasma folate concentrations between the CC and TT genotypes was significant ($P = 0.04$). Plasma folate concentration was moderately correlated with plasma riboflavin (Spearman's correlation coefficient = 0.31, $P < 0.001$) and weakly correlated with FMN + FAD (Spearman's correlation coefficient = 0.11, $P = 0.02$). Plasma riboflavin and FMN + FAD concentrations were also correlated (Spearman's correlation coefficient = 0.25, $P < 0.001$).

Riboflavin status was inversely associated with tHcy concentrations in the overall sample (**Table 2**). Individuals in the lowest riboflavin tertile category (<6.89 nmol/L) had significantly higher tHcy concentrations than individuals in either the second ($P = 0.02$) or third ($P = 0.03$) tertile categories. We tested to see whether the relationship between riboflavin and tHcy was modified by folate status. There was a significant interaction between riboflavin and folate concentrations ($P < 0.001$). When we stratified individuals on the basis of their folate status, we observed that the relationship between riboflavin and tHcy was present only among those with lower folate status (*P*-trend = 0.03). Among those with lower folate status, individuals with riboflavin levels in the lowest third of the sample had

TABLE 1

Characteristics of subjects

Characteristic	Overall	MTHFR[1] 677 C → T genotype		
		CC	CT	TT
n	450	149	152	149
Women, %	50.0 (45.4–54.5)[1]	50.3 (42.3–58.4)	49.3 (41.4–57.3)	50.3 (42.3–58.4)
Age, *y*	56.6 (55.8–57.5)	56.5 (54.9–58.0)	56.9 (55.4–58.4)	56.6 (55.0–58.1)
Homocysteine,[2] *μmol/L*	9.8 (9.5–10.1)	9.4 (8.9–9.9)	9.6 (9.0–10.1)	10.4[3] (9.8–11.0)
Riboflavin,[2] *nmol/L*	9.2 (8.7–9.8)	9.0 (8.1–10.0)	9.1 (8.2–10.1)	9.5 (8.5–10.6)
FMN + FAD,[2] *nmol/L*	45.6 (43.3–48.0)	44.7 (40.9–48.9)	45.8 (41.9–50.1)	46.2 (42.2–50.5)
Folate,[2] *nmol/L*	12.7 (11.8–13.6)	14.3 (12.5–16.1)	12.5 (10.9–14.0)	11.8 (10.4–13.4)

[1] Mean (95% confidence interval).
[2] Geometric mean.
[3] Mean for TT is significantly different from the means for CC ($P = 0.01$) and CT ($P = 0.04$).
Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; MTHFR, methylenetetrahydrofolate reductase.

significantly higher tHcy concentrations than those with riboflavin concentrations in the upper two thirds of the sample. There was no significant interaction between riboflavin and genotype ($P = 0.17$), and none of the trend tests within the genotype groups was significant. However, among individuals with the TT genotype, those in the lowest riboflavin category had higher homocysteine concentrations that those in the middle riboflavin tertile category ($P = 0.008$) and marginally higher than those in the upper tertile category ($P = 0.08$).

We further examined the association between riboflavin and tHcy stratified by both folate status and genotype (**Table 3**). The interaction between folate and riboflavin status was not significant in persons with either the CC ($P = 0.27$) or CT ($P = 0.72$) genotypes, but was significant in persons with the TT genotype ($P = 0.02$). Those with low folate and the TT genotype demonstrated a significant association between riboflavin and tHcy (P-trend = 0.03), but those with high folate and the TT genotype did not (P-trend = 0.20). The tHcy concentrations in the lowest riboflavin category averaged 14.5 μmol/L, which was ≥25% higher than concentrations in the upper two tertile categories.

We observed no significant associations between FMN + FAD concentrations and tHcy in the overall sample (**Table 4**). There were no significant interactions between FMN + FAD and either MTHFR C677T genotype or folate status.

DISCUSSION

We have demonstrated that low plasma riboflavin concentrations are associated with higher plasma total homocysteine concentrations. This is consistent with earlier work that demonstrated inverse associations between tHcy concentrations and both dietary and plasma riboflavin levels (12,22–24). We have further demonstrated that the relationship is confined to individuals who have both lower circulating folate concentrations and are homozygotes for the MTHFR C677T polymorphism.

Hustad and co-workers (12) previously demonstrated the influence of the MTHFR thermolabile polymorphism on the relationship between tHcy and riboflavin in a Norwegian sample, but they observed no effect of folate levels on this relationship. One potential explanation for this difference between their findings and ours might be differences in riboflavin and folate status between the Norwegian and Framingham samples. If riboflavin status were lower in Norway than in the United States, the protection of the MTHFR thermolabile variant by folate substrates would be less effective. Similarly, a lower distribution of folate status in Norway than in the United States might attenuate the protection offered by higher folate to those individuals with low riboflavin and the CT/TT genotype.

In spite of the fact that there were no apparent differences in circulating riboflavin and folate levels, it is possible that nutritional differences do exist. The study of Hustad and colleagues was based on nonfasting blood samples (12), whereas the present study used fasting samples; however, even without the difference in fasting status, the absolute folate and riboflavin values derived from the two laboratories may not be comparable. Furthermore, one might expect that the intakes of riboflavin and folate would be lower in Norway than in the United States based on the addition of these vitamins to foods through the enrichment of grain products and fortification of many breakfast cereals in the United States. The recent addition of folic acid to enriched grain products did not occur until after the fifth Framingham Offspring examination from which the data for the current analyses were taken (20,25), but products made from refined flour have been enriched with riboflavin in the United States since the 1940s, and many breakfast cereals were fortified with folic acid before the addition of the latter to enriched grain products.

The failure to observe an overall association with FMN + FAD, or any interactions with folate or MTHFR genotype, is consistent with the findings of Hustad and coworkers (12), but the reason for the lack of association is not clear. It may reflect the fact that plasma FMN + FAD is not a sensitive indicator of riboflavin status because most of the flavins present in the blood are found in erythrocytes.

Although we failed to note a significant interaction between riboflavin and genotype, our observation that the association between riboflavin and tHcy was largely confined to those with low folate and the TT genotype is consistent with the model of Guenther et al. (26), which is based on a variant form of the *Escherichia coli* MTHFR that expressed a modification similar to the human C677T mutation. They showed that the diminished activity of the modified, thermolabile *E. coli* enzyme is attributable to diminished FAD binding, which affects the equilibrium

TABLE 2

Plasma total homocysteine concentration classified by plasma riboflavin category in the Framingham Offspring Study Cohort[1]

	Plasma riboflavin category, *nmol/L*			
	<6.89	6.89–10.99	≥11.0	*P*-trend
All subjects				
n	147	151	152	
Mean tHcy, *μmol/L*	10.3	9.5	9.5	
95% CI[2]	9.8–10.8	9.1–10.0	9.1–10.0	
P-value	—[2]	0.02	0.03	0.20
Plasma folate <12.5 nmol/L				
n	107	69	61	
Mean tHcy, *μmol/L*	11.7	10.3	10.6	
95% CI	11.1–12.4	9.6–11.0	9.8–11.4	0.03
P-value	—	0.006	0.04	
Plasma folate ≥12.5 nmol/L				
n	40	82	91	
Mean tHcy, *μmol/L*	8.9	8.6	8.4	
95% CI	8.1–9.6	8.1–9.1	7.9–8.9	
P-value	—	0.56	0.33	0.76
MTHFR[2] CC genotype				
n	50	53	46	
Mean tHcy, *μmol/L*	9.8	9.2	9.2	
95% CI	9.1–10.5	8.6–9.9	8.5–9.9	
P-value	—	0.26	0.25	0.22
MTHFR CT genotype				
n	52	45	55	
Mean tHcy, *μmol/L*	9.8	9.7	9.2	
95% CI	9.1–10.5	9.0–10.4	8.6–9.8	
P-value	—	0.85	0.24	0.66
MTHFR TT genotype				
n	45	53	51	
Mean tHcy, *μmol/L*	11.6	9.6	10.3	
95% CI	10.5–12.9	8.8–10.5	9.3–11.3	0.40
P-value	—	0.008	0.08	

[1] Geometric means adjusted for age, sex, MTHFR C677T genotype (except when stratified by genotype) and logarithm of plasma folate concentrations.

[2] Reference category.

Abbreviations: CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine.

between the more stable tetramer and the less stable dimeric form of the protein. Folate derivatives protected both the wild-type and mutant *E. coli* enzymes against flavin loss. The human enzyme is a dimer rather than a tetramer, and contains an allosteric domain that binds the inhibitor *S*-adenosylmethionine, a domain that is lacking in the *E. coli* protein. In spite of structural differences between the human and the *E. coli* enzymes, interaction of these enzymes with the FAD and folate substrates appears similar.

The present study helps to expand our understanding of the role of nutrition and its interaction with genetic makeup in determining circulating tHcy concentrations. This study provides evidence that riboflavin is a determinant of tHcy in combination with the MTHFR C677T mutation in persons with low folate levels. However, given a MTHFR C677T homozygote frequency of ~0.12 in North American population samples (4,11), and given the fact that higher tHcy was observed only in those who fell in the lowest third of riboflavin status and the lowest half of folate status, one would expect only ~1–3% of the population to be at risk of increased homocysteine concentrations associated with low riboflavin status. Furthermore, the data for the present study were collected before the implementation of FDA-mandated folic acid fortification of enriched grain products in the United States (25). The folic acid fortification of grain products increased circulating folate concentrations by >100% (20,27). Therefore, the proportion of Americans with lower folate status has decreased dramatically, further lowering the risk of higher homocysteine concentrations associated with lower riboflavin status in the United States. Although riboflavin appears to play a role in homocysteine metabolism, riboflavin status would not appear to be an important determinant of circulating tHcy concentration in the United States where enriched flour and grain products include riboflavin. However, the importance of riboflavin as a determinant of homocysteine should not be underestimated in other countries, particularly where the prevalence of inadequate folate and riboflavin intakes is more common.

TABLE 3

Plasma total homocysteine concentration classified by plasma riboflavin category stratified by MTHFR C677T genotype and folate status in the Framingham Offspring Study Cohort[1]

	Plasma riboflavin categories, *nmol/L*			
	<6.89	6.89–10.99	≥11.0	*P*-trend
MTHFR[2] CC genotype				
Folate <12.5 nmol/L				
n	37	20	14	
Mean tHcy, *μmol/L*	10.9	9.5	10.6	
95% CI[2]	10.0–11.8	8.5–10.6	9.2–12.2	
P-value	—[2]	0.08	0.79	0.44
Folate ≥12.5 nmol/L				
n	13	33	32	
Mean tHcy, *μmol/L*	8.6	8.8	8.3	
95% CI	7.6–9.8	8.1–9.5	7.7–9.0	
P-value	—	0.83	0.62	0.41
MTHFR CT genotype				
Folate <12.5 nmol/L				
n	40	20	24	
Mean tHcy, *μmol/L*	10.4	10.4	9.9	
95% CI	9.7–11.2	9.4–11.5	9.0–10.9	
P-value	—	0.97	0.43	0.74
Folate ≥12.5 nmol/L				
n	12	25	31	
Mean tHcy, *μmol/L*	9.2	9.0	8.4	
95% CI	7.9–10.8	8.1–9.9	7.6–9.2	
P-value	—	0.77	0.31	0.75
MTHFR TT genotype				
Folate <12.5 nmol/L				
n	30	29	23	
Mean tHcy, *μmol/L*	14.5	11.2	11.6	
95% CI	12.8–16.4	9.9–12.7	10.0–13.3	
P-value	—	0.007	0.03	0.03
Folate ≥12.5 nmol/L				
n	15	24	28	
Mean tHcy, *μmol/L*	8.6	8.2	8.5	
95% CI	7.3–10.2	7.2–9.3	7.6–9.6	
P-value	—	0.62	0.91	0.20

[1] Geometric means adjusted for age, sex and logarithm of plasma folate concentrations.

[2] Reference category.

Abbreviations: CI, confidence interval; MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine.

TABLE 4

Plasma total homocysteine concentration classified by plasma flavin nucleotide (FMN + FAD) category in the Framingham Offspring Study Cohort[1]

	Plasma FMN + FAD, *nmol/L*			
	<35.7	35.7–65.9	≥65.9	*P*-trend
All subjects				
n	149	148	153	
Mean tHcy, *μmol/L*	10.2	9.6	9.5	
95% CI	9.7–10.8	9.1–10.0	9.1–10.1	
P-value	—[3]	0.09	0.14	0.19
Plasma folate <12.5 nmol/L				
n	88	72	77	
Mean tHcy, *μmol/L*	11.8	10.7	10.5	
95% CI	10.9–12.7	10.0–11.4	9.7–11.4	
P-value	—	0.08	0.09	0.19
Plasma folate ≥12.5 nmol/L				
n	61	76	76	
Mean tHcy, *μmol/L*	8.7	8.4	8.6	
95% CI	8.0–9.5	7.9–8.9	8.0–9.2	
P-value	—	0.54	0.82	0.64
MTHFR CC genotype				
n	50	50	49	
Mean tHcy, *μmol/L*	9.6	9.6	9.0	
95% CI	8.8–10.4	9.0–10.3	8.3–9.7	
P-value	—	0.93	0.38	0.23
MTHFR CT genotype				
n	50	49	53	
Mean tHcy, *μmol/L*	9.9	9.5	9.3	
95% CI	9.0–10.7	8.8–10.2	8.6–10.1	
P-value	—	0.52	0.44	0.94
MTHFR TT genotype				
n	49	49	51	
Mean tHcy, *μmol/L*	11.2	9.5	10.6	
95% CI	9.9–12.7	8.7–10.5	9.4–11.8	0.28
P-value	—	0.06	0.57	

[1] Geometric means adjusted for age, sex, MTHFR C677T genotype (except when stratified by genotype) and logarithm of plasma folate concentrations.

[2] Reference category.

Abbreviations: CI, confidence interval; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; MTHFR, methylenetetrahydrofolate reductase; tHcy, total homocysteine.

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