Riboflavin lowers blood pressure in cardiovascular disease patients homozygous for the 677C→T polymorphism in *MTHFR*

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Objective **The purpose was to examine the effect of intervention with riboflavin (a cofactor for MTHFR) on blood pressure in patients homozygous (TT genotype) for the common 677C→T polymorphism in MTHFR.**

Methods **We investigated 197 premature cardiovascular disease patients, prescreened for the *MTHFR* 677C→T polymorphism, from an original cohort of 404 to select those with the TT genotype ($n = 60$) and a similar number with heterozygous (CT; $n = 85$) or wild-type (CC; $n = 75$) genotypes. Of these, 181 completed an intervention in which participants were randomized within each genotype group to receive 1.6 mg per day riboflavin or placebo for 16 weeks.**

Results **Among patients taking one or more antihypertensive drugs at recruitment (82%), we observed that target blood pressure (<140/90 mmHg) had been achieved in only 37% patients with the TT genotype compared with 59% with the CT and 64% with the CC genotype ($P < 0.001$). Riboflavin intervention reduced mean blood pressure specifically in those with the TT genotype (from 144/87 to 131/80 mmHg; $P < 0.05$ systolic; $P < 0.05$ diastolic), with no response observed in the other genotype groups.**

Conclusion **Riboflavin is effective in reducing blood pressure specifically in patients with the MTHFR 677 TT genotype. The findings, if confirmed, may have important implications for the prevention and treatment of hypertension. *J Hypertens* 28:478–486 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Abbreviations: EGRac, erythrocyte glutathione reductase activity coefficient; FAD, flavin adenine dinucleotide; MTHFR, methylenetetrahydrofolate reductase; PLP, pyridoxal phosphate; tHcy, total homocysteine

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Introduction

An estimated 10% people worldwide are homozygous (TT genotype) for the common 677C→T polymorphism in the folate-metabolising enzyme MTHFR, but the frequency can be as high as 26% (southern Italy) and 32% (Mexico) in some areas [1]. In these people, there is a reduction in MTHFR activity *in vivo*, which has been shown in molecular studies to be a result of the variant enzyme having a lower affinity for its riboflavin cofactor [i.e. flavin adenine dinucleotide (FAD)] [2]. Because MTHFR is required for the formation of 5-methyltetrahydrofolate needed to convert homocysteine to methionine, the most obvious manifestation of this polymorphism is an elevation in plasma homocysteine (tHcy) concentration [3], a phenotype that is particularly pronounced if folate status is low [4]. Apart from folate, recent studies show that tHcy levels in those with the TT genotype are highly sensitive to riboflavin status [5–7]. Enhancing riboflavin appears to stabilize the variant form of the enzyme [2] and results in marked lowering in tHcy, specifically in people with the TT genotype [8].

The question as to whether the *MTHFR* 677C→T polymorphism is associated with a higher risk of CVD was investigated by three recent meta-analyses involving over 25 000 cases, which showed an overall significantly higher (14 to 21%) CVD risk in people with, compared to those without, this polymorphism [9–11]. The trend toward increasing risk of CVD among individuals with none, one or two copies of the variant gene is consistent with the increasing gradient in tHcy typically found among the three genotypes. Thus, the results of these meta-analyses suggest a causal relationship between tHcy and CVD, which one recent meta-analysis of clinical trials has identified for stroke [12], but which some original reports of secondary trials of homocysteine-lowering had failed to confirm for CVD events generally [13–15]. However, analysis of the odds ratios among countries shows a large geographical variation in the association between this polymorphism and CVD risk, an inconsistency generally assumed to be the result of differences in folate status [10,11]. Riboflavin, the cofactor for MTHFR, is often overlooked as a potential modulator of the CVD risk

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associated with this polymorphism. On the basis of the marked genotype-specific decrease in tHcy with riboflavin intervention [8], it could be predicted that people with the TT genotype who also have low riboflavin status would have an excess risk of CVD, whereas those with optimal riboflavin status would not carry the expected risk. No study, however, has addressed the relationship between the *MTHFR* 677C→T polymorphism and riboflavin in relation to either CVD or a major CVD risk factor.

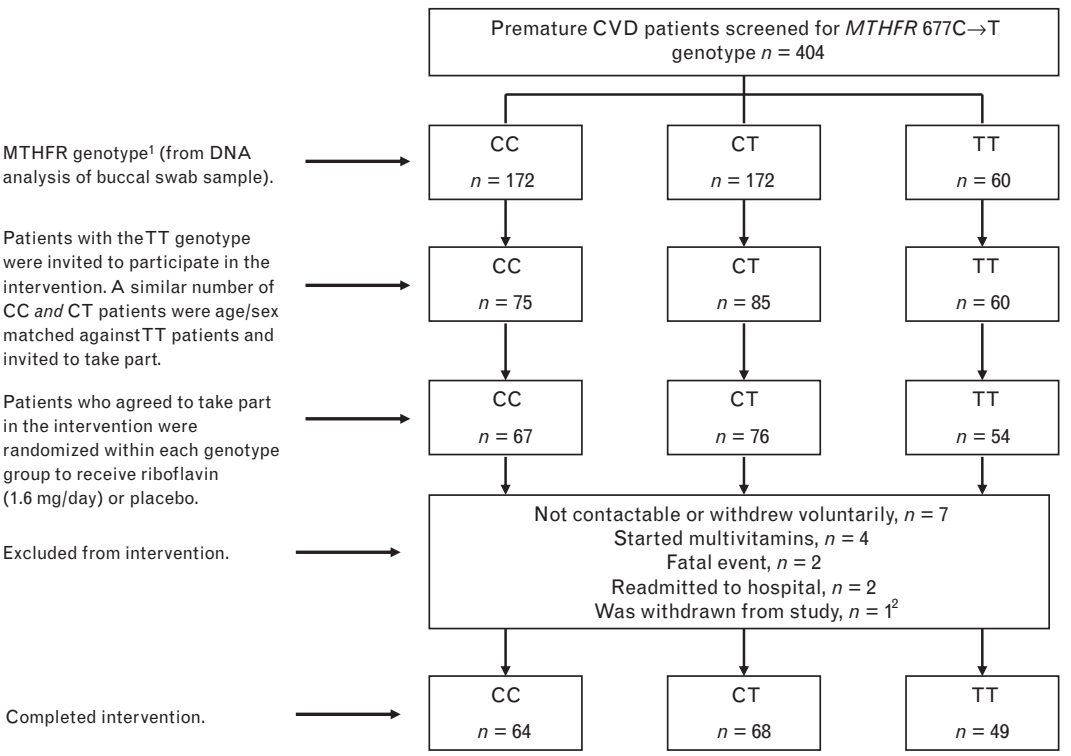
The association of this polymorphism with blood pressure has previously been examined with inconsistent results [16–20]. A recent meta-analysis of 26 studies in Asian and white populations reported a significant association between the polymorphism and hypertension, but again noted a large degree of heterogeneity between studies, which was most likely explained by ethnicity [21]. Given the genotype-specific effect of riboflavin shown in our previous intervention study [8], we hypothesized that riboflavin status might also have a role in modulating the elevated blood pressure reported in people with the TT genotype. In this study, we examined whether riboflavin status was a determinant of blood pressure in patients with the TT genotype and whether improving the status of riboflavin would correct any genotype-related elevation in blood pressure.

Methods

Study sample

We recruited premature CVD patients (men aged 55 years and women aged 65 years and under at the time of event) from the Cardiology Unit at Altnagelvin Area Hospital, Western Health and Social Care Trust, Northern Ireland, between May 2003 and March 2005. CVD was identified by a previous myocardial infarction diagnosed on the basis of ECG changes or angina diagnosed by an exercise stress test. Potential participants ($n = 404$; Fig. 1) were screened for *MTHFR* genotype from DNA analysis of a buccal swab sample. Of those identified as having the TT genotype, 54 agreed to participate in the intervention. Those with the TT genotype were age and sex matched with a similar number of patients with the CC or CT genotypes, resulting in a total of 197 who proceeded to the intervention. Exclusion criteria were history of gastrointestinal, hepatic or renal disease, consumers of B-vitamin supplements, use of medication known to interfere with B-vitamin metabolism (e.g. methotrexate, sulfasalazine, anticonvulsants), less than 3 months since myocardial infarction. Ethical approval was granted by

Fig. 1



Flow diagram of study design and completion rates. 1, CC (wild-type), CT (heterozygous) and TT (homozygous) genotypes for the *MTHFR* 677 C→T polymorphism. 2, One patient had a markedly elevated homocysteine value at baseline (130.4 μmol/l), suggesting a metabolic disorder; the patient's physician was notified and the patient was removed from the current investigation.

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both the Research Ethical Committee of the University of Ulster and Altnagelvin Area Hospital. All patients provided written informed consent and completed a health and medical/lifestyle questionnaire.

Study design

The study was a 16-week placebo-controlled double-blind randomized controlled trial. No previous study had examined blood pressure response to riboflavin and the only riboflavin intervention conducted specifically in people with the *MTHFR* 677 TT genotype was a study of tHcy response completed at our centre [8]. Thus, power calculations for the current study were based on the tHcy response to riboflavin in the TT genotype group in our earlier study [8] and estimated a sample size of 60 per genotype group. Patients were stratified within each of the three genotype groups on the basis of their initial plasma tHcy and subsequently randomized within each stratum to receive either riboflavin (1.6 mg per day) or placebo. In order to maximize compliance, patients were provided with supplements every 4 weeks in 7-day pill boxes and asked to return any unused pills, which were then recorded. Medication usage was recorded at recruitment and remained unchanged throughout the intervention period. Blood pressure was measured and nonfasting blood samples were collected before and after intervention at Altnagelvin Area Hospital, or workplace or home of the patient.

Procedures

Blood samples were analysed for plasma total homocysteine [22], red cell folate [23], serum vitamin B12 [24] and plasma pyridoxal phosphate (PLP, vitamin B6) [25]. Riboflavin status was determined by erythrocyte glutathione reductase activity coefficient (EGRac), a functional assay that measures the activity of glutathione reductase before and after in-vitro reactivation with its prosthetic group FAD [26]; EGRac is calculated as a ratio of FAD-stimulated and un-stimulated enzyme activity, with values at or above 1.3 generally indicative of suboptimal riboflavin status. Sample preparation and fractionation were performed within 0.5–2.5 h of the time of sampling and fractions were stored at -70°C for batch analysis at the end of the study. For all assays, samples were analysed blind, in duplicate and within 1 year of collection. Quality control for the various assays was provided by repeated analysis of stored batches of pooled samples covering a wide range of values. Standard clinical laboratory tests were used for coagulation screening and lipid profiles at the Department of Pathology, Altnagelvin Area Hospital.

Blood pressure measurements were recorded at baseline and post-intervention by the same researcher blinded to the genotype and treatment status of the patient. At each time point, two separate measurements were taken 15 min apart, with the patient at rest (10–15 min) in the sitting position, using an Omron 705CP electronic blood pressure monitor (Medisave, Dorset, UK). Weight (kg), height (m), BMI (weight (kg)/height^2 (m)) and waist circumference (cm) were also recorded.

Dietary evaluation

Dietary intakes at baseline were assessed by means of a 4-day food diary. Patients were given detailed advice, both written and oral, on how to complete the food diaries and were asked to do so over two weekdays and two weekend days. Portion sizes were estimated using household measurements and subsequently quantified using published food portion sizes. The food diaries were then analysed for daily nutrient intakes using the dietary analysis programme WISP (version 1.28; Tinuviel Software, Warrington, UK).

Statistical analysis

All statistical analysis was performed using the SPSS statistical package for the social sciences (version 15.0; SPSS UK Ltd, Chertsey, UK). One-way ANOVA with Tukey posthoc test was used to compare baseline characteristics among the genotype groups. Categorical data were assessed using χ^2-tests. The response to riboflavin intervention was examined by conducting a within–between repeated measures ANOVA for both systolic and diastolic blood pressures (mmHg). The between-patient factors were genotype (CC vs. CT vs. TT) and intervention group (placebo vs. riboflavin) with time (before and after) as the within-patient factor. Adjustments for multiple comparisons using Bonferroni were incorporated in the analyses. Posthoc independent *t*-tests were carried out to determine significant differences between groups for interaction effects. In all analyses, P values less than 0.05 were considered significant.

Prior to hypothesis testing in relation to the response to riboflavin intervention, the distributions for all variables were examined for violations to assumptions of normality. Outliers were found in the systolic analyses (CC group $n=1$; TT group $n=1$) and diastolic analyses (CC group $n=1$; TT group $n=1$). Owing to extreme data points and nonrepresentativeness of the sample, these outliers were eliminated from the analyses. Tests for skewness revealed departure from normality for both systolic and diastolic blood pressures. Data for these variables were transformed using logarithmic (SQRT) transformations [27]. Subsequent analyses using transformed and untransformed data produced comparable results; therefore, untransformed scores were used in all analyses.

Results

Baseline results

All study participants were white. The CC, CT and TT genotype groups were comparable for baseline characteristics (Table 1). Seventy-five percent of patients were men and 68% had a family history of CVD. In total, 82%

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Table 1 Baseline characteristics of premature cardiovascular disease patients prescreened to select those with the TT genotype

	MTHFR 677C→T genotype			
	CC ($n=67$)	CT ($n=76$)	TT ($n=54$)	P^a
Age now (years)	53.4 (6.1)	52.6 (5.0)	54.0 (6.4)	0.382
Age at time of event (years)	47.1 (5.6)	46.9 (5.4)	47.1 (7.5)	0.891
Male (%)	78.1	69.7	78.2	0.303
Family history of CVD (%)	68.7	65.8	70.4	0.763
Current smoker (%)	31.3	27.6	38.9	0.209
BMI (kg/m^2)	29.3 (4.8)	28.7 (4.5)	29.3 (4.5)	0.724
Waist circumference (cm)	96.3 (11.9)	94.2 (11.4)	95.0 (13.4)	0.625
Prothrombin time (s)	13.7 (2.8)	14.1 (3.4)	13.6 (2.3)	0.700
Fibrinogen concentration (g/l)	3.93 (0.84)	3.97 (1.05)	3.88 (0.83)	0.853
Total cholesterol (mmol/l)[b]	4.4 (0.81)	4.5 (0.8)	4.5 (1.0)	0.849
Blood pressure[c]				
Systolic blood pressure (mmHg)	131.1 (18.0)[a]	133.0 (19.7)[a]	142.8 (19.5)[b]	0.002
Diastolic blood pressure (mmHg)	80.3 (12.5)[a]	83.3 (11.5)[a,b]	86.0 (12.3)[b]	0.038
B-vitamin status				
Plasma homocysteine (μmol/l)	9.8 (3.3)[a]	10.5 (3.9)[a,b]	12.8 (8.0)[b]	0.007
EGRac[d]	1.37 (0.17)	1.39 (0.22)	1.37 (0.19)	0.854
Red cell folate (nmol/l)	1030 (518)[a]	1011 (400)[a]	796 (408)[b]	0.009
Serum vitamin B12 (pmol/l)	328 (192)	276 (125)	282 (179)	0.146
Plasma pyridoxal phosphate (nmol/l) (vitamin B6)	68.0 (35.0)[a]	63.2 (40.4)[a,b]	49.4 (32.0)[b]	0.017

Data are expressed as mean (SD) unless otherwise indicated. CVD, cardiovascular disease. [a] Statistical significance for comparison between genotype groups by the χ^2-test or one-way ANOVA or as appropriate. Values in a row with different letters indicate significant difference ($P<0.05$) by a Tukey posthoc test. [b] 90% of patients were taking lipid-lowering medication. [c] 82% of patients were taking antihypertensive medication. [d] EGRac, Erythrocyte glutathione reductase activation coefficient (biomarker of riboflavin status).

of the patients were taking one or more antihypertensive drugs. At baseline, mean systolic blood pressure was found to be 12 mmHg higher in patients with the TT compared with those with the CC genotype, whereas the difference between CT and CC groups was not significant. In the case of diastolic blood pressure, there was a mean difference of almost 6 mmHg between CC and TT groups; the value observed for the CT genotype was intermediate relative to the other two genotypes. When we examined B-vitamin status at baseline, folate status, as expected, was significantly lower and correspondingly tHcy significantly higher in patients with the TT compared with those with other genotypes. The TT genotype group was also found to have significantly lower biomarker status of vitamin B6 (PLP), whereas no genotype differences were observed in vitamin B12 or riboflavin status (i.e. EGRac).

In Tables 2 and 3, we examine whether there were differences in the effectiveness of antihypertensive drugs according to MTHFR genotype among those patients (82% of the total sample) taking one or more antihypertensive drug on recruitment. There were no significant differences in drug usage among the MTHFR genotype groups for the three most predominant drug categories (which accounted for 74% of drug usage): beta-blockers only (53% CC, 46% CT, 52% TT), angiotensin-converting enzyme (ACE) inhibitors only (25, 19, 29%), beta-blockers + ACE inhibitors (22, 35, 19%), ($P=0.478$, χ^2-test) (Table 3). We calculated the percentage of patients in each genotype group who had achieved goal blood pressure on treatment (i.e. blood pressure <140/90 mmHg; JNC 7) [27]. Far fewer patients with the TT genotype (37%) had managed to achieve this target compared with patients with CT (59%) or CC (64%) genotypes.

We then examined whether riboflavin status had a role in determining blood pressure among the genotype groups at baseline (Table 4). Using the median value for the

Table 2 Achievement of goal blood pressure at baseline in those patients being treated for hypertension[a] according to *MTHFR* 677C→T genotype[b]

	MTHFR 677C→T genotype			
	CC ($n=57$)	CT ($n=63$)	TT ($n=43$)	P^c
Systolic blood pressure (mmHg)	132.7 (18.0)[a]	133.1 (20.1)[a]	145.5 (19.6)[b]	0.001
Diastolic blood pressure (mmHg)	81.5 (12.6)[a]	83.6 (12.1)[a,b]	87.9 (12.3)[b]	0.038
Hypertension (%)[d]	36[a]	41[b]	63[c]	<0.001

[a] 82% of the overall sample were taking one or more routine antihypertensive drug (angiotensin-converting enzyme inhibitor, calcium-channel blocker, beta-blocker or diuretic) on recruitment (see Table 3 for details). [b] Data are expressed as mean (SD) unless otherwise indicated. Individual blood pressure values are based on the mean of two separate measurements taken 15 min apart. [c] Statistical significance for comparison between genotype groups by one-way ANOVA. Values in a row with different letters indicate significant difference ($P<0.05$) by a Tukey posthoc test. The χ^2-test was used to compare percentage hypertension among the groups. [d] Hypertension (stage 1 or stage 2) defined as systolic blood pressure more than 140 mmHg or diastolic blood pressure more than 90 mmHg. The treatment of hypertension is aimed at achieving the goal blood pressure of less than 140/90 mmHg (JNC 7, seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure) [28].

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Table 3 Antihypertensive drugs being taken by patients at time of sampling

Drug treatment	Percentage of patients
Beta-blockers only	38
ACE inhibitors only	18
Calcium-channel blockers only	6
Diuretics only	3
ACE inhibitors + beta-blockers	18
ACE inhibitors + diuretics	4
ACE inhibitors + calcium-channel blockers	1
Beta-blockers + calcium-channel blockers	2
Beta-blockers + diuretics	6
Calcium-channel blockers + diuretics	0.6
ACE inhibitors + calcium-channel blockers + diuretics	0.6
Beta-blockers + calcium-channel blockers + diuretics	0.6

There was no significant differences in drug usage among the MTHFR genotype groups for the three most predominant drug categories (which accounted for 74% of drug usage): beta-blockers only (53% CC, 46% CT, 52% TT); ACE inhibitors only (25, 19, 29%); beta-blockers + ACE inhibitors (22, 35, 19%); ($P = 0.478$, χ^2-test). ACE, angiotensin-converting enzyme.

biomarker of riboflavin status (i.e. EGRac) as a cut-off, we arbitrarily categorized patients in each genotype group into those with lower or higher riboflavin status. Patients with the combination of the TT genotype and lower riboflavin status at baseline were found to have a mean systolic blood pressure that was 16 mmHg higher than in CC patients; the CT group showed intermediate values that were not significantly different from those of the CC group. In contrast, in patients with higher riboflavin status, systolic blood pressure was not significantly elevated in the TT compared with other genotypes. Riboflavin status also appeared to be a determinant of diastolic blood pressure among the three genotypes. Apart from riboflavin, we observed no interactive effect on blood pressure at baseline between this polymorphism and the biomarker status of any other B-vitamin or tHcy.

Response to riboflavin intervention

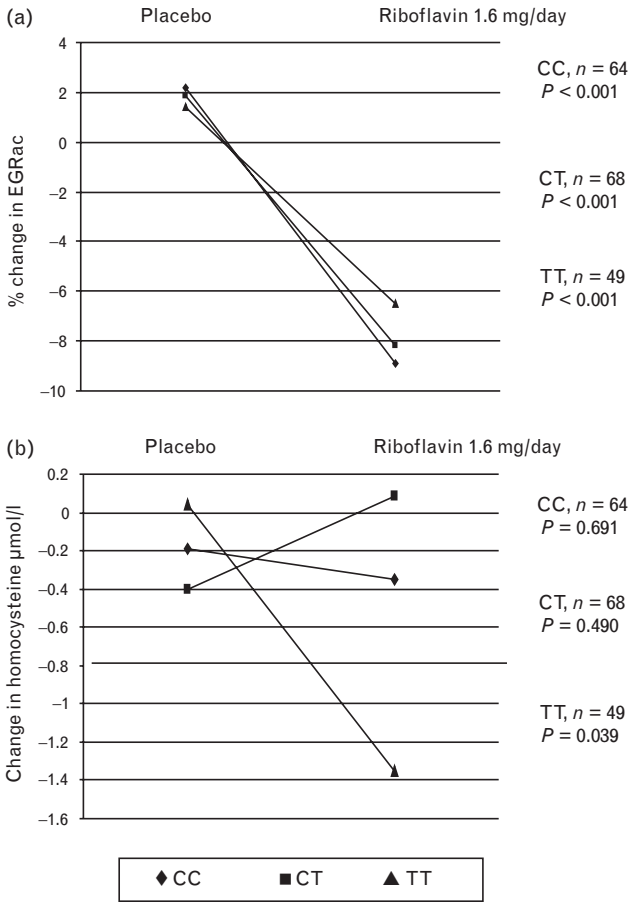
Of the 197 who started intervention (1.6 mg per day riboflavin for 16 weeks), 181 completed it (Fig. 1). Compliance (as determined by pill counts) was found to be excellent (>99%) and similar among treatment and genotype groups. There was a significant response to riboflavin treatment in all genotype groups, as indicated by a significant decrease in the riboflavin biomarker EGRac (Fig. 2). In contrast, tHcy showed a significant response to intervention only in the TT genotype group, with no response observed in either the CT or CC genotype groups (Fig. 2).

Table 4 Influence of the *MTHFR* 677C→T polymorphism on blood pressure at baseline by status of riboflavin

	MTHFR 677C→T genotype			
	CC ($n = 67$)	CT ($n = 76$)	TT ($n = 54$)	P^a
Systolic blood pressure (mmHg)				
Total	131.1 (18.0)[a]	133.0 (19.7)[a]	142.8 (19.5)[b]	0.002
Lower riboflavin[b]	131.2 (20.4)[a]	135.8 (19.0)[a]	147.4 (19.8)[b]	0.005
Higher riboflavin	131.0 (14.8)	129.6 (20.4)	138.6 (19.2)	0.172
Diastolic blood pressure (mmHg)				
Total	80.3 (12.5)[a]	83.3 (11.5)[a,b]	86.0 (12.3)[b]	0.038
Lower riboflavin	80.8 (13.5)	84.6 (11.8)	88.1 (12.7)	0.076
Higher riboflavin	79.6 (11.2)	81.9 (11.1)	84.1 (12.2)	0.381

Data are expressed as mean (SD). Individual blood pressure values are based on the mean of two separate measurements taken 15 min apart. [a] Statistical significance for comparison between genotype groups by one-way ANOVA. Values in a row with different letters indicate significant difference ($P < 0.05$) by a Tukey posthoc test. [b] 'Lower' and 'higher' riboflavin categories were established arbitrarily by splitting each genotype group in two using the median value for the biomarker of riboflavin status erythrocyte glutathione reductase activity coefficient.

Fig. 2



Response of riboflavin status (a) and homocysteine (b) to intervention with riboflavin (1.6 mg per day) for 16 weeks. Riboflavin status was measured as erythrocyte glutathione reductase activation coefficient (EGRac), a functional assay for riboflavin, with higher values indicating lower riboflavin status. *P* values refer to independent *t*-tests within each genotype group to compare response to intervention between treatment and placebo.

The corresponding blood pressure response is shown in Table 5. For systolic blood pressure, there was a time × genotype interaction (mmHg) [$F_{(2173)} = 5.14$, $P = 0.007$], which was influenced by treatment to produce a three-way interaction (time × genotype × treatment) [$F_{(2173)} = 3.04$, $P = 0.05$], indicating that genotype exerts an influence on the treatment response for systolic pressure. Posthoc independent *t*-tests indicated that within the TT group, systolic blood pressure decreased significantly by −13.4 (15.2) mmHg ($P = 0.02$). No significant

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Table 5 Blood pressure response to riboflavin intervention (1.6 mg per day, 16 weeks)

	MTHFR 677C→T genotype[a]					
	CC ($n=63$)		CT ($n=68$)		TT ($n=48$)	
	Placebo	Riboflavin	Placebo	Riboflavin	Placebo	Riboflavin
Systolic blood pressure (mmHg)						
Before	125.7 (20.0)	134.3 (14.9)^{NS}	133.7 (21.5)	136.3 (16.9)^{NS}	143.8 (18.2)	144.0 (21.8)^{NS}
After	130.0 (21.9)	133.4 (15.4)^{NS}	129.9 (21.5)	135.5 (15.4)^{NS}	141.6 (22.6)	130.7 (20.8)^{NS}
Change[b,c,d,e]	4.4 (13.6)	−0.9 (14.9)^{NS}	−3.7 (18.0)	−0.8 (12.5)^{NS}	−2.2 (18.5)	−13.4 (15.2)*
Diastolic blood pressure (mmHg)						
Before	78.1 (13.3)	80.1 (8.9)^{NS}	82.0 (12.1)	86.0 (10.9)^{NS}	84.5 (10.7)	87.1 (13.6)^{NS}
After	81.8 (15.1)	80.8 (15.1)^{NS}	81.9 (14.3)	84.2 (9.3)^{NS}	85.4 (12.8)	79.5 (10.6)^{NS}
Change[b,d,f]	3.7 (9.9)	0.03 (11.7)^{NS}	−0.8 (10.7)	−0.09 (10.8)^{NS}	0.9 (13.3)	−7.5 (11.1)*

Data are expressed as mean (SD). At each time-point, individual blood pressure values were based on the mean of two separate measurements taken 15 min apart. NS, not significant. [a] Patients were randomized within each genotype group to treatment or placebo. [b] Probability values refer to interactions obtained from a two-way repeated measures ANOVA ($P \leq 0.05$; with Bonferroni adjustments for multiple comparisons). [c] Significant time effect. [d] Significant time × genotype interaction. [e] Significant time × genotype × treatment interaction. [f] Significant time × treatment interaction. * Posthoc independent *t*-tests were used to compare placebo and riboflavin within each genotype group.

response in systolic blood pressure, however, was observed in patients with CC or CT genotypes. For diastolic blood pressure, there was an interaction between time × genogenotype [$F_{(2173)}=3.16$, $P=0.045$] and an interaction between time × treatment [$F_{(1173)}=7.11$, $P=0.008$]. However, there was no interaction between the three factors (i.e. time × genotype × treatment). Posthoc independent *t*-tests indicated that within the TT group, diastolic blood pressure decreased significantly by −7.5 (11.1) mmHg ($P=0.02$), but no significant response in diastolic blood pressure was observed in patients with CC or CT genotypes. These results were observed despite the fact that the majority of patients (82% overall and 81% of the TT genotype group) were taking one or more antihypertensive drugs at the time of intervention. When we repeated the analysis this time including only those patients taking antihypertensive drugs, the results were similar, showing a lowering of both diastolic (by 9 mmHg, $P=0.011$) and systolic (by 10 mmHg, $P=0.096$) blood pressure in response to riboflavin (results not shown).

In the subsample of participants (38%) who provided dietary information, mean (SD) dietary riboflavin intakes compared favourably with the value of 1.1–1.8 mg per day currently recommended worldwide and were similar among the three genotypes groups: CC, 1.63 (0.78) mg per day; CT, 1.51 (0.52) mg per day; TT, 1.55 (0.68) mg per day.

Discussion

Previous studies have found an association between the 677C→T polymorphism in *MTHFR* and hypertension [16–18,21]. We examined whether riboflavin has a role in this relationship because it is known to have an important modulating effect on the typical (elevated homocysteine) phenotype found in people with the homozygous mutant TT genotype [8].

At baseline, we observed significantly higher blood pressure in patients with the TT genotype compared with those with the CC genotype. Furthermore, among the patients being treated with one or more antihypertensive agents, 63% with the TT genotype were found to be clinically hypertensive on treatment and failed to achieve goal blood pressure (<140/90 mmHg) [28]. This was almost twice the prevalence of hypertension (36%) observed in patients with the CC genotype, suggesting that the predicted therapeutic effect (from clinical trials) of a given antihypertensive drug is less likely to be achieved in patients with this genetic factor. In our study, the TT genotype group was overrepresented (by design) in order to get comparable patient numbers across the three genotype groups and we may, therefore, have observed more marked effects than reported by previous studies that also found higher blood pressure among patients with the TT genotype [16–18].

The major focus of this study was to examine the role of riboflavin in determining blood pressure in patients with the TT genotype. The baseline data indicated that the previously reported genetic predisposition to hypertension appeared to be sensitive to riboflavin status. By arbitrarily splitting the sample on the basis of higher and lower riboflavin status at baseline (using the median value for the riboflavin biomarker EGRac), we observed that the elevated blood pressure in the TT genotype group compared with the other genotypes was marked in those with lower riboflavin status. Among those with higher riboflavin status, the trend towards higher blood pressure in the TT genotype was not significant. Of greater importance, we demonstrated that intervention to increase riboflavin status resulted in reducing systolic blood pressure by 13 mmHg and diastolic blood pressure by almost 8 mmHg, specifically in patients with the TT genotype to values similar to those in the CC genotype group. It is known that there is a continuous relationship between blood pressure and CVD risk across a wide range of values [29]. The extent of lowering of diastolic blood pressure we observed in the TT genotype group in response to riboflavin can be estimated from a

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meta-analysis to correspond to a lowering of the risk of heart disease by 29% and stroke by 46% [30]. Furthermore, one-third of the TT patient group achieved a 20 mmHg or greater reduction in systolic blood pressure with riboflavin treatment, a magnitude of change previously associated with at least a two-fold difference in death rates from stroke, ischaemic heart disease and other vascular causes in a meta-analysis of 1 million adults [31]. Of note, this response was achieved with a riboflavin level of only 1.6 mg per day, equivalent to the dietary reference intake value. Thus, a diet that provides a higher status of riboflavin, or intervention with low dose riboflavin as a supplement, would appear to negate this apparent genetic predisposition to hypertension.

Neither the mechanism explaining the effect of riboflavin on blood pressure in patients with the TT genotype is clear nor whether this effect is independent of tHcy. Elevated blood pressure has been associated with higher tHcy in several studies [32–35]; however, other observational studies have found no association [36,37]. We previously showed that riboflavin (1.6 mg per day, as used in the current study) lowers tHcy in healthy adults with the TT genotype but not in those with CT or CC genotypes [8]. In the current study, we again show a genotype-specific lowering effect of riboflavin on tHcy, this time in premature CVD patients. Our current and earlier findings indicate that riboflavin can correct the function of the flawed MTHFR enzyme in hepatocytes, the major homocysteine-metabolizing cells. It is likely that with suboptimal riboflavin, this genotype would lead to impaired function of the enzyme not only in hepatocytes but also in all cells including those of the arterial system, which, in turn, might result in decreased vascular function leading to hypertension. Thus, riboflavin may interact with MTHFR to influence blood pressure independently of tHcy, and elevated plasma tHcy may merely be a marker of impaired MTHFR activity in cells rather than being causatively implicated in hypertension. In support of this view, two recent studies showed that despite a marked homocysteine-lowering response to high-dose B-vitamin supplementation (folic acid, vitamin B12, vitamin B6, but not riboflavin) for 2 years, there was no corresponding lowering of blood pressure in healthy older adults [38] or in stroke patients in the Vitamin Intervention for Stroke Prevention (VISP) trial [13]. In contrast, two other intervention trials reported small but significant lowering of blood pressure in response to combined high-dose folic acid and vitamin B6 for 2 years [39] or high-dose folic acid alone for 4 weeks in smokers [40]. Another small study found modest reductions in pulse pressure and large artery stiffness with folic acid supplementation for 3 weeks and concluded (despite very small numbers) that this was independent of *MTHFR* genotype [41]. Furthermore, a large prospective cohort study found that higher folate intake was associated with a decreased risk of incident hypertension, particularly in younger women [42]. However, no previous intervention study examined the potential of B-vitamins to lower blood pressure in participants preselected for the TT genotype and none showed the extent of reduction in blood pressure demonstrated here. Apart from riboflavin, we observed no interactive effect on blood pressure at baseline between this polymorphism and the biomarker status of any other B-vitamin or tHcy.

There may be important implications of our findings, if confirmed, for the 10% of the general population, and the higher proportions of some ethnic groups, who carry the TT genotype [1]. Furthermore, although only patients with the TT genotype showed a significant blood pressure response to riboflavin, it is possible that a response could be achieved in CT patients (which our intervention failed to demonstrate), given that the CT group at baseline showed blood pressure levels and rates of hypertension that were intermediate compared with the other genotypes. Such an effect would be predicted to be much smaller than that shown in the TT genotype, but potentially important at a population level, given that over 40% of people worldwide have the CT genotype [1]. Our results suggest the potential for the primary prevention of hypertension by increasing riboflavin intakes of individuals (with supplements or riboflavin-fortified foods) or populations (with mandatory fortification). Any expected benefit would of course be greatest for the many populations worldwide with no riboflavin fortification policies and some with evidence of generalized low biomarker status of riboflavin [43], but there may be some smaller benefit in North American populations, as current fortification levels may not necessarily be sufficient to optimize MTHFR enzyme activity in all people with this polymorphism. However, the more immediate issue suggested by this study is that the response to blood pressure treatment may be improved by riboflavin administration in a substantial proportion of patients intractable to current antihypertensive drugs, with potential savings in healthcare costs. Finally, our findings raise the possibility that the previously unrecognized modulating effect of riboflavin on blood pressure in those with the TT genotype shown here may help to explain the large geographical variation in the extent of CVD risk generally associated with the *MTHFR* 677C→T polymorphism. Of note, the excess risk of CVD associated with this polymorphism was not found to be significant in the United States [10,11], where mandatory riboflavin fortification has existed for over 50 years. In other countries without fortification, however, riboflavin intakes are generally much lower and more variable depending on food habits, consistent with the finding that this polymorphism carries a significantly increased but variable risk of CVD outside of the United States [10,11].

In conclusion, we confirm the findings of other reports that the *MTHFR* 677TT genotype is associated with

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hypertension and report for the first time that patients with this genotype appear to be more resistant to routine antihypertensive therapy. The most important finding is that the higher blood pressure in patients with this common genetic variant is responsive to low-dose riboflavin. The findings if confirmed will have important implications for the prevention and treatment of hypertension, with potential savings in healthcare costs.

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There are no other conflicts of interest.

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