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Riboflavin Lowers Homocysteine in Individuals Homozygous for the *MTHFR* 677C→T Polymorphism

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Background—Meta-analyses predict that a 25% lowering of plasma homocysteine would reduce the risk of coronary heart disease by 11% to 16% and stroke by 19% to 24%. Individuals homozygous for the methylenetetrahydrofolate reductase (*MTHFR*) 677C→T polymorphism have reduced *MTHFR* enzyme activity resulting from the inappropriate loss of the riboflavin cofactor, but it is unknown whether their typically high homocysteine levels are responsive to improved riboflavin status.

Methods and Results—From a register of 680 healthy adults 18 to 65 years of age of known *MTHFR* 677C→T genotype, we identified 35 with the homozygous (TT) genotype and age-matched individuals with heterozygous (CT, n=26) or wild-type (CC, n=28) genotypes to participate in an intervention in which participants were randomized by genotype group to receive 1.6 mg/d riboflavin or placebo for a 12-week period. Supplementation increased riboflavin status to the same extent in all genotype groups (8% to 12% response in erythrocyte glutathione reductase activation coefficient; $P<0.01$ in each case). However, homocysteine responded only in the TT group, with levels decreasing by as much as 22% overall (from 16.1 ± 1.5 to 12.5 ± 0.8 $\mu\text{mol/L}$; $P=0.003$; n=32) and markedly so (by 40%) in those with lower riboflavin status at baseline (from 22.0 ± 2.9 and 13.2 ± 1.0 $\mu\text{mol/L}$; $P=0.010$; n=16). No homocysteine response was observed in the CC or CT groups despite being preselected for suboptimal riboflavin status.

Conclusions—Although previously overlooked, homocysteine is highly responsive to riboflavin, specifically in individuals with the *MTHFR* 677 TT genotype. Our findings might explain why this common polymorphism carries an increased risk of coronary heart disease in Europe but not in North America, where riboflavin fortification has existed for >50 years. (*Circulation*. 2006;113:74-80.)

Key Words: cardiovascular diseases ■ homocysteine ■ methylenetetrahydrofolate reductase (NADPH2) ■ nutrition ■ riboflavin

Considerable evidence from numerous prospective and retrospective case-control studies has emerged in recent years to link elevated plasma homocysteine levels with increased cardiovascular risk. Meta-analyses of such studies predict that lowering homocysteine by 3 $\mu\text{mol/L}$ (or $\approx 25\%$ for an average of 12 $\mu\text{mol/L}$) would reduce the risk of coronary heart disease (CHD) by 11% to 16% and stroke by 19% to 24%.^{1,2} Homocysteine levels are determined by both nutritional and genetic factors. Folate, the focus of much current debate on food fortification worldwide, is the major determinant of homocysteine, and folic acid supplementation can lower homocysteine levels in healthy and diseased populations by $\approx 25\%$.³ Vitamins B₁₂ and B₆ also play key roles in homocysteine metabolism, with evidence showing that both vitamins are important determinants of homocysteine, particularly when folate status has been optimized.⁴⁻⁶

Thus, all 3 vitamins arguably should be included in any emerging fortification policy aimed at lowering homocysteine in the general population.

The most important genetic determinant of homocysteine in the general population is the common 677C→T variant in the gene coding for the enzyme methylenetetrahydrofolate reductase (*MTHFR*), required for the formation of 5-methyltetrahydrofolate, which in turn is required to convert homocysteine to methionine. People who are homozygous for this polymorphism (TT genotype, the frequency of which varies from 3% to 32% in populations worldwide⁷) have reduced *MTHFR* activity, resulting in elevated homocysteine levels,⁸ an effect that is particularly marked in those with lower folate status.⁹ In the absence of positive results from an appropriately designed and sufficiently powered clinical trial to confirm that lowering homocysteine can reduce the risk of

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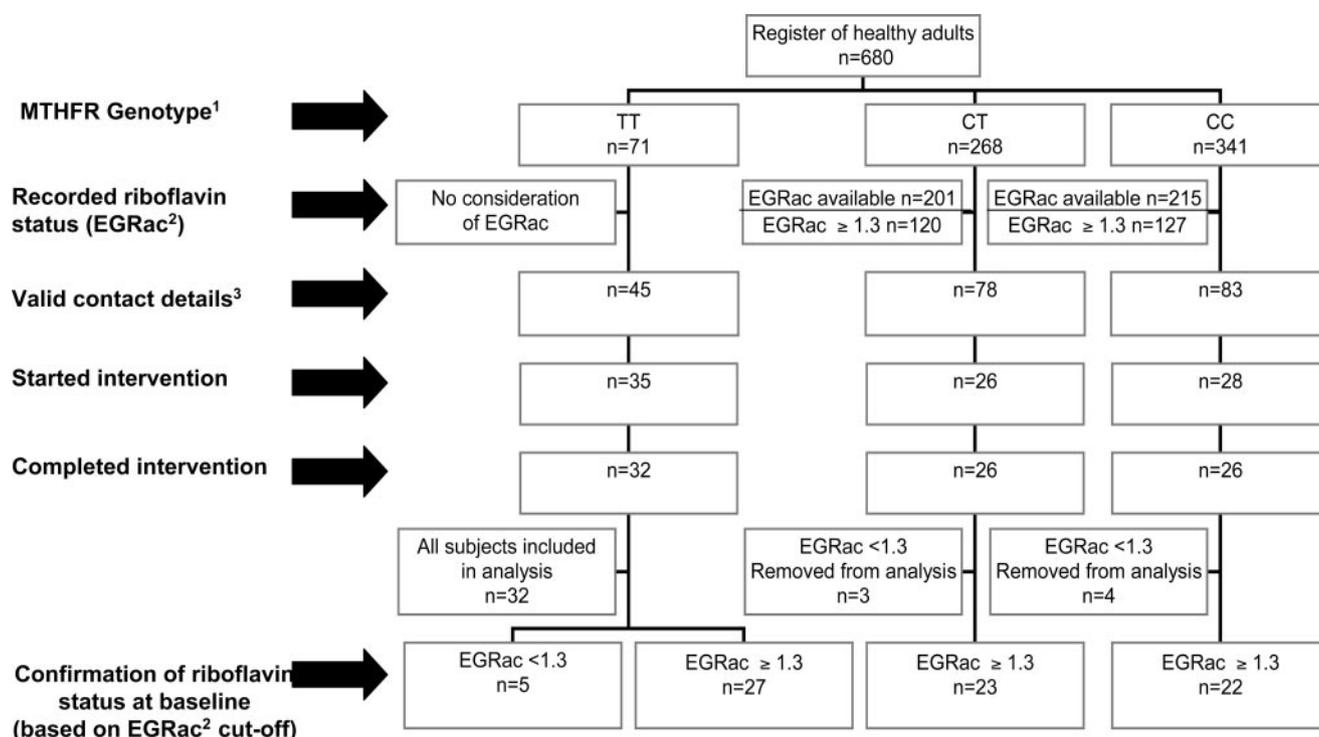


Figure 1. Flowchart of study participants. ¹TT (homozygous), CT (heterozygous), and CC (wild-type) genotypes for the *MTHFR* 677C→T polymorphism. ²EGRac is a functional assay for riboflavin. A higher EGRac ratio indicates lower riboflavin status (values ≥ 1.3 generally indicate suboptimal status). ³Valid contact details refers to subjects who were contactable and found to be still residing within the study zone.

vascular disease, the most convincing evidence for a cause-and-effect relationship comes from studies examining the impact on disease risk of this genetic variant. Two recent meta-analyses involving $\approx 12\,000$ cases reported a significantly higher risk of CHD in people with compared with those without the 677C→T polymorphism in *MTHFR*.^{2,10} The trend toward increasing risk of disease shown among individuals with wild-type (CC), heterozygous (CT), and homozygous mutant (TT) genotypes is consistent with the increasing gradient in homocysteine levels typically found among the 3 genotypes,^{2,8} as would be expected if there is a causal relationship between elevated homocysteine and risk of disease. However, there appears to be some inconsistency of findings between European and North American populations as to the relationship of this polymorphism with disease risk.¹⁰

A fourth, much overlooked, B vitamin is also involved in homocysteine metabolism. In addition to folate, riboflavin (in its coenzymatic form flavin adenine dinucleotide [FAD]) is required as a cofactor for the MTHFR enzyme. The reduced activity of the thermolabile variant of MTHFR has been shown to result from the inappropriate loss of its FAD cofactor.¹¹ What is unclear, however, is whether improving riboflavin status can affect the activity of the abnormal enzyme in vivo and consequently normalize plasma homocysteine levels in individuals with the TT genotype. We hypothesized that homocysteine levels will be responsive to improved riboflavin status, specifically in individuals with the TT genotype. The aim of this study was to examine the effect of riboflavin supplementation on homocysteine levels

in individuals with and without the *MTHFR* 677C→T polymorphism.

Methods

Design, Participants, and Intervention

We accessed an existing register of healthy adults previously screened for B vitamin studies at this center between January 1997 and September 2002. Subjects had originally been recruited to the various studies using identical criteria and excluding the following: those suffering from (or with a history of) gastrointestinal disease, hepatic disease, vascular disease, renal disease, or hematological disorders; those taking supplements containing B vitamins or drugs known to interfere with the metabolism of folate or related B vitamins; and those receiving treatment for vitamin B₁₂ deficiency (ie, intramuscular injections) or with a serum concentration <111 pmol/L (150 ng/L). Ethical approval for both the current and original investigations was granted by the Research Ethical Committee of the University of Ulster, and subjects gave written, informed consent. As part of their original signed consent, subjects had agreed to be contacted and invited to participate in future nutritional investigations at the center.

From our register of 680 healthy men and women 18 to 65 years of age with known *MTHFR* genotype (Figure 1), we identified 45 homozygotes for the *MTHFR* 677C→T polymorphism (TT genotype) with valid contact details (ie, contactable and still residing within the study zone). A further 161 aged-matched individuals who were not homozygous (CC and CT genotypes) and had a recorded suboptimal status of riboflavin (erythrocyte glutathione reductase activation coefficient [EGRac] ≥ 1.3) and valid contact details were also identified. These subjects (n=206) were invited to participate in a double-blind placebo-controlled intervention trial with riboflavin. A screening blood sample for homocysteine determination was collected from all subjects who agreed to take part in the study. Participants were stratified on the basis of homocysteine levels within each genotype group; subjects in each stratum were then

Baseline Participant Characteristics

Variables	<i>MTHFR</i> Genotype				<i>P</i> *
	All (n=89)	CC (n=28)	CT (n=26)	TT (n=35)	
Age, y	38.7±9.1	40.1±7.2	35.9±9.1	39.7±10.1	0.176
BMI	25.8±3.8	26.5±3.5	25.6±3.5	25.5±4.2	0.483
tHcy, μmol/L	13.8±7.8	10.7±2.8 ^a	12.1±3.4 ^{ab}	17.6±10.8 ^b	0.001
Red cell folate, nmol/L	814±306	1015±310 ^a	793±261 ^b	668±246 ^b	<0.001
Serum folate, nmol/L	21.3±14.2	24.3±9.5 ^a	19.4±10.0 ^{ab}	20.4±19.1 ^b	0.043
Serum B ₁₂ , pmol/L	197±90	216±114	178±72	195±177	0.463
Riboflavin, EGRac	1.44±0.15	1.43±0.15	1.46±0.17	1.43±0.14	0.638

BMI indicates body mass index; tHcy, plasma total homocysteine concentration. Values are mean±SD.

*Statistical significance for comparison between genotype groups by 1-way ANOVA. Values in a row with different superscript letters indicate significant difference ($P<0.05$) by a Bonferroni post hoc test. Values were log transformed before analysis for normalization purposes as appropriate.

randomized to receive riboflavin (1.6 mg/d, equal to the European Union *Population Reference Intake* value for men¹²) or placebo for an intervention period of 12 weeks. To monitor compliance, participants were provided supplements every 2 weeks in 7-day pill organizer boxes and asked to return the box at each visit; any missed doses were recorded.

Blood samples were collected at screening, before and after intervention at the University of Ulster, or at the subject's place of work or home first thing in the morning after an overnight fast. Samples were analyzed blind by standard laboratory assays for plasma total homocysteine,¹³ red cell folate,¹⁴ serum folate,¹⁴ and serum vitamin B₁₂.¹⁵ The *MTHFR* 677C→T genotype was identified by polymerase chain reaction amplification, followed by *Hinf*I restriction digestion.⁸ Riboflavin status was determined by EGRac, a functional assay that measures the activity of glutathione reductase before and after in vitro reactivation with its prosthetic group FAD.¹⁶ EGRac is calculated as the ratio of FAD-stimulated to unstimulated enzyme activity, with values ≥ 1.3 generally indicative of suboptimal riboflavin status. Samples (baseline and follow-up for both placebo and treatment) for each assay were analyzed blind, in 1 batch, in duplicate, and within 9 months of sampling. Quality control was provided by repeated analysis of stored batches of pooled samples covering a wide range of values.

Statistical Analysis

Statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS version 9.0.1, SPSS Inc). Data were transformed to natural logs for normalization purposes before analysis as appropriate. One-way ANOVA (with Bonferroni's post hoc test) was used to examine the differences in relevant participant characteristics at baseline between the *MTHFR* genotype groups. The effect of riboflavin intervention within each genotype group was examined with independent *t* tests (without correction for multiple testing) to compare percent response to intervention (posttreatment minus pretreatment value expressed as a percentage of pretreatment value) between treatment and placebo. Two-factor ANOVA was carried out to examine folate and riboflavin as determinants of homocysteine at baseline among those with the TT genotype and to examine the impact of baseline riboflavin status on the homocysteine response to treatment within the TT group. For all statistical analysis, values of $P<0.05$ were considered significant.

Results

Participant Characteristics at Baseline

Homocysteine levels and related B vitamin status by *MTHFR* 677C→T genotype in the 89 subjects who started intervention are listed in the Table. The TT genotype group had

significantly higher homocysteine and lower folate levels compared with the CC group; the CT group had intermediate concentrations. No other differences between genotype groups were found. In the total sample, homocysteine was found to be significantly correlated (Pearson's correlation coefficient) with red cell folate ($r=-0.484$, $P<0.0001$), serum folate ($r=-0.480$, $P<0.0001$), serum B₁₂ ($r=-0.356$, $P=0.001$), and riboflavin status (EGRac: $r=0.349$, $P=0.001$). In the TT genotype group, similar correlations were found, except for riboflavin status, which was related much more strongly to homocysteine (EGRac: $r=0.629$, $P<0.0001$; $n=35$) than in the total sample. Among non-TT genotype groups (CC and CT combined), riboflavin status was not found to be a significant determinant of homocysteine (EGRac: $r=0.210$, $P=0.128$; $n=54$).

Examination of the relative influence at baseline of riboflavin and folate status as determinants of homocysteine among those with the TT genotype revealed that riboflavin was a much more potent modulator than folate of homocysteine levels (not shown). Subjects in the TT group ($n=35$) were assigned to 1 of 4 categories on the basis of "low" or "high" status for each nutrient using median EGRac and red cell folate values for the group as cutoffs. Those subjects in the TT group with a low status of both nutrients ($n=14$) had a median homocysteine level of 21.3 μmol/L compared with 9.7 μmol/L in those with a high status of both nutrients ($n=13$). In the small number of individuals with low status of riboflavin and high status of folate ($n=4$), the median homocysteine level was 18.2 μmol/L, whereas in those with low status of folate and high status of riboflavin ($n=4$), the value was 11.7 μmol/L. Two-factor ANOVA showed riboflavin ($P=0.030$) but not folate ($P=0.395$) status to be a significant determinant of homocysteine, with no significant interaction between the 2 factors ($P=0.435$).

Response to Riboflavin Intervention

Of the 89 subjects who started intervention, 84 completed it (Figure 1); the remainder withdrew voluntarily or were discontinued on the basis of failing to return pillboxes or to provide a final blood sample despite repeated reminders. Of

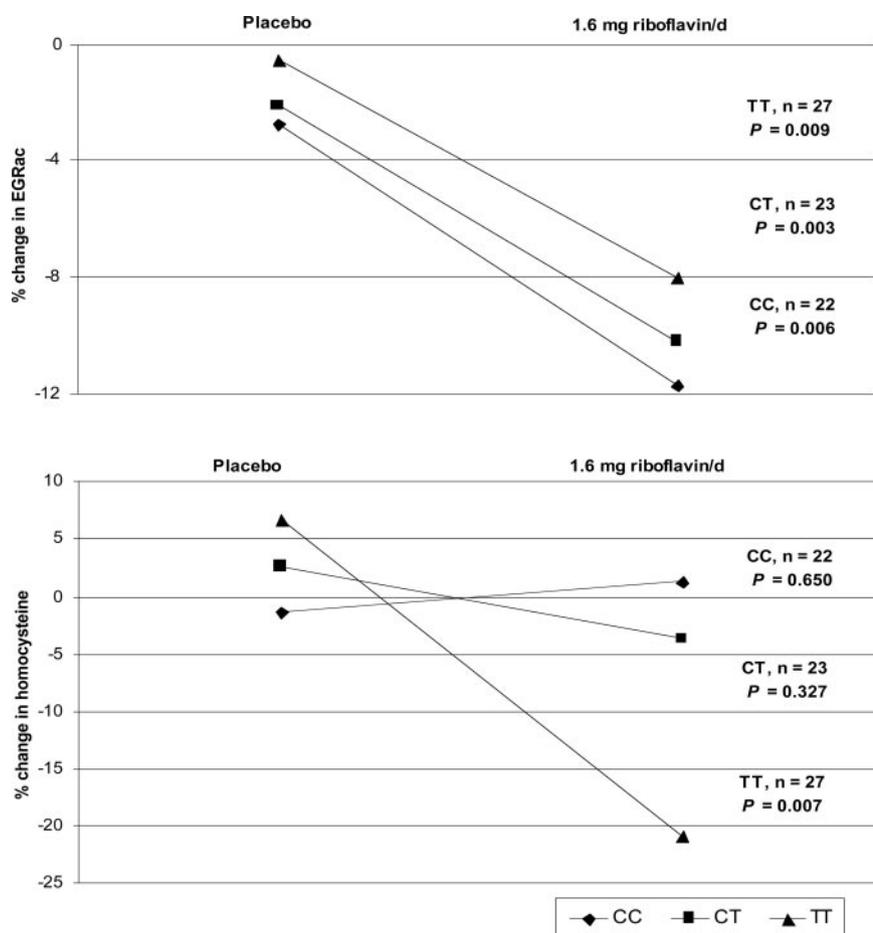


Figure 2. Response of riboflavin status (top) and homocysteine (bottom) to intervention with riboflavin for 12 weeks. Riboflavin status was measured as EGRac, a functional assay for riboflavin, with higher values indicating lower riboflavin status. Probability values refer to independent *t* tests within each genotype group (without correction for multiple testing) to compare response with intervention between treatment and placebo. Values are mean percent change (post-treatment minus pretreatment value expressed as a percentage of pretreatment value).

these, 7 (non-TT genotype) were removed from analysis because measurement (on completion of the study) of their riboflavin status revealed that, although their historical sample had indicated suboptimal status, they had in fact normal riboflavin status at the start of intervention and were therefore ineligible for inclusion. The remaining 77 subjects were included in the statistical analysis.

Intervention among those preselected for suboptimal riboflavin status resulted in a significant improvement in riboflavin status, the extent of which was similar across the 3 genotype groups (Figure 2, top). However, homocysteine responded only in the TT group, with levels lowering by 22%; no homocysteine response was observed in the CC or CT groups despite having suboptimal riboflavin status before intervention (Figure 2, bottom). The response to intervention in the total TT group was then examined according to whether subjects had low or high riboflavin status (ie, split on the basis of the median EGRac value for the TT group) at baseline (Figure 3). Those with the lower riboflavin status showed markedly higher homocysteine levels before intervention. Furthermore, it was clear that the homocysteine response in the overall TT group was driven by those who started intervention with the lower riboflavin status, among whom homocysteine levels decreased by 40% (from 22.0 ± 2.9 and 13.2 ± 1.0 $\mu\text{mol/L}$; $P=0.010$; $n=16$). Two-factor ANOVA with homocysteine as the response showed a significant interaction between treatment and baseline ribo-

flavin ($P=0.017$), demonstrating that the homocysteine response to treatment was dependent on baseline riboflavin status. Correspondingly, riboflavin status (EGRac) was significantly enhanced in response to treatment only in the subgroup with lower riboflavin status at baseline ($P=0.007$) (not shown). Probability values were not corrected for multiple testing because the effect of riboflavin intervention was examined in each genotype group separately.

Discussion

If the many large-scale randomized trials currently testing whether lowering homocysteine can reduce the risk of cardiovascular disease¹⁷ prove positive, then decisions about the most appropriate means of optimizing nutritional status to prevent homocysteine accumulation in healthy populations will have to be urgently addressed. Dietary requirements to achieve optimal nutritional status are increasingly recognized as being not only age and sex specific but also dependent on genetic factors, with some individuals likely to have higher requirements for specific nutrients in the face of common polymorphisms in nutrient metabolism.^{18,19} Here, we report for the first time significant lowering of homocysteine in response to riboflavin supplementation in individuals homozygous for the *MTHFR* 677C→T polymorphism, with levels decreasing by as much as 22% overall and markedly so (by 40%) in those with lower riboflavin status at baseline. No homocysteine response to intervention was observed in those

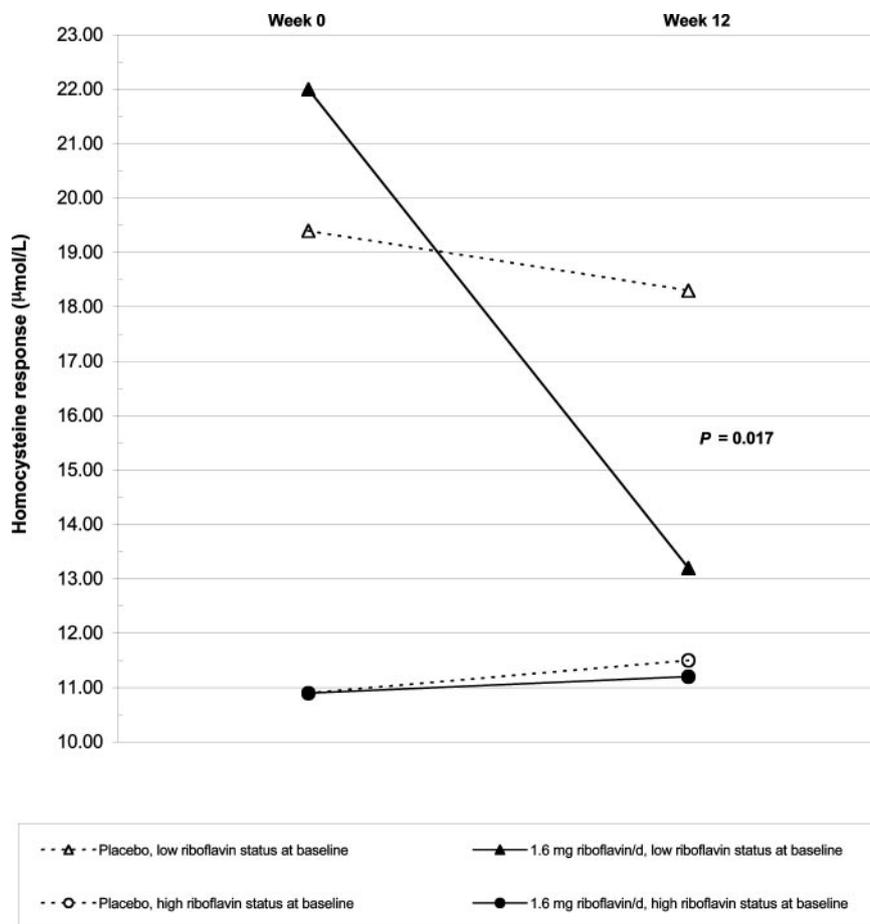


Figure 3. Homocysteine response to intervention in the TT genotype group according to riboflavin status at baseline. Subjects were categorized into “low” or “high” riboflavin status ($n=16$ in each case) at baseline on the basis of having an EGRac value above and equal to 1.39 or below 1.39, the median value for the TT group. The probability value refers to the significant interaction between baseline riboflavin status and treatment (2-factor ANOVA, without correction for multiple testing), demonstrating that the homocysteine response to treatment was dependent on baseline riboflavin status. Values are mean homocysteine concentrations ($\mu\text{mol/L}$) at weeks 0 and 12 (before and after intervention with riboflavin).

without the polymorphism or heterozygotes (CC or CT genotypes, respectively), despite a significant improvement in riboflavin status in both cases and the preselection of subjects with suboptimal riboflavin status at baseline. The lack of a homocysteine-lowering effect of riboflavin has previously been reported in an intervention study of healthy elderly people who also were prescreened for suboptimal riboflavin status but not for *MTHFR* genotype.²⁰ The responsiveness of homocysteine to riboflavin is therefore specific to individuals with the *MTHFR* 677TT genotype and represents a new gene-nutrient interaction. Emerging food policy in different countries should consider the riboflavin requirements of people with this common genetic predisposition to elevated homocysteine, the frequency of which is $\approx 10\%$ but can be as high as 20% (Northern China), 26% (Southern Italy), and 32% (Mexico) in some areas worldwide.⁷

The marked homocysteine-lowering effect of riboflavin in the TT genotype group shown here was demonstrated with a riboflavin dose of 1.6 mg/d. This is the European Union *Population Reference Intake* value for men¹²; official riboflavin recommendations for men and women are similar elsewhere in the world, ranging 1.1 to 1.8 mg/d.^{21,22} Such a low dose is easily achievable on a population basis through food fortification or on an individual basis via increased dietary selection of riboflavin-rich foods such as milk and dairy products. After its introduction in recent years in the United States and Canada, mandatory fortification of food with folic acid has become the subject of much current debate

in Europe and elsewhere. In the United Kingdom, for example, folic acid fortification was proposed by the Department of Health in 2000.²³ A formal consultation process has since taken place, but the final decision has been delayed. Although driven primarily by the proven role of folic acid in the prevention of neural tube defects, the potential benefit of fortification in reducing the risk of CHD and stroke via homocysteine lowering is also considered to be highly relevant to the current debate. As has been experienced in the United States since its introduction,²⁴ there is no doubt that folic acid fortification, if introduced in a population, would have a major homocysteine-lowering effect regardless of genotype. Individuals with the *MTHFR* 677 TT genotype are considered to have increased dietary folate requirements because they have lower red cell folate levels compared with those without this genetic variant¹⁹ and are found to have higher homocysteine levels only when folate is below the median within a TT genotype population.^{9,25} However, the baseline data in the present study show that riboflavin status may be a much more potent modulator than folate status of the expected (high homocysteine) phenotype among individuals with the TT genotype. Thus, to cover the needs of appreciable subgroups (3% to 32%) of populations worldwide with this genotype,⁷ riboflavin may need to be considered for inclusion along with folic acid in fortification programs under discussion.

To date, 3 observational studies in humans have investigated the relationship between riboflavin status and homo-

cysteine in individuals with different *MTHFR* genotypes^{26–28} with somewhat inconsistent results. In the United States, an association between riboflavin status and homocysteine was reported in people with the TT genotype in the Framingham cohort,²⁶ but this was confined only to those with the TT genotype and low folate status. Thus, riboflavin did not seem to be the limiting nutrient. The 2 studies conducted in healthy European populations (Norway and Northern Ireland), however, both identified riboflavin as an important determinant of homocysteine among individuals with the TT genotype and indicated that this effect was not explained by folate.^{27,28} The question of whether riboflavin is in fact an independent modifier of homocysteine in people with the TT genotype was therefore unresolved.²⁹ The present results showing a genotype-specific response of homocysteine to riboflavin supplementation now confirm that it is. The most likely explanation for the inconsistencies in the aforementioned studies is that mandatory fortification of flour with riboflavin has been in place since the 1940s in the United States. The impact of this policy in optimizing riboflavin status in the general US population would reduce the extent to which riboflavin is found to be a limiting nutrient in determining homocysteine levels in people with the TT genotype.

The critical question, however, is whether the *MTHFR* 677C→T polymorphism is associated with a higher risk of cardiovascular disease. Again, there are inconsistencies in the literature in this regard that could relate to the role of riboflavin. One recent meta-analysis involving 40 studies showed clearly that, although there was an overall significantly (16%) higher risk of CHD in individuals with the TT (compared with the CC) genotype, analysis of the odds ratios between continents showed that CHD risk was significantly increased for people with the TT genotype in Europe but not in North America.¹⁰ The authors suggested that their observations were most likely a result of differences in folate status,¹⁰ a factor well recognized to influence homocysteine levels among individuals with the TT genotype.⁹ However, the impact of folic acid fortification in the United States is likely to have had a minimal effect on these results, given that it was introduced only in 1998. Although many of the studies included the meta-analysis had a publication date of 1998 or later,¹⁰ the period under investigation in terms of risk/development of CHD would have been far earlier. Riboflavin fortification, in contrast, was introduced in the United States >50 years ago. In European countries in which no such food policy exists, riboflavin intakes are likely to be lower overall and much more variable among individuals, depending on food choices. For example, a diet that includes dairy foods and/or fortified breakfast cereals would generate considerably higher riboflavin intakes than one that does not include these foods. The present findings show that, among individuals with the TT genotype, there is a major impact on homocysteine levels of enhancing riboflavin status by a modest increase in riboflavin intake that is achievable on a population basis through food fortification. Riboflavin has been largely overlooked as a modulator of homocysteine in the face of the *MTHFR* 677C→T polymorphism, but the present findings indicate that higher riboflavin intakes can contribute to neutralizing the effect of this genetic variant on homocysteine

levels. We suggest, therefore, that the reported differences among countries as to whether this polymorphism represents an increased risk of cardiovascular disease relate not only to differences in folate, as commonly suggested,^{2,10} but also to differences in the prevailing riboflavin status. From the homocysteine data presented here, we could predict that people with the TT genotype who also have low riboflavin status would have an excess risk of cardiovascular disease, whereas TT homozygotes with optimal riboflavin status may not show the expected risk. No study, however, has addressed this issue.

Finally, it is highly likely that a gene-nutrient interaction that influences homocysteine would impinge on the risk of not only cardiovascular disease but also neural tube defects and other diseases linked to the *MTHFR* 677C→T polymorphism. The modulating effect of riboflavin might explain, for example, why the TT genotype carries an increased risk of a neural tube defect in some populations³⁰ but not in others.³¹ Furthermore, although only mutant homozygotes (TT genotype) showed a significant homocysteine response to riboflavin in the present study, it is possible that a weak response to riboflavin exists among heterozygotes (CT genotype), which our study has failed to detect. Although we predict that the size of any effect (if it existed) would be much smaller among heterozygotes, even a small effect could be important in the latter subgroup, given its much higher representation (typically 40%) in the general population,⁷ as demonstrated recently in the case of neural tube defects.³² It might be anticipated, therefore, that any benefit of riboflavin fortification on cardiovascular risk (estimated from the population-attributable fraction) could affect an even greater proportion of a population than the typical 10% who carry the TT genotype.⁷

In conclusion, the present study shows for the first time a marked homocysteine-lowering effect of riboflavin supplementation specifically in individuals with the *MTHFR* 677 TT genotype. Our findings have important implications for emerging food fortification policies aimed at the primary prevention of disease. In countries now considering mandatory folic acid fortification similar to that introduced in 1998 by the US Food and Drug Administration, consideration should be given to the addition of riboflavin to cover the specific requirements of substantial proportions of healthy populations that are homozygous for this polymorphism. Further studies should confirm whether riboflavin status can modulate the genetic risk for cardiovascular and other diseases linked to this polymorphism. However, the genotype-specific effect of riboflavin intervention on homocysteine levels shown here may partly explain why the *MTHFR* 677C→T polymorphism carries an increased risk of CHD in Europe but not in North America where riboflavin fortification has existed for >50 years.

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Disclosures

None.

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