

# Retinal Vein Occlusion, Homocysteine, and Methylene Tetrahydrofolate Reductase Genotype

Stuart J. McGimpsey,<sup>1</sup> Jayne V. Woodside,<sup>2</sup> Louise Bamford,<sup>1</sup> Sarah E. C. M. Gilchrist,<sup>2</sup> Ryan Graydon,<sup>2</sup> Gareth C. McKeeman,<sup>2</sup> Ian S. Young,<sup>2</sup> Anne E. Hughes,<sup>3</sup> Chris C. Patterson,<sup>4</sup> Dermot O'Reilly,<sup>4</sup> David McGibbon,<sup>5</sup> and Usha Chakravarthy<sup>6</sup>

**PURPOSE.** The aim of this case-control study was to investigate the relationship between homocysteine (tHcy), 5,10 methylene tetrahydrofolate reductase (MTHFR) C677T genotype, folate and vitamin B12 status, and retinal vein occlusion (RVO).

**METHODS.** Subjects with RVO ( $n = 106$ ) were recruited from outpatient and inpatient sources. Controls ( $n = 98$ ) were selected to achieve a similar age and sex distribution. Full ocular examination was performed and medical history was taken for each study participant. Plasma and serum samples were analyzed for tHcy level and folate and vitamin B12 status, and extracted DNA was assessed for the MTHFR C677T genotype.

**RESULTS.** There was no significant difference in plasma tHcy level or thermolabile MTHFR allele frequency between subjects and controls. Similarly, there was no significant difference in folate or vitamin B12 status between subjects and controls. MTHFR genotype did not affect folate or vitamin B12 concentrations in subjects or controls. However, tHcy was significantly higher in thermolabile homozygotes than in nonthermolabile homozygotes (ratio of geometric means, 1.35; 95% confidence interval [CI], 1.04–1.74;  $P = 0.024$ ).

**CONCLUSIONS.** Hyperhomocysteinemia, the MTHFR C677T mutation, and folate and vitamin B12 status are not important risk factors for RVO in this population. (*Invest Ophthalmol Vis Sci.* 2005;46:4712–4716) DOI:10.1167/iovs.04-1229

It is generally accepted that a mildly elevated homocysteine level is a risk factor for atherosclerosis.<sup>1</sup> Similarly, several studies have suggested elevated homocysteine level to be a potential risk factor in retinal vascular occlusive disease.<sup>2–5</sup> However, not all studies demonstrate a relationship between elevated plasma homocysteine (tHcy) and retinal vascular occlusive disease.<sup>6–8</sup>

Homocysteine, a sulfur-containing amino acid, is an intermediary product in methionine metabolism. It is metabolized

by two major pathways. When methionine is in excess, homocysteine follows the transsulfuration pathway, where it is irreversibly conjugated to serine by cystathionine  $\beta$ -synthase in a process requiring vitamin B6 as a cofactor. Under conditions of low methionine, homocysteine is primarily metabolized through the methionine-conserving remethylation pathway. In most tissues, homocysteine is remethylated in a process requiring methionine synthase, vitamin B12 as cofactor, and methyltetrahydrofolate as cosubstrate. The pathway requires the enzyme methylene tetrahydrofolate reductase (MTHFR) and an adequate supply of folic acid. Genetic and acquired abnormalities in the function of any of these enzymes or deficiencies in folic acid, vitamin B6, or vitamin B12 cofactors can lead to elevated total plasma homocysteine (tHcy) levels.<sup>9</sup> One important cause of elevated plasma homocysteine level is a polymorphism in the *MTHFR* gene that is common in Western populations. Persons homozygous for the thermolabile variant of *MTHFR* show higher plasma levels of homocysteine, particularly when serum folate levels are low.

Among northern European populations, it is recognized that northern Ireland has a higher than average incidence of cardiovascular events, and evidence indicates that abnormalities of homocysteine metabolism may be contributing factors.<sup>10</sup> A cross-sectional study in northern Ireland has found that 11.5% of males of working age are homozygous for the thermolabile allele (TT) of the *MTHFR* gene, confirming that this is a common polymorphism in this geographic region.<sup>11</sup> This study also established that the polymorphism was a major determinant of homocysteine levels at the upper end of the range. Therefore, we explored the relationship among the *MTHFR* polymorphism, tHcy, and B-group vitamin status in subjects with retinal vein occlusion (RVO) and in age- and sex-matched healthy controls.

## METHODS

### Study Design, Procedures, and Sample Size Calculations

The present study used a case-control design, recruiting participants between August 1999 and March 2004. Institutional ethics review board approval was obtained, and the study was conducted in full accord with the tenets of the Declaration of Helsinki. Each participant received a detailed information leaflet and provided informed written consent before inclusion.

Subjects with RVO were recruited from ophthalmic casualty, outpatient clinics, and inpatient sources. Controls were either patients undergoing cataract surgery who were free of retinal disease or accompanying relatives or friends of patients. Exclusion criteria for controls were history of vasculitic, inflammatory, and overt ischemic retinal diseases. All controls underwent detailed assessment of the anterior segment and the posterior segment after pupillary dilation. Controls were recruited concurrently during the subject recruitment period. Exclusion criteria for subjects and controls included age <18

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From the Departments of <sup>1</sup>Ophthalmology and <sup>3</sup>Medical Genetics, Royal Group of Hospitals, Belfast, Ireland, United Kingdom; and the Departments of <sup>2</sup>Medicine, <sup>4</sup>Epidemiology and Public Health, <sup>5</sup>Medical Genetics, and <sup>6</sup>Ophthalmology, Queen's University, Belfast, Ireland, United Kingdom.

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Corresponding author: Stuart J. McGimpsey, Department of Medicine, Royal Group of Hospitals, Mulhouse Building, Grosvenor Road, Belfast BT12 6BJ, UK; stuart@mcgimpsey.fsworld.co.uk.

years, inability to give consent, and current medication with vitamin B12, B6, or folic acid.

Previous studies of tHcy and RVO have been relatively small, and it was considered important that this study be adequately powered. Sample size calculation was based on the variation noted in plasma homocysteine in a pilot study in a similar population. It was calculated that 103 patients in each group would be necessary to detect with 90% power a difference between subjects and controls on the log scale that was equivalent to a 20% difference in tHcy levels on the original scale.

Two hundred four subjects gave consent and entered the study (106 controls, 98 subjects). Acute RVO was defined as that occurring within 3 months of recruitment; chronic RVO was classified as that lasting  $\geq 3$  months.

A systematic data collection procedure was used during which age, sex, and smoking status were recorded. Medical conditions, including diabetes, cardiovascular status (presence of angina, atrial fibrillation, myocardial infarction, transient ischemic attacks, stroke, deep vein thrombosis, pulmonary embolism), relevant drug history (use of aspirin, warfarin, vitamin supplementation), presence of blood dyscrasias, and any life-threatening disorder were also recorded. Subjects were considered to have hypertension if they had already been treated with antihypertension drugs or if their blood pressure was  $>140$  mm Hg systolic or  $>90$  mm Hg diastolic (as defined by the World Health Organization-International Society of Hypertension).

For subjects, the duration of visual symptoms, ocular medication, and ocular history were noted. A full ophthalmic examination of both eyes was carried out, and distance visual acuity, intraocular pressure, clarity of the fundal media, and morphologic details of vein occlusion (central or branch) were recorded.

A blood sample (30 mL) was taken from each participant. Blood for homocysteine was collected in an EDTA tube, transported on ice, and centrifuged at 4°C within 45 minutes of collection. A clotted sample was kept in the dark for 1 hour before centrifugation for the analysis of vitamin B12 and folate. Plasma and serum samples were stored at -80°C until analysis. Samples were coded for blinded analysis.

## Laboratory Methods

tHcy was analyzed by high-performance liquid chromatography with fluorometric detection according to Ubbink et al.<sup>12</sup> Quality control samples were run daily. At a plasma homocysteine concentration of 7.87  $\mu\text{M}$ , the interassay coefficient of variation (CV) was 6.8%, whereas the intra-assay CV was 2.6%. Serum vitamin B12 and folate levels were measured by radioassay using a kit from ICN Pharmaceuticals (Costa Mesa, CA). At a serum folate concentration of 7.86 ng/mL, the interassay CV was 9.6% and the intra-assay CV was 6.0%. At a serum B12 concentration of 169.6 pg/mL, the interassay CV was 9.6% and the intra-assay CV was 7.3%.

DNA was extracted from whole blood using standard methods. Forward and reverse primers for the polymorphism were synthesized, and amplification of the selected region of the *MTHFR* gene was performed by PCR. The amplified DNA was restricted using *HinfI* and was run on polyacrylamide gels to determine genotype, as described by Frosst et al.<sup>13</sup>

## Statistical Analysis

Statistical analysis was performed on logarithmically transformed homocysteine, folate, and vitamin B12 values because all three had positively skewed distributions. Comparisons were obtained using the independent samples *t*-test and one-way and two-way analyses of variance;  $\chi^2$  tests were used to compare the characteristics of the groups, including genotype and allele distributions. Tests for departure of genotype frequencies from Hardy-Weinberg equilibrium were obtained using the  $\chi^2$  goodness-of-fit test.

TABLE 1. Demographic and Cardiovascular Risk Status of RVO Cases and Healthy Controls

	Subjects (n = 106)	Controls (n = 98)	P
Sex			
Male	55 (52)	45 (46)	0.39
Female	51 (48)	53 (54)	
Age (y)	67.9 (14.2)	68.4 (13.8)	0.21
Smoking			
Never	79 (74.5)	82 (83.7)	0.11
Current or former	27 (25.5)	16 (16.3)	
Diabetes			
No	100 (94)	85 (87)	0.06
Yes	6 (6)	13 (13)	
Hypertension			
No	54 (50.9)	73 (74.5)	0.001
Treated	52 (49.1)	25 (25.5)	
Cardiovascular history			
No	84 (79.2)	76 (77.6)	0.77
Angina/myocardial infarction	22 (20.8)	22 (22.4)	
Thrombosis/embolism			
No	98 (92.5)	91 (92.9)	0.91
Yes	8 (7.5)	7 (7.1)	
Anticoagulant medication			
No	59 (55.7)	64 (65.3)	0.16
Yes	47 (44.3)	34 (34.7)	

Data are presented as N (%), except age, which is presented as mean (SD). Groups compared using chi-square test with Yates correction, except age, which was compared using an independent samples *t*-test.

## RESULTS

Subjects and controls were well matched for age and sex. They were examined for differences using the Townsend material deprivation score, which again were found to be similar (mean difference between subjects and controls, 0.91; 95% CI, -0.09-1.19;  $P = \text{NS}$ ). Demographic details of subjects and controls are shown in Table 1. Among subjects, 46 (43%) had branch vein occlusions, and 60 (57%) had central vein occlusions. Examination of duration of vein occlusion indicated that 47 (47%) had occurred within the last 3 months and that 52 (53%) had occurred  $>3$  months before inclusion in the study. One hundred six subjects and 98 controls were available for analysis, but blood samples were only available for 100 subjects and 91 controls for tHcy analysis, for 99 subjects and 89 controls for folate and vitamin B12 analysis, and for 103 subjects and 94 controls for MTHFR analysis.

A history of hypertension ( $P = 0.001$ ) was more frequently recorded in subjects than in controls. There was no significant difference in anticoagulant (warfarin) use between subjects and controls. The incidence of cardiovascular and cerebrovascular events was not significantly different between subjects and controls (Table 1).

## tHcy

There were no significant differences in mean plasma tHcy (geometric mean) between subjects and controls (Table 2), nor were there any statistically significant differences when subjects were categorized by type of vein occlusion (branch vein occlusion [ $n = 42$ ], 11.3  $\mu\text{M}$  [7.9-15.1  $\mu\text{M}$ ]; central vein occlusion [ $n = 57$ ], 10.4  $\mu\text{M}$  [8.3-12.7  $\mu\text{M}$ ]; controls [ $n = 91$ ], 9.5  $\mu\text{M}$  [7.3-15.2  $\mu\text{M}$ ]; geometric mean [interquartile range],  $P = 0.23$ , one-way ANOVA). Categorization on the basis of retinal ischemia also did not result in significant differences between groups (data not shown). When segregated by dura-

TABLE 2. tHcy, Folate, and Vitamin B12 Concentrations in Patients with RVO and in Healthy Controls

	Subjects, Median (range) <i>n</i>	Controls, Median (range) <i>n</i>	Ratio of Geometric Means (95% CI)	<i>P</i>
tHcy ( $\mu\text{mol/L}$ )	10.8 (8.3–14.3) 100	9.5 (6.8–14.9) 91	1.13 (0.97–1.33)	0.12
Folate (ng/mL)	6.25 (4.24–8.49) 99	6.9 (4.84–8.71) 89	0.91 (0.77–1.07)	0.24
Vitamin B12 (pg/mL)	415 (298–558) 99	372 (264–494) 89	1.11 (0.96–1.29)	0.15

Data are geometric means (interquartile range) for plasma tHcy, serum folate, and vitamin B12. Subjects and controls were compared using an independent samples *t*-test.

tion of vein occlusion, the geometric mean plasma tHcy was 10.0 (7.7–13.4)  $\mu\text{M}$  in 47 subjects with vein occlusions who presented within 3 months of onset compared with 11.6 (8.7–14.6)  $\mu\text{M}$  in the 52 subjects with occlusions who presented later ( $P = 0.12$ ).

### Serum Folate and Vitamin B12

There were no statistically significant differences between subjects and controls with respect to serum folate and vitamin B12 levels (Table 2). Folate and vitamin B12 did not differ in subjects when classified by type or duration of vein occlusion (data not shown).

### MTHFR Genotype

There was no evidence of departure from Hardy-Weinberg equilibrium in the distribution of alleles among subjects or controls. Genotype distribution did not differ significantly between subjects and controls (Table 3), and the frequency of the thermolabile (T) allele was similar in the two groups (37.4% in subjects, 35.1% in controls).

tHcy levels in the three MTHFR genotypes are compared in Table 4. Tests for interaction in the two-way analysis of variance were not significant, providing no evidence to suggest that the pattern of genotype differences in tHcy levels varied between subjects and controls. Although heterozygotes showed no elevation in tHcy status compared with nonthermolabile homozygotes (ratio of geometric means, 1.04; 95% CI, 0.88–1.23), thermolabile homozygotes showed a significant ( $P = 0.024$ ) elevation (ratio of geometric means, 1.35; 95% CI, 1.04–1.74). Neither folate nor vitamin B12 level differed by MTHFR genotype (Table 4).

## DISCUSSION

Opinion is still divided on the role of elevated plasma tHcy level on retinal vascular occlusion disease. In terms of tHcy, seven case-control studies have shown tHcy to be significantly higher in subjects than in controls,<sup>2–5,14–16</sup> whereas three others using a similar approach failed to demonstrate an association.<sup>6–8</sup> With respect to MTHFR genotype, only 3<sup>5,17,18</sup> of 10<sup>5,6,8,15–21</sup> studies have identified the presence of the ther-

molabile polymorphism as a risk factor for RVO. The present study did not identify elevated mean plasma tHcy level or homozygosity for the thermolabile T677 variant of the *MTHFR* gene as risk factors for RVO.

Age is an important confounding factor when analyzing the potential role of plasma tHcy in vascular disorders. In a large, apparently healthy population ( $N = 11,941$ ), it was shown that increasing age is correlated with elevated homocysteine level ( $r = .23$ ;  $P < 0.05$ , after adjustment for sex, smoking habit, coffee consumption, and folate status).<sup>22</sup> Although many of the studies in the literature state that subjects and controls were age-matched, the precision of matching and a statistical test of the outcome of matching are rarely presented. It is noteworthy that in two of the four studies that identified tHcy as a risk factor for RVO, controls were significantly younger than subjects.<sup>3,4</sup> In these studies, the use of controls who were on average 5 years and 14 years younger, respectively, than the subject group could account for the finding of significant differences.

Two other important determinants of tHcy are serum vitamin B12 and folate levels.<sup>2</sup> Higher plasma levels of folate and B12 counteract the tendency to elevated plasma tHcy, potentially reducing the risk for endothelial damage. Thus, there is a sound rationale for the inclusion of serum B12 and folate status in any model examining a role for plasma tHcy and MTHFR genotype in a vascular event or disorder.

A potential limitation of the present study is its retrospective design. Patients were recruited at variable intervals after the onset of RVO; therefore, the measured concentrations may be different from those that existed at the time of the event. Several studies have demonstrated a clear relationship between plasma tHcy and adverse cardiovascular outcome over a relatively short time scale.<sup>23–25</sup> However, we found no link between plasma tHcy and RVO, even when we compared tHcy in subjects in whom RVO had occurred within 3 months of presentation (defined as acute cases) with those who had a longer history (chronic cases).

Although the controls in the present study were similar to subjects in age and sex distribution, they were recruited from a hospital setting. We did not match for other factors that are potentially able to alter tHcy levels, such as smoking, hypertension, renal function, and diabetes. We noted the increased number of patients with diabetes in our control population. Although this number was higher than in the subject population, the difference was not statistically significant. When the data were reanalyzed with these patients excluded, the study findings remained unchanged. Interestingly, the present study, while showing no relationship between subject status and plasma tHcy, was in accord with previous studies demonstrating that hypertension is a significant risk factor for RVO.<sup>10,26,27</sup> Of note, there was no statistical difference between subjects

TABLE 3. Frequency of MTHFR Genotypes in Subjects and Controls

	Subjects <i>n</i> (%)	Controls <i>n</i> (%)
CC	37 (35.9)	42 (44.7)
CT	55 (53.4)	38 (40.4)
TT	11 (10.7)	14 (14.9)

TABLE 4. Effect of MTHFR Genotype on tHcy, Folate, and Vitamin B12 Levels in RVO Subjects and Healthy Controls

Status	Genotype	tHcy ( $\mu\text{mol/L}$ )	Folate (ng/mL)	B12 (pg/mL)
		Geometric Mean (interquartile range) <i>n</i>	Geometric Mean (interquartile range) <i>n</i>	Geometric Mean (interquartile range) <i>n</i>
Subjects	CC	10.1 (8.0-14.3) 36	6.64 (4.54-8.27) 35	379 (277-521) 35
	CT	10.9 (8.3-13.8) 52	5.99 (4.24-8.55) 51	439 (299-638) 51
	TT	12.7 (9.3-18.8) 11	6.72 (3.88-10.84) 11	408 (369-535) 11
Controls	CC	9.3 (5.7-14.3) 37	6.58 (4.66-8.53) 37	389 (305-531) 37
	CT	9.0 (6.9-13.6) 36	7.49 (5.47-9.64) 34	368 (247-497) 34
	TT	13.3 (9.8-18.0) 14	5.74 (3.75-6.79) 14	330 (255-465) 14

tHcy, folate, and vitamin B12 were compared between subjects and controls and among the three genotypes by two-way ANOVA.

Comparison of subjects and controls: tHcy,  $F = 2.29$ ,  $df = 1,182$ ;  $P = 0.13$ ; folate,  $F = 0.80$ ,  $df = 1,178$ ;  $P = 0.37$ ; vitamin B12,  $F = 1.69$ ,  $df = 1,178$ ;  $P = 0.20$ .

Comparison of three genotypes: tHcy,  $F = 3.10$ ,  $df = 2,182$ ;  $P = 0.05$ ; folate,  $F = 0.19$ ,  $df = 2,178$ ;  $P = 0.83$ ; vitamin B12,  $F = 0.45$ ,  $df = 2,178$ ;  $P = 0.64$ .

and controls in the numbers of patients with a history of smoking, cardiovascular disease, thromboembolism, or anticoagulant medication.

Another potential source of bias in the present study was the method of collection of blood samples, which depended on when patients attended the ophthalmic unit. Thus, blood was not taken when patients were in the fasting state, and the effect on tHcy, folate, and vitamin B12 measurements might have been significant. Therefore, we selected a second group of people ( $n = 20$ ) from a large local database with no known retinal disorder and in whom plasma samples had been collected in a standardized manner in the fasting state. This group was matched for age and sex to our subject population and was found to have a mean plasma tHcy concentration that was nearly identical to that of our original control population.

The present study is one of the largest studies published to date and shows that neither plasma tHcy nor homozygosity for the thermolabile allele of the *MTHFR* gene was an important risk factor for RVO. It is also the first to examine the possible interaction between duration of vein occlusion and plasma tHcy. However, we agree that large, well-designed, prospective studies are required to definitively show whether tHcy plays a role in the etiology of RVO.

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