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Riboflavin as a Determinant of Plasma Total Homocysteine: Effect Modification by the Methylenetetrahydrofolate Reductase C677T Polymorphism

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Background: **Plasma total homocysteine (tHcy) is a risk factor for cardiovascular disease. tHcy concentrations are partly determined by folate, cobalamin, and vitamin B_6 status, and methylenetetrahydrofolate reductase (MTHFR) and other flavoenzymes are important for the biotransformation of these vitamins. This motivates the investigation of the possible relationship between riboflavin status and tHcy.**

Methods: **The study had a cross-sectional design and included 423 healthy blood donors, ages 19–69 years. We determined plasma tHcy, serum folate, serum cobalamin, serum creatinine, and *MTHFR* C677T genotype. In addition, we measured riboflavin and its two coenzyme forms, flavin mononucleotide and flavin adenine dinucleotide, in EDTA plasma by capillary electrophoresis and laser-induced fluorescence detection.**

Results: **Riboflavin determined tHcy independently in a multiple linear regression model with adjustment for sex, age, folate, cobalamin, creatinine, and *MTHFR* genotype ($P = 0.008$). tHcy was 1.4 μmol/L higher in the lowest compared with the highest riboflavin quartile. The riboflavin-tHcy relationship was modified by genotype ($P = 0.004$) and was essentially confined to subjects with the C677T transition of the *MTHFR* gene.**

Conclusions: **Plasma riboflavin is an independent determinant of plasma tHcy. Studies on deficient populations are needed to evaluate the utility of riboflavin supplementation in hyperhomocysteinemia.**

Increased plasma total homocysteine (tHcy)[1] is a risk factor for occlusive disease in the coronary, cerebral, and peripheral arteries and for venous thrombosis *(1, 2)*. It also is associated with neural tube defects and pregnancy complications *(3)*.

Homocysteine is an important intermediate in one-carbon metabolism. Intracellular homocysteine is either converted to cysteine via the vitamin B_6-dependent transsulfuration pathway or is remethylated to methionine *(4)*. In most tissues, the latter reaction is catalyzed by the ubiquitous enzyme methionine synthase (EC 2.1.1.13), which requires cobalamin as a cofactor and 5-methyltetrahydrofolate as a methyl donor *(4)*. This explains why folate and cobalamin are major determinants of plasma tHcy *(5)*.

The formation of 5-methyltetrahydrofolate is catalyzed by the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR; EC 1.7.99.5) *(4)*. The commonly occurring C677T polymorphism of the *MTHFR* gene confers reduced enzyme activity and is associated with a moderate increase in tHcy *(6)*, particularly in subjects with impaired folate status *(7–10)*.

Riboflavin is the precursor of flavin mononucleotide (FMN) and FAD *(11)*, which serve as cofactors for enzymes involved in the metabolism of vitamin B_6, folate, and cobalamin *(12–16)*. FMN serves as a cofactor for pyridoxine-5′-phosphate oxidase (EC 1.4.3.5), which is important for the formation of the active form of vitamin B_6, pyridoxal-5′-phosphate *(12, 14)*, whereas FAD is a cofactor for MTHFR *(13, 16)*. Both flavin coenzymes are involved in cobalamin metabolism *(12)* and serve as cofactors for methionine synthase reductase (EC 2.1.1.135)

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[1] Nonstandard abbreviations: tHcy, total homocysteine; MTHFR, 5,10-methylenetetrahydrofolate reductase; and FMN, flavin mononucleotide.

(15). The role of flavoenzymes in the metabolism of several B vitamins points to the possibility that riboflavin status may influence homocysteine metabolism and thereby plasma tHcy concentration.

Riboflavin status in humans is usually assessed by the erythrocyte glutathione reductase activity coefficient *(17, 18)*. We recently developed a capillary electrophoresis method for the determination of riboflavin, FMN, and FAD in plasma *(19)*, and we used this assay to determine the possible relationship between riboflavin status and plasma tHcy in 423 blood donors. Folate and *MTHFR* status were also determined because MTHFR is a FAD-dependent enzyme with major effects on intracellular folate distribution *(20)*.

Materials and Methods

STUDY POPULATION

We included 423 blood donors (263 men and 160 women; age range, 19–69 years; Table 1) in the study. The subjects were predominantly Caucasian and were recruited at the Blood Bank of Haukeland University Hospital, Bergen, Norway. No medication was allowed except inhaled drugs and hormonal substitution. The subjects were not fasting.

The procedures followed were in accordance with the Helsinki Declaration, and all participants gave informed consent.

BLOOD SAMPLING

Blood samples were collected into Venoject tubes with EDTA (Terumo Europe). The samples were immediately put on ice, and plasma was obtained by centrifugation within 30 min. Venoject plain silicon-coated tubes (Terumo Europe) were centrifuged within 1 h after sampling to obtain serum. EDTA plasma and serum were stored at −70 °C until analysis.

BIOCHEMICAL ANALYSES

Plasma tHcy was analyzed by HPLC and fluorescence detection *(21)*.

Riboflavin, FMN, and FAD were determined in EDTA plasma by a modification of the method described by Hustad et al. *(19)*. A plasma volume of 25 μL was used for the analysis. Trichloroacetic acid (75 μL of a 100 g/L solution) containing 15 nmol/L isoriboflavin was added to the samples, and 75 μL of the supernatant was neutralized by the addition of 24 μL of 2 mol/L K_2HPO_4. The neutralized trichloroacetic acid-treated plasma was subjected to solid-phase extraction using C_{18} columns as described in the original publication *(19)*, except that doubly distilled water was used instead of phosphate buffer. The eluate was lyophilized overnight (Lyovac GT2; Leybold-Heraeus), and the analytes were then dissolved in 25 μL of water. The vitamers were separated by capillary electrophoresis on a Beckman P/ACE System 2210 (Beckman Instruments) and detected by laser-induced fluorescence.

Serum folate was determined by a *Lactobacillus casei* microbiological assay *(22)* and serum cobalamin by a *L. leichmannii* microbiological assay *(23)*. Both the folate and cobalamin assays were adapted to a microtiter plate formate and carried out by a robotic workstation (Microlab AT plus 2; Hamilton Bonaduz).

Serum creatinine was determined using the alkaline picrate method for the CHEM 1 system (Technicon).

MTHFR C677T genotyping was performed according to the method described by Ulvik et al. *(24)*. Whole blood (1 μL) was added directly to the reaction vessel. The blood was overlaid with 50 μL of PCR master mixture and subjected to 33 thermocycles. The allele-specific PCR products were analyzed by multiple-injection capillary electrophoresis.

Serum thyrotropin was measured by a fluoroimmunoassay (autoDELFIA; Wallac Oy).

STATISTICAL METHODS

Medians with 5th and 95th percentiles and geometric means with 95% confidence intervals were used for descriptive statistics. Means were compared using the Student *t*-test and one-way ANOVA. Multiple linear regres-

Table 1. Characteristics of the study population.[a]

	Male (n = 263)	Female (n = 160)	*P* [b]
Age, median (range), years	42 (20–69)	40 (19–64)	0.04
Plasma tHcy, μmol/L	9.7 (6.3–15.0)	8.0 (5.3–12.5)	<0.001
Plasma riboflavin, nmol/L	12.7 (6.0–52.4)	12.2 (4.6–42.9)	0.7
Plasma FMN, nmol/L	7.9 (3.9–16.3)	6.9 (3.6–15.2)	0.06
Plasma FAD, nmol/L	61.2 (45.7–83.2)	57.6 (40.6–78.7)	0.01
Serum folate, nmol/L	15.0 (8.6–29.7)	15.5 (8.5–28.4)	0.5
Serum cobalamin, pmol/L	393 (246–677)	356 (200–546)	<0.001
Serum creatinine, μmol/L	90.0 (77.2–108.0)	78.0 (64.0–91.0)	<0.001
MTHFR C677T genotype, %			
CC	49	47	0.7
CT	44	42	0.7
TT	7	11	0.2

[a] Data are given as median and 5–95th percentiles, when not otherwise indicated.
[b] Student *t*-test.

sion models were used to assess the simultaneous relationship between the various predictors and tHcy. The independent variables were represented in the model as indicator variables denoting membership to one of four categories for age, riboflavin, folate, cobalamin, and creatinine. Thus, the regression coefficients estimated the difference in mean tHcy between the reference category and the other categories for each factor. tHcy concentrations across categories of each factor were tested for linear trend.

We investigated the possible interaction between plasma riboflavin and *MTHFR* C677T genotype (coded as 0 for CC, 1 for CT, and 2 for TT) by including a product term between the two variables in a multiple linear regression model with tHcy as the dependent variable, retaining riboflavin and genotype as independent variables in the model. Similarly, we investigated the possible interaction between plasma riboflavin and serum folate.

To further investigate the interaction between plasma riboflavin and *MTHFR* C677T genotype, we studied the relationship between riboflavin and tHcy in the CC and the CT/TT groups separately. For these analyses, we used generalized additive gaussian regression with adjustment for sex, age, folate, cobalamin, and creatinine. This method gives a graphical presentation of the relationship between riboflavin and tHcy. The estimated adjusted difference in tHcy between any two riboflavin concentrations can be read from the graph. Because of the low number of subjects with the TT genotype, CT and TT individuals were combined into one group. We also investigated the riboflavin-tHcy relationship at serum folate concentrations above and below median.

SPSS, Ver. 9.0 for Windows (SPSS Inc.), was used for all statistical analyses except generalized additive gaussian regression, which was performed by S-PLUS 2000 for Windows (MathSoft). All tests were 2-tailed, and $P < 0.05$ was considered statistically significant.

Results

POPULATION CHARACTERISTICS AND BLOOD INDICES

The study population consisted of 263 men and 160 women (Table 1) with a mean age of 42 years (range, 19–69 years). Men had higher median plasma tHcy (9.7 μmol/L) than women (8.0 μmol/L). Plasma FAD and serum cobalamin concentrations were higher in men than in women, whereas plasma riboflavin, plasma FMN, and serum folate concentrations were not different between genders (Table 1) *(19)*. All subjects had folate and cobalamin concentrations above the lower reference limits, except one subject with serum folate <4.5 nmol/L and one subject with serum cobalamin <150 pmol/L.

tHcy, riboflavin, and FMN *(19)* showed a skewed distribution, whereas FAD, folate, cobalamin, creatinine, and age were more symmetrically distributed (Table 1).

The *MTHFR* C677T genotype frequencies for CC, CT, and TT were 48%, 43%, and 9%, respectively. tHcy and folate concentrations were significantly related to genotype, and subjects with the TT genotype had higher tHcy and lower folate than subjects with the CC and CT genotypes (Table 2). Concentrations of riboflavin, FMN, FAD, and cobalamin were not related to *MTHFR* status (Table 2).

We measured thyrotropin in 42 subjects with riboflavin in the lowest decile to investigate the possibility of subclinical hypothyroidism. Their mean value was 1.5 mIU/L (range, 0.4–4.7 mIU/L), which is within the reference range (0.3–5.0 mIU/L) of our laboratory.

BIVARIATE CORRELATIONS

Plasma riboflavin, serum folate, serum creatinine, and age correlated significantly with tHcy (Table 3). Spearman correlation coefficients for the riboflavin-tHcy relationship were calculated separately for the CC, CT, and TT genotypes and were −0.05 ($P = 0.5$), −0.13 ($P = 0.07$), and −0.31 ($P = 0.07$), respectively. When the CT and the TT groups were combined, the correlation coefficient was −0.18 ($P = 0.009$).

There was a positive correlation between riboflavin and cobalamin, whereas the correlation between riboflavin and folate was not significant. Compared with riboflavin, FMN showed a weaker correlation to tHcy and a correlation similar to cobalamin. In contrast, FAD was positively correlated to tHcy as well as age and creatinine (Table 3).

Table 2. Plasma tHcy and vitamin status according to *MTHFR* C677T genotype.

	Genotype[a]			
	CC (n = 204)	CT (n = 182)	TT (n = 37)	*P* [b]
Plasma tHcy, μmol/L	8.6 (8.3–9.0)	9.1 (8.8–9.5)	11.5 (9.7–13.5)	0.03
Plasma riboflavin, nmol/L	13.3 (12.1–14.6)	13.7 (12.6–15.0)	12.1 (10.0–14.7)	0.6
Plasma FMN, nmol/L	7.5 (7.0–8.0)	7.9 (7.5–8.4)	7.1 (5.9–8.4)	0.2
Plasma FAD, nmol/L	59.3 (57.7–60.9)	60.7 (59.0–62.5)	56.6 (52.7–60.8)	0.2
Serum folate, nmol/L	16.4 (15.6–17.2)	15.0 (14.3–15.9)	13.2 (11.6–15.0)	0.02
Serum cobalamin, pmol/L	381 (365–397)	379 (362–398)	360 (325–400)	0.9

[a] Data are given as geometric mean with 95% confidence intervals in parentheses.
[b] By ANOVA, adjusted for age and sex.

Table 3. Spearman correlation coefficients for tHcy and determinants of tHcy.

	tHcy	Riboflavin	FMN	FAD	Folate	Cobalamin	Creatinine
Riboflavin	−0.10[a]						
FMN	−0.04	0.64[b]					
FAD	0.10[a]	0.19[b]	0.25[b]				
Folate	−0.32[b]	0.08	0.06	0.01			
Cobalamin	−0.09	0.22[b]	0.22[b]	0.05	−0.01		
Creatinine	0.32[b]	0.10	0.13[b]	0.18[b]	−0.02	0.12[a]	
Age	0.15[b]	0.08	−0.04	0.14[b]	0.19[b]	−0.01	0.09

[a] $P < 0.05$.
[b] $P < 0.01$.

MULTIPLE REGRESSION ANALYSES

Plasma riboflavin, serum folate, serum cobalamin, serum creatinine, *MTHFR* C677T genotype, and age were associated with plasma tHcy in a multiple linear regression model (Table 4). Riboflavin was significantly inversely related to tHcy after adjustment for sex and age and after additional adjustment for folate, cobalamin, creatinine, and genotype. Plasma tHcy was 1.4 μmol/L higher in the lowest compared with the highest riboflavin quartile (Table 4). When subjects with the TT genotype were

Table 4. Determinants of tHcy.

	Simple adjustment		Multiple adjustment	
	Estimated change in tHcy, μmol/L	*P*[b]	Estimated change in tHcy, μmol/L	*P*[b]
Model 1[a]				
Sex (vs female; n = 160)				
Male (n = 263)	1.5	<0.001	0.5	0.2
Age, years (vs 19–33; n = 108)				
34–41 (n = 101)	0.2		0.5	
42–50 (n = 113)	0.7	0.06	1.3	0.002
51–69 (n = 101)	1.0		1.4	
Plasma riboflavin, nmol/L (vs >17.3; n = 106)				
12.6–17.3 (n = 105)	0.3		0.3	
9.1–12.5 (n = 106)	0.5	0.002	0.6	0.008
<9.1 (n = 106)	1.7		1.4	
Serum folate, nmol/L (vs >19.3; n = 107)				
15.2–19.3 (n = 105)	0.8		0.7	
12.5–15.1 (n = 105)	1.3	<0.001	1.2	<0.001
<12.5 (n = 106)	3.2		2.8	
Serum cobalamin, pmol/L (vs >462; n = 106)				
380–462 (n = 106)	0.7		0.0	
310–379 (n = 105)	1.0	0.004	0.8	0.01
<310 (n = 106)	1.6		1.0	
Serum creatinine, μmol/L (vs <79; n = 104)				
79–85 (n = 103)	0.6		0.9	
86–92 (n = 113)	1.9	0.004	1.9	0.001
>92 (n = 103)	1.8		2.1	
MTHFR C677T genotype (vs CC; n = 204)				
CT (n = 182)	0.5	<0.001	0.2	<0.001
TT (n = 37)	4.6		3.9	
Model 2				
Riboflavin-*MTHFR* genotype product term			−0.7	0.004
Model 3				
Riboflavin-folate product term			0.2	0.3

[a] Model 1 is adjusted for sex and age (simple adjustment) and for sex, age, plasma riboflavin, serum folate, serum cobalamin, serum creatinine, and *MTHFR* C677T genotype (multiple adjustment). Models 2 and 3 are identical to model 1 with multiple adjustment, except that a product term between riboflavin and *MTHFR* C677T genotype (model 2) or riboflavin and folate (model 3) is included.
[b] *P* for trend.

excluded from the analysis, the corresponding tHcy difference was 0.8 μmol/L ($P = 0.02$). Thus, the magnitude of the effect was similar to that observed for cobalamin but less than for folate (Table 4).

We used generalized additive gaussian regression to estimate the adjusted dose–response curve between plasma riboflavin and plasma tHcy in the CC and CT/TT groups. We found a significant inverse relationship between plasma riboflavin and tHcy in the CT/TT group, but not in the CC group (Fig. 1). This was further investigated in four groups defined by MTHFR genotype and serum folate concentrations above and below median. The riboflavin-tHcy association was present both at high and low folate in the CT/TT but not the CC group (Fig. 1). This modification of the riboflavin-tHcy relationship by *MTHFR* status was statistically significant ($P = 0.004$; Table 4, model 2). No corresponding effect of folate status on the riboflavin-tHcy relationship was detected (Table 4, model 3).

We found no significant association between plasma concentrations of flavin coenzymes and tHcy. FMN was inversely related to tHcy in a multiple regression model, but the association failed to reach significance after adjustment for sex and age ($P = 0.06$) and after additional

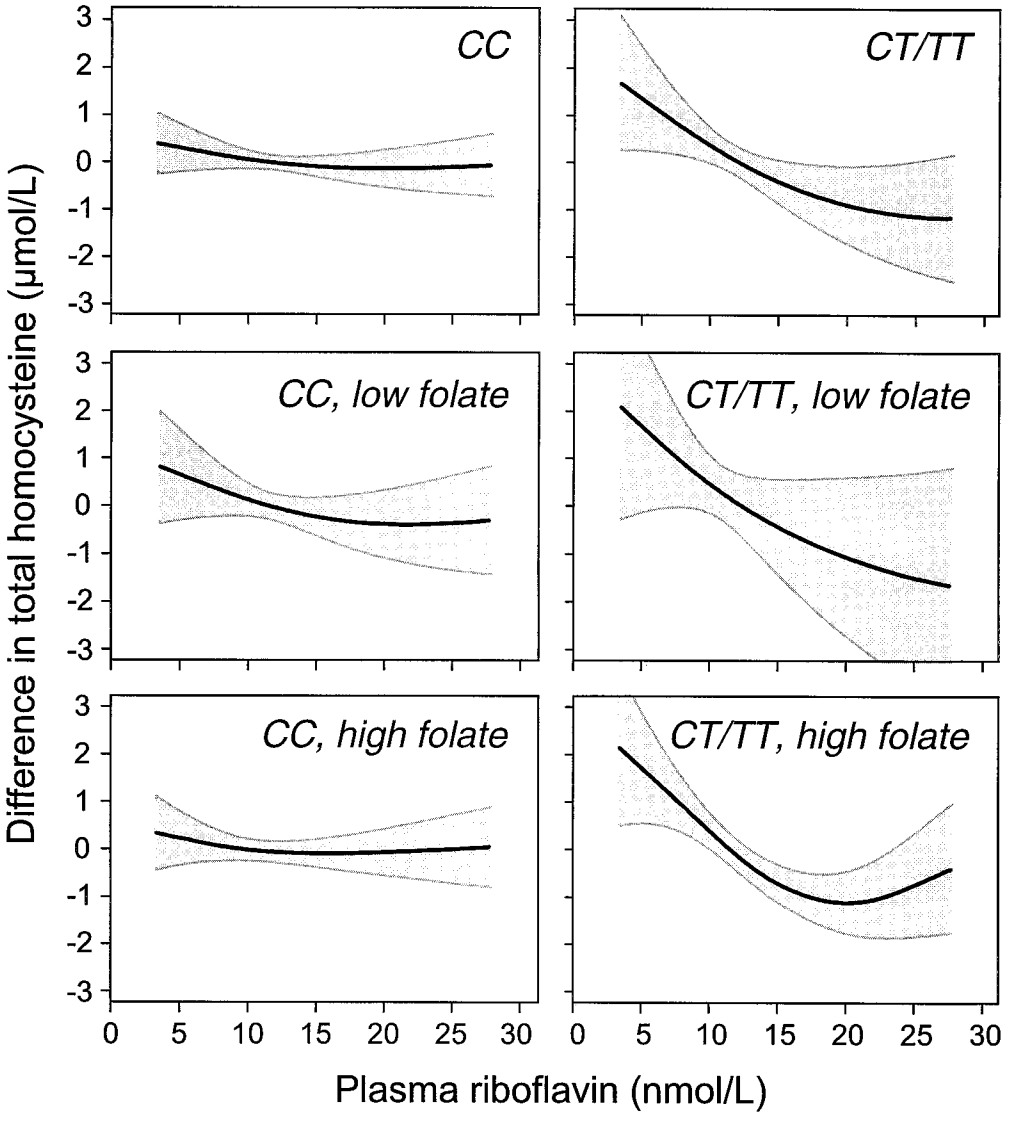

Fig. 1. Dose–response curves for the relationship between plasma riboflavin and plasma tHcy according to *MTHFR* C677T genotype and folate status.

The *top panels* show dose–response curves for riboflavin and tHcy according to *MTHFR* genotype. The *middle panels* show the relationships in subjects with low serum folate (<15.2 nmol/L) in the CC and the CT/TT groups, respectively. The *bottom panels* depict the relationships in subjects with high serum folate (≥15.2 nmol/L). The curves are obtained by additive gaussian regression analysis. *Solid lines* indicate estimated dose-response curves, and *shaded areas* represent 95% confidence intervals. The model is adjusted for sex, age, serum folate (*top panels* only), serum cobalamin, and serum creatinine.

adjustment for folate, cobalamin, creatinine, and *MTHFR* C677T genotype ($P = 0.3$). No relationship was observed between plasma FAD and tHcy in a corresponding regression model with adjustment for age and creatinine ($P = 0.9$).

Discussion

Flavoenzymes are involved in the metabolism of vitamins that are important determinants of tHcy *(12–16)*, and this motivates the investigation of the relationship between riboflavin status and plasma tHcy. In the present study, we demonstrated that plasma riboflavin is an independent determinant of plasma tHcy and that these two show an inverse dose–response relationship (Tables 3 and 4). The association is essentially confined to subjects with the CT/TT genotype of the MTHFR C677T polymorphism (Fig. 1).

The study had a cross-sectional design and included 423 healthy male and female blood donors, 19–69 years of age. Their nutritional status was adequate as judged by blood vitamin concentrations (Table 1). Only 3.8% of the subjects had tHcy concentrations >15 μmol/L, compared with 9.3% in the Hordaland homocysteine cohort *(25)*, and this probably reflects a healthy life-style and adequate B-vitamin status in our study population. The types and strengths of the associations between tHcy and folate, cobalamin, creatinine, *MTHFR* genotype, sex, and age (Tables 1–4) were in agreement with published data *(26, 27)*. Thus, we have demonstrated a riboflavin-tHcy relationship in a healthy population that is not vitamin B-deficient. Conceivably, this relationship may be even stronger in riboflavin-deficient subjects.

The inverse relationship between tHcy and plasma riboflavin remained significant after adjustment for other tHcy predictors, including folate (Table 4). Because low folate is associated with high tHcy, particularly in subjects with the TT genotype (Table 2) *(7–10)*, there might be residual confounding from folate status. This possibility seems unlikely, however, because the riboflavin-tHcy relationship was also observed in subjects with high serum folate (Fig. 1).

The association between riboflavin and tHcy is most likely mediated by the MTHFR enzyme. This is supported by the observation that the riboflavin effect is dependent on *MTHFR* genotype (Table 4 and Fig. 1). Thus, riboflavin deficiency may reduce MTHFR activity, which in turn decreases the availability of 5-methyltetrahydrofolate *(10, 20)* and thereby homocysteine remethylation. This idea is supported by the finding that MTHFR activity *(13, 28)* and relative amounts of 5-methyltetrahydrofolate *(13, 28, 29)* are reduced in the liver of riboflavin-deficient rats.

The recent finding of Guenther et al. *(16)* provides an explanation for the increased tHcy responsiveness to low riboflavin in subjects with the CT/TT genotype (Fig. 1). The authors showed that the A177V mutation of bacterial MTHFR, which is homologous to the A222V (C677T) substitution in human MTHFR, was associated with an enhanced FAD dissociation rate *(16)*. Thus, subjects with the T allele may require higher concentrations of FAD for maximal catalytic activity.

We suggest that the riboflavin-tHcy relationship is mediated by the FAD-dependent enzyme MTHFR. This raises the question of why plasma riboflavin, but not FAD, is associated with tHcy. Published data indicate that plasma/serum concentrations of riboflavin may reflect tissue riboflavin status better than FAD *(30, 31)*. In a group of riboflavin-deficient men maintained on restricted riboflavin intake for several months, the sum of plasma riboflavin and plasma FMN was lower than in the control group, whereas plasma FAD was not significantly different *(31)*. In riboflavin-deficient rats whose growth was improved by successive addition of dietary riboflavin, serum riboflavin increased proportionally much more than serum FAD during supplementation *(30)*. Furthermore, in tissues of riboflavin-deficient rats, riboflavin decreased to very low concentrations *(32–34)* with partial preservation of flavin coenzymes, in particular FAD *(33–35)*.

The distribution of riboflavin, FMN, and FAD in tissues is probably under strict metabolic control *(33, 34, 36)*. The existence of such regulatory processes is indicated by a selective preservation of some flavoenzyme-dependent metabolic pathways in riboflavin deficiency *(13, 34)*. Notably, in riboflavin-deficient rats, hepatic MTHFR activity is markedly reduced, demonstrating that this enzyme is sensitive to riboflavin deficiency *(13, 28)*. Our data suggest that this response may be modulated by the C677T polymorphism in humans. Reduced MTHFR activity may in turn alter folate distribution by directing folate species toward the synthesis of purines and pyrimidines at the expense of 5-methyltetrahydrofolate synthesis. We hypothesize that this mechanism may secure DNA and RNA synthesis of proliferating cells in folate deficiency *(13, 28)*.

In conclusion, plasma riboflavin is an independent predictor of tHcy. Our results indicate that riboflavin status may affect tissue distribution and economy of reduced folate by modifying MTHFR activity, particularly in subjects with the C677T transition. Studies on riboflavin-deficient populations are needed to evaluate the utility of riboflavin supplementation in hyperhomocysteinemia.

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