Impaired functioning of thermolabile methylenetetrahydrofolate reductase is dependent on riboflavin status: implications for riboflavin requirements[1–3]

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ABSTRACT

Background: Methylenetetrahydrofolate reductase (MTHFR; EC 1.7.99.5) supplies the folate needed for the metabolism of homocysteine. A reduction in MTHFR activity, as occurs in the homozygous state for the 677C→T (so-called thermolabile) enzyme variant (*TT* genotype), is associated with an increase in plasma total homocysteine (tHcy).

Objective: In vitro studies suggest that the reduced activity of thermolabile MTHFR is due to the inappropriate loss of its riboflavin cofactor. We investigated the hypothesis that MTHFR activity in the *TT* genotype group is particularly sensitive to riboflavin status.

Design: We studied tHcy and relevant B-vitamin status by *MTHFR* genotype in a cross-sectional study of 286 healthy subjects aged 19–63 y (median: 27 y). The effect of riboflavin status was examined by dividing the sample into tertiles of erythrocyte glutathione reductase activation coefficient, a functional index of riboflavin status.

Results: Lower red blood cell folate ($P = 0.0001$) and higher tHcy ($P = 0.0082$) concentrations were found in the *TT* group than in the heterozygous (*CT*) or wild-type (*CC*) groups. However, these expected relations in the total sample were driven by the *TT* group with the lowest riboflavin status, whose mean tHcy concentration (18.09 μmol/L) was almost twice that of the *CC* or *CT* group. By contrast, adequate riboflavin status rendered the *TT* group neutral with respect to tHcy metabolism.

Conclusions: The high tHcy concentration typically associated with homozygosity for the 677C→T variant of *MTHFR* occurs only with poor riboflavin status. This may have important implications for governments considering new fortification policies aimed at the prevention of diseases for which this genotype is associated with increased risk.   *Am J Clin Nutr* 2002;76:436–41.

INTRODUCTION

There is considerable evidence implicating elevated plasma total homocysteine (tHcy) as an independent risk factor for occlusive cardiovascular disease (CVD) (1). Homocysteine metabolism is rigorously controlled by 2 pathways—catabolism to cysteine and remethylation to methionine, which in turn are dependent on an adequate supply of one or more of the B vitamins folate, vitamin B-12, and vitamin B-6. Concentrations of tHcy are therefore inversely related to the status of these vitamins, in particular folate (2), the supplementation with which lowers tHcy by ≈25% (3). On the basis of typical tHcy concentrations (12 μmol/L) in Western populations (2, 4), this value is equivalent to an absolute reduction of 3–4 μmol/L. A prolonged lowering of tHcy by this amount is suggested to correspond to a 30–40% reduction in vascular disease (5).

Concentrations of tHcy are also influenced by genetic factors. In the extreme, patients with the inborn error homocysteinuria have profoundly elevated tHcy concentrations in plasma and urine and develop occlusive vascular disease in early adulthood or even childhood (6). Although such inborn errors are rare, polymorphic variants of the enzymes involved in homocysteine metabolism are commonly found in the general population. The most notable of these is the so-called thermolabile variant (a 677C→T transition) of the gene for the enzyme methylenetetrahydrofolate reductase (FADH) (MTHFR; EC 1.7.99.5), which catalyzes the production of 5-methyltetrahydrofolate, which in turn is required for the remethylation of homocysteine to methionine. Persons who are homozygous for thermolabile *MTHFR* (*TT* genotype) have reduced enzyme activity and a corresponding elevation in tHcy (7). This genetic polymorphism, which affects ≈12% of the healthy white population (8), also appears to have a significant effect on folate requirements. We previously showed lower red blood cell folate concentrations in persons with the *TT* genotype, suggesting that there is a higher dietary folate requirement because of this genetic predisposition (9). Others have concluded that persons with the *TT* genotype may require folic acid

supplementation to prevent fasting hyperhomocysteinemia (10), on the basis of results showing that such persons have elevated tHcy concentrations when plasma folate is below (but not when it is above) the population median. This finding was later confirmed in a meta-analysis (11). However, one unresolved inconsistency in the homocysteine theory of CVD is that although some studies (12, 13) showed that the *TT* genotype is associated with an increased risk of CVD, others (14, 15) failed to show this expected relation.

There is a fourth B vitamin involved in homocysteine metabolism, albeit its role to date has received little attention. In addition to folate, MTHFR requires riboflavin in the coenzyme form of flavin adenine dinucleotide (FAD). In vitro studies have shown that thermolabile MTHFR is ≈10 times as likely as the wild-type enzyme to dissociate from its FAD prosthetic group and thus become inactivated (16). What is unclear, however, is whether riboflavin status affects the activity of the abnormal enzyme in vivo, and consequently, tHcy concentrations. The hypothesis addressed in this study, therefore, is that tHcy concentrations in persons with the *TT* genotype (and therefore reduced MTHFR activity) would be more sensitive to riboflavin status than would those without the variant. We examined the effect of riboflavin status on the interrelation between thermolabile *MTHFR* and tHcy concentrations in healthy persons.

SUBJECTS AND METHODS

Subject recruitment

Healthy men and women aged 19–63 y (median: 27 y) were recruited between January 1997 and January 1999 from the staff and student population of the University of Ulster at Coleraine. All potential subjects were interviewed with the use of a short medical questionnaire regarding general health and drug and supplement use. Subjects excluded from the study were those with gastrointestinal disease or hematologic disorders, those taking any supplements containing B vitamins, and those with a history of vascular, hepatic, or renal disease. The vitamin B-12 status of all potential subjects was also investigated; any subject receiving treatment for vitamin B-12 deficiency (ie, intramuscular injections) or having a serum concentration < 111 pmol/L (150 ng/L) was excluded. Ethical approval was granted by the Research Ethical Committee of the University of Ulster, and subjects gave their written, informed consent.

Blood sampling and laboratory measurements

Blood samples were collected at the University of Ulster, first thing in the morning after an overnight fast. Each subject provided a 20-mL blood sample. The blood samples were collected into 3 tubes: one 8-mL EDTA-containing tube for plasma and red blood cell extraction, one 4-mL EDTA-containing tube for the preparation of red blood cell lysates and the measurement of packed cell volume, and one 8-mL serum separation tube for serum extraction. Fresh samples were initially treated within 0.5–2.5 h of the time of sampling as described in detail elsewhere (17) and were then stored at −20 °C for subsequent batch analysis at the end of the study.

The *MTHFR* 677C→T genotype was identified by polymerase chain reaction amplification followed by *Hin*F1 restriction diges-

tion (7). Plasma tHcy was measured by immunoassay (18). Red blood cell folate (19), serum folate (19), and serum vitamin B-12 (20) were measured by microbiological assay. Plasma pyridoxal-*P* (vitamin B-6) was measured by reversed-phase HPLC with fluorescence detection (21). Riboflavin status was determined on the basis of the erythrocyte glutathione reductase activation coefficient (EGRac), a functional assay in which the activity of flavin-dependent glutathione reductase (NADPH) (EC 1.6.4.2) is measured before and after in vitro reactivation with its prosthetic group FAD (22). EGRac is then calculated as the ratio of FAD-stimulated to -unstimulated activity of erythrocyte glutathione reductase, with values ≥1.2 generally considered to represent suboptimal riboflavin status. The EGRac assay requires washed red blood cells that are prepared from a fresh EDTA sample in the following manner. After centrifugation at $719 \times g$ (2000 rpm) for 15 min at 4 °C and removal of the plasma layer and a small amount of cells (for later identification of the *MTHFR* 677C→T genotype), the remaining red blood cells were washed 3 times with phosphate-buffered saline. The saline and buffy layer were removed after each centrifugation, and the resulting washed red blood cells were stored for EGRac determination.

For all assays, samples were analyzed blind, in duplicate (except for EGRac, where triplicate samples were measured) and within 6 mo of sampling. Quality control was provided by repeated analysis of stored batches of pooled washed red blood cells (for EGRac), plasma (for tHcy and plasma pyridoxal-*P*), serum (for folate and vitamin B-12), and red blood cell folate lysates, covering a wide range of values in each case.

Statistics

Statistical analyses were performed with the use of the DATA DESK statistics package (Data Description Inc, Ithaca, NY). In all statistical calculations, tHcy and vitamin variables (except EGRac) were transformed to natural logs for normalization purposes. One-way analysis of variance (with Bonferroni post hoc tests) was used to examine the effect of genotype on subject characteristics of relevance to tHcy concentration. General-linear-model techniques were used to examine the relative effects of different variables as determinants of tHcy, after which a two-way analysis of variance with interaction was carried out with the use of genotype and riboflavin status as main effects. Tertiles of riboflavin status were determined from the 33.3rd and 66.6th percentiles of the range of EGRac values. On the basis of these tertiles, a low riboflavin status was established at ≥1.19, medium status at < 1.19 to >1.13, and high status at ≤1.13. Concentrations of tHcy between genotype groups in each category of riboflavin status were compared by using analysis of variance with Bonferroni post hoc tests. For all statistical analysis, $P < 0.05$ was considered significant.

RESULTS

Population characteristics and determinants of homocysteine

Subject characteristics and the nutrient status of relevance to tHcy by *MTHFR* 677C→T genotype are listed in **Table 1**. There was no significant difference in either age or sex distribution between genotype groups (ANOVA). The *TT* group had significantly higher tHcy concentrations than did either the *CC* or *CT*

TABLE 1
Subject characteristics of relevance to plasma total homocysteine (tHcy) by thermolabile methylenetetrahydrofolate (*MTHFR*) 677C→T[1]

	Genotype				
	All (*n* = 286)	CC (*n* = 123)	CT (*n* = 130)	TT (*n* = 33)	P[2]
Age (y)[3]	29.5 (9.3)	29.5 (9.6)	29.5 (8.8)	32.5 (9.3)	0.14
Sex					0.23
Men	107	45	47	15	
Women	179	78	83	18	
tHcy (μmol/L)[3]	7.87 (18.4) [286]	7.49 (16.5)[a] [123]	7.85 (16.6)[a] [130]	9.33 (25.0)[b] [33]	0.0082
Red blood cell folate (ng/mL)[3]	331 (6.5) [283]	354 (5.8)[a] [121]	323 (6.4)[a] [129]	269 (8.3)[b] [33]	0.0001
Serum folate (ng/mL)[3]	7.59 (29.5) [276]	8.22 (28.4) [119]	7.31 (28.9) [126]	6.43 (35.9) [31]	0.093
Riboflavin (EGRac)[4]	1.16 ± 0.07 [260]	1.16 ± .06 [110]	1.16 ± .07 [119]	1.16 ± .10 [31]	0.89
Vitamin B-12 (pg/mL)[3]	353 (8.6) [278]	354 (8.2) [121]	353 (9.0) [125]	346 (8.6) [32]	0.97
Vitamin B-6 (nmol/L)[3,5]	95.2 (11.5) [269]	98.4 (11.5) [115]	90.7 (11.9) [124]	103.2 (9.0) [30]	0.34

[1] *TT*, homozygous; *CT*, heterozygous; and *CC*, wild type. EGRac, erythrocyte glutathione reductase activation. All variables except EGRac were transformed to natural logs for normalization purposes. Values within a row with different superscript letters are significantly different, *P* < 0.05 (Bonferroni post hoc *t* test).
[2] One-way ANOVA by genotype.
[3] Geometric $\bar{x}$; CV% in parentheses.
[4] $\bar{x}$ ± SD.
[5] Pyridoxal-*P*.

groups (*P* = 0.006 and 0.04, respectively, Bonferroni post hoc tests). Red blood cell folate was the only vitamin indicator that was significantly different by genotype; concentrations were significantly lower in the *TT* than in the *CC* or *CT* genotype groups.

General linear modeling enabled a ranking of the factors explaining the variation in tHcy to be made and tested the effects of each factor after the effects of the other factors were removed. Serum folate, age, and serum vitamin B-12 were log transformed for the analysis. The order of factor importance was as follows: serum folate (*P* < 0.0001), sex (*P* = 0.0003), red blood cell folate (*P* = 0.0008), riboflavin (*P* = 0.0173), vitamin B-12 (*P* = 0.1023), age (*P* = 0.2155), *MTHFR* 677C→T genotype (*P* = 0.4166), and vitamin B-6 (*P* = 0.6988).

Thermolabile MTHFR, riboflavin status, and homocysteine

Two-way analysis of variance showed significant effects of *MTHFR* genotype (*P* = 0.0097) and riboflavin (*P* = 0.0001) on tHcy and a significant interaction between genotype and riboflavin (*P* = 0.0023). The tHcy concentrations of the *TT* group were then compared with those of the *CT* and *CC* groups in the sample categorized by tertiles of riboflavin status (**Figure 1**). Results indicated that the significant effect (*P* = 0.0082) of genotype on tHcy concentration seen in the total sample (Table 1) appeared to be driven by the subjects with the lowest riboflavin status (*P* = 0.0003). Among those subjects in the lowest tertile of riboflavin status, the mean tHcy concentration in the *TT* genotype group was approximately twice the concentration (18.09 μmol/L) in either the *CT*
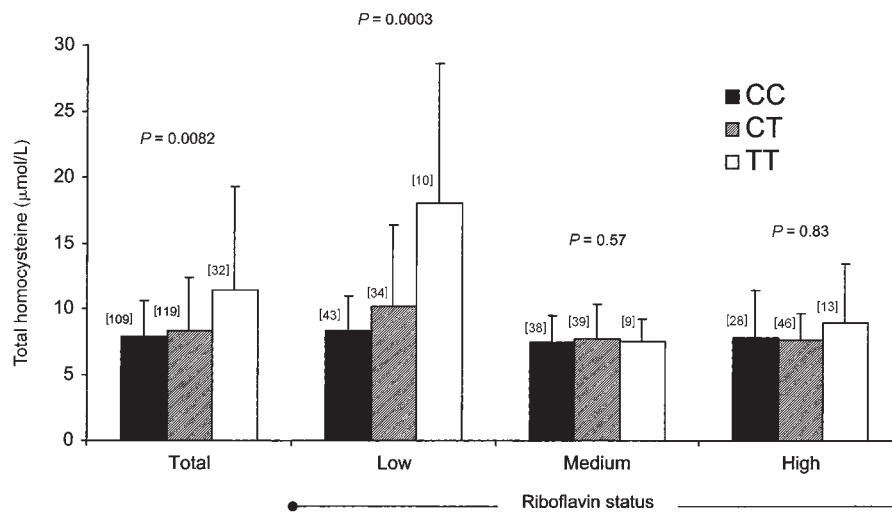

FIGURE 1. Mean (± SD) concentrations of plasma total homocysteine among wild-type (*CC*), heterozygous (*CT*), and homozygous (*TT*) genotypes for the methylenetetrahydrofolate reductase 677C→T gene polymorphism by tertiles of riboflavin status (on the basis of erythrocyte glutathione reductase activation coefficients): low status, ≥1.19; medium status, <1.19 to >1.13; and high status, ≤1.13. The statistical evaluation of the total sample is given in Table 1 and the Results. The *P* values in the figure were determined with ANOVA. Low riboflavin category (Bonferroni post hoc tests): *P* = 0.54 for *CC* compared with *CT*, *P* = 0.002 for *CT* compared with *TT*, and *P* = 0.0002 for *CC* compared with *TT*. *n* in brackets.

(10.15 μmol/L) or the *CC* (8.32 μmol/L) group ($P = 0.002$ and $P = 0.0002$, respectively, Bonferroni post hoc tests). In contrast, there were no significant differences in tHcy concentration between the genotype groups in either the medium or high tertile of riboflavin status. About 28.6% of the sample fell at or above the EGRac threshold value of 1.2, which generally indicates a sub-optimal riboflavin status (23).

Riboflavin status was found to not be related to folate status because of the lack of a significant correlation (Spearman rank-order correlation) between EGRac and red blood cell folate in the total sample ($r = -0.120$, $P = 0.075$; data not shown). However, both nutrients were significantly correlated with tHcy: EGRac ($r = 0.171$, $P = 0.0009$) and red blood cell folate ($r = -0.472$, $P = 0.0001$). Of the subjects with a red blood cell folate status below the median, those with the *TT* genotype ($n = 23$) had a mean tHcy concentration of 12.03 μmol/L compared with 9.17 μmol/L for those with the *CT* ($n = 63$) and 8.86 μmol/L for those with the *CC* ($n = 55$) genotype ($P = 0.0011$, ANOVA). In subjects with a higher red blood cell folate status (ie, at or above the median), tHcy concentrations were not significantly different between the genotype groups ($P = 0.28$, ANOVA); mean tHcy concentrations were 9.57, 7.72, and 7.17 μmol/L in the *TT* ($n = 10$), *CT* ($n = 66$), and *CC* ($n = 66$) genotype groups, respectively.

DISCUSSION

Previous studies have shown significantly lower folate (9–11) and, consequently, higher tHcy concentrations (11, 24, 25) in homozygotes for thermolabile *MTHFR* and that the phenotypic presentation of elevated tHcy in persons with this genotype is most pronounced if they have folate concentrations below the population median (10, 11). Such studies have led to the conclusion that folate requirements may be increased to maintain normal homocysteine metabolism in the face of the *TT* genotype (9, 10). The current study confirms previous findings of low folate status and elevated tHcy concentrations in association with the *TT* genotype, and shows the significant interaction between genotype and folate status reported by others. The importance of this study, however, is that it indicates that these effects in the total sample may be ultimately driven by riboflavin status. When we examined our sample by tertiles of riboflavin status, we showed that tHcy concentrations were elevated only in subjects with the *TT* genotype who had poor riboflavin status. The mean tHcy concentration (18.09 μmol/L) in this group was almost twice that of the other genotype groups, and it represents a very profound elevation in tHcy concentration for a young, healthy, adult population (26). In other persons with the same (*TT*) genotype but with moderate or good riboflavin status, tHcy concentrations were not elevated compared with wild-type and heterozygous genotypes for thermolabile *MTHFR*. Also, in contrast with folate, which is a strong predictor of tHcy within all genotype groups (11), riboflavin status appeared to be relatively unimportant in determining tHcy concentrations in the absence of the thermolabile variant of MTHFR. Our findings suggest, therefore, that a riboflavin-dependent mechanism contributes to the interrelation between folate and plasma tHcy among persons with the *TT* genotype.

Our hypothesis—that MTHFR activity in persons with the *TT* genotype would be particularly sensitive to riboflavin status—was based on the known properties of thermolabile MTHFR established in vitro, namely, that it loses its riboflavin (FAD) cofactor and then becomes inactive (16). Although the precise mechanism is unclear, the present findings indicate a high sensitivity to riboflavin status among persons with the *TT* genotype with respect to tHcy concentrations. Our results suggest that, specifically in persons with the *TT* genotype, the thermolabile MTHFR enzyme can become inactive and cause low folate status by a riboflavin-dependent mechanism. One could speculate that persons with the *TT* genotype who have optimal riboflavin status may have a higher capacity to replace inactivated enzyme than do persons with the *TT* genotype, who have low riboflavin status. Alternatively, a higher riboflavin status may prevent the FAD cofactor from leaving the active site or may allow its quick replacement, thus stabilizing the variant form of the enzyme. Further work should confirm that elevated tHcy concentrations in persons with the *TT* genotype are responsive to riboflavin supplementation, alone or in combination with folic acid.

Although a poor dietary status may well lead to deficiencies of both riboflavin and folate, it does not explain our observations. The 2 nutrients have different dietary sources (27), and we found no correlation between them in the total sample. Thus, we suggest that any relation between riboflavin and folate is a functional one (arising only in association with the thermolabile variant of MTHFR) rather than a simple dietary one. To our knowledge, only one other study in humans has investigated the metabolic interrelation between riboflavin and tHcy. Hustad et al (28) showed a significant inverse relation between tHcy and the concentration of certain analytes of riboflavin in a sample of Norwegian blood donors, a relation that was present in subjects with both low and high serum folate concentrations. Although the validity of these analytes has yet to be confirmed, the results of that study agree well with those of the current study, showing the importance of riboflavin status as measured by EGRac—the generally regarded gold standard index of riboflavin status. In both studies, the riboflavin-tHcy relation was found to be genotype dependent.

The most important observation from the present study is that optimal riboflavin status appears to render the effect of being homozygous for thermolabile *MTHFR* neutral with respect to tHcy concentration. This might explain why some studies (11, 14, 15) failed to show the expected increased risk of vascular disease in association with the *TT* genotype as seen in other studies (12, 13). Our results suggest that tHcy concentrations are not necessarily elevated in persons with the *TT* genotype, but rather the elevation depends on riboflavin status. Studies investigating the *TT* genotype as a risk factor for vascular disease have not considered riboflavin status, which may vary considerably depending on the population under investigation. In the United States, for example, mandatory fortification of flour with riboflavin for many years—and more recently with folic acid—ensures high intakes in the whole population (irrespective of individual dietary practices), which could reduce the extent to which the *TT* genotype is found to carry an increased risk of vascular disease. Results of the current study have a greater public health relevance for many other countries in which the fortification of foods is not so widespread, particularly in countries such as Italy, Spain, and Mexico, where the prevalence of the *TT* genotype for *MTHFR* is high (8). Likewise, it is clear that in some countries such as Ireland, the *TT* genotype carries an increased risk of a neural tube defect (29), whereas in other studies this is not apparent (30). Again, differences in the prevailing riboflavin status in a particular population could affect the activity of thermolabile MTHFR in cells of the closing neural tube, thereby influencing the extent to which this genotype is associated with the risk of neural tube defect.

This study has important implications for governments worldwide now considering new legislation for the introduction of mandatory fortification with folic acid. In the United Kingdom, for example, the Department of Health recently recommended such a policy, primarily aimed at the prevention of neural tube defects but with the possible additional benefit of reducing the risk of CVD via homocysteine lowering (31). No consideration is given to riboflavin under the proposed fortification policy; however, our results suggest that it has the potential to be a critical factor in the prevention of these diseases in the 12% of healthy persons who carry the *TT* genotype. In addition, current dietary recommendations have not considered the gene-nutrient interactions described herein, which may result in certain groups having dietary requirements different from those of the rest of the population. Previous studies indicate that a substantial proportion of healthy populations have suboptimal riboflavin status, with a reported prevalence of 49% (32) to 78% (33) in noninstitutionalized elderly people. In the current study of young adults, 28.6% of the sample was found to be outside the normal range for riboflavin status. In the general population, the elevated homocysteine concentrations of persons who carry the *TT* genotype for *MTHFR* would be expected to decrease by increasing riboflavin status. A smaller but possibly important benefit of increased riboflavin status might also be anticipated among heterozygotes, who make up >40% of healthy populations.

In conclusion, this study confirms our a priori expectations based on evidence from molecular biology (16) and suggests that persons with thermolabile MTHFR are particularly sensitive to riboflavin status to maintain adequate folate status and to prevent the accumulation of homocysteine. Although several studies have addressed the association between thermolabile MTHFR and the risk of disease (eg, neural tube defects and vascular disease), none has considered riboflavin status. We showed that adequate riboflavin status renders the *TT* genotype neutral with respect to tHcy concentrations, suggesting that it may be highly relevant in the prevention of such diseases among the 12% of the population who have this genetic predisposition. Most healthy people are unaware of their riboflavin status or their *MTHFR* genotype (because neither measurement is routinely performed), making riboflavin an important but unrecognized consideration in the ongoing debate about the optimization of nutritional status in the general population to produce the most effective homocysteine lowering.

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