

# Plasma homocysteine, methylene tetrahydrofolate reductase C677T and factor II G20210A polymorphisms, factor VIII, and vWF in central retinal vein occlusion

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## Abstract

**Aims**—To determine whether plasma homocysteine, methylene tetrahydrofolate reductase (MTHFR) C677T and factor II G20210A polymorphisms, factor VIII, and vWF are risk factors for central retinal vein occlusion (CRVO).

**Method**—Prospective comparison of 63 consecutive patients with central retinal vein occlusion and 63 age matched controls. Plasma homocysteine and vWF were estimated by ELISA, the MTHFR and factor II G20210A polymorphisms determined by polymerase chain reaction with restriction enzyme product digestion and factor VIII by one stage automated clotting assay.

**Results**—Plasma homocysteine (patients: median 12.4  $\mu\text{mol/l}$ , controls: median 11.6  $\mu\text{mol}$  OR = 1.05,  $p=0.20$ ), factor VIII (patients: median = 115 U/dl, controls: median = 113 U/dl), and vWF (patients: median = 115 U/dl, controls: median = 108 U/dl) were not statistically higher in patients than in controls. Five CRVO patients and seven controls were homozygous for the MTHFR C677T mutation. One control was heterozygous for the factor II G20210A mutation.

**Conclusion**—This study has not identified new risk factors for CRVO.

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Although prothrombotic tendencies have been implicated, the pathogenesis of central retinal vein occlusion (CRVO) remains unclear. Recently mild hyperhomocysteinaemia has been established as a risk factor for cardiovascular disease,<sup>1</sup> plasma levels being influenced by environmental and genetic factors.<sup>2</sup> Mild hyperhomocysteinaemia may result from the thermolabile variant of the methylenetetrahydrofolate reductase (MTHFR) enzyme, caused by a C677T mutation, in the presence of low but probably not normal serum folate.<sup>3</sup> Several studies have suggested hyperhomocysteinaemia is a risk factor for non-arteritic ischaemic optic neuropathy and central retinal artery occlusion<sup>4,5</sup> and although it has been implicated in central retinal vein occlusion not all studies have confirmed this.<sup>4–9</sup> A genetic variation has been identified in the 3' untranslated region of the prothrombin gene (factor II G20210A) in 18% of familial venous thrombosis cases.<sup>10</sup> A prevalence of 8.3% has been reported for the mutation in patients with

CRVO compared with 0% for controls<sup>11</sup> although a further study did not support this finding.<sup>6</sup>

APCr and the ntG1691A polymorphism (factor V Leiden) have been extensively investigated in CRVO with several studies showing an increased prevalence of APCr, although results overall have been inconclusive.<sup>12,13</sup>  $\beta$ -Thromboglobulin, platelet factor 4, fibrinopeptide A, and protein S and C deficiency have been identified in occasional patients<sup>14</sup>; antithrombin III deficiency recognised in cases progressing to iris neovascularisation, but factor VIII and von Willebrand factor (vWF) have not been fully evaluated.

Therefore, to determine whether plasma homocysteine, the MTHFR C677T and factor II G20210A polymorphisms, and factor VIII, and vWF levels predispose to CRVO, we prospectively analysed these variables in a cohort of CRVO patients and age matched controls.

## Methods

### PATIENTS AND CONTROLS

Consecutive patients presenting with CRVO, defined as dilation and tortuosity in all four quadrants of the fundus, of less than 3 months' duration were enrolled. CRVO was classified as non-ischaemic (VA > 6/36, no relative afferent pupillary defect (RAPD) and  $\leq 30$  disc areas of non-perfusion on fundus fluorescein angiography) or ischaemic (VA  $\leq 6/36$ , RAPD and > 30 disc areas of non-perfusion). Exclusion criteria were branch or hemispheric retinal vein occlusion, co-existent central or branch retinal artery occlusion, significant change in lifestyle since the onset of CRVO—for example, substantial dietary change, commencement of medication (for example, methotrexate), or diagnosis of disease (for example, hypothyroidism, chronic renal failure) known to affect homocysteine levels. Controls were patients attending the same clinic during the enrolment period excluding those with branch retinal vein occlusion. Patients were managed according to Central Retinal Vein Occlusion Study guidelines. Blood was taken at presentation for plasma homocysteine, MTHFR C677T, factor II G20210A, factor VIII, and vWF. In addition, patients were screened for protein S, protein C, antithrombin III, activated partial thromboplastin time, prothrombin time, plasma fibrinogen, plasma IgG, and IgM anticardiolipin antibody, CRP, total protein, albumin, plasma triglycerides, cholesterol, rheumatoid factor, and antinuclear antibodies.

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Table 1 Age adjusted odds ratio of each study factor

Study factor	Odds ratio	p Value	95% CI	No of pairs
Plasma homocysteine ( $\mu\text{mol/l}$ )	1.06	0.2	(0.97, 1.14)	63
Factor VIII ( $\mu\text{mol/l}$ )	1.01	0.2	(1.00, 1.01)	63
von Willebrand factor ( $\mu\text{mol/l}$ )	1	0.32	(1.00, 1.01)	63

Table 2 Study factor by case-control status

Study factor	Cases Median (IQR)	Controls Median (IQR)	No of pairs
Plasma homocysteine ( $\mu\text{mol/l}$ )	12.4 (9.4, 15.7)	11.6 (9.8, 13.9)	63
Factor VIII ( $\mu\text{mol/l}$ )	115 (98, 145)	113 (90, 140)	63
von Willebrand factor ( $\mu\text{mol/l}$ )	115 (93, 141)	108 (87, 157)	63
Homozygosity for MTHFR C677T	5	7	63
Heterozygosity for factor II G20210A	1	0	63

## PLASMA HOMOCYSTEINE

Whole blood was collected into potassium EDTA tubes, centrifuged at 2000 *g* for 10 minutes at room temperature and stored at 70°C within 1 hour of collection until the time of assay. Plasma homocysteine was measured by automated enzyme linked immunosorbent assay (Biorad Laboratories).

## MTHFR C677T POLYMORPHISM

A fragment of the MTHFR gene was amplified by polymerase chain reaction (PCR) in a mixture of 10 mM TRIS-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100  $\mu\text{g}$  gelatine/ml, 1 mg triton X-100/ml, 200  $\mu\text{M}$  dNTPs, 100 ng of each primer (sense 5'-TGAAGGAGAAGGTGTCTGCGG GA-3' and antisense 5'-AGGACGGTGCAGGTGAGAGTG-3'), genomic DNA and 1U TAQ polymerase in a total volume of 100  $\mu\text{l}$ . Reaction conditions were: 5 minutes' initial denaturation at 96°C, 35 cycles of denaturation for 50 seconds at 93°C, primer annealing for 50 seconds at 55°C, primer extension for 30 seconds at 72°C, and final extension for 7 minutes at 72°C. The 198 bp product was digested using 0.5U of endonuclease Hinf I, the mutated MTHFR gene (allele 677T) only, giving 175 and 23 bp fragments.

## FACTOR II G20210A MUTATION

A 345 bp fragment from exon 14 and the 3' untranslated region of the prothrombin gene was amplified by PCR using primers 5'-TCTAGAAACAGTTGCCTGGC-3' (nucleotides 19889–19908) and a mutagenic primer 5'-ATAGCACTGGGAGCATTGAA\*GC-3' (nucleotides 20333–20212) [\*=mutagenic base] introducing a new Hind III site in the amplified fragment from the less frequent allele (A2:AAG) yielding two fragments (322 bp and 23 bp) compared to the more frequent allele (A1:GAG) (345 bp product only). The reaction mix was 1  $\mu\text{g}$  DNA, 1.5 mM MgCl<sub>2</sub>, 5  $\mu\text{l}$  10 $\times$  PCR buffer, 50 pmoles each primer, and 1 unit of thermostable DNA polymerase in a volume of 50  $\mu\text{l}$ . Reaction conditions were 5 minutes' initial denaturation at 94°C, 35 cycles of 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 60 seconds. Ten  $\mu\text{l}$  PCR product were digested using 0.5U of endonuclease Hind III.

## FACTOR VIII AND VWF

Factor VIII was measured by a one stage clotting method and vWF antigen and activity by ELISA.

## STATISTICS

Based on reported homocysteine levels of 10.25 (SE 1.88)  $\mu\text{mol/l}$  in patients versus 8.7 (SE 0.5)  $\mu\text{mol/l}$  for controls,<sup>4</sup> our study had greater than 90% power to detect a difference of this size. Conditional logistic regression was used to assess the association between each continuous study factor and the odds of being a case. Results are presented as odds ratios together with 95% CIs (see Table 1). Analyses were conducted using STATA statistical software.

## Results

Sixty six patients with CRVO were identified but three were excluded because of previous retinal artery occlusion (two), and one because of the recent onset of type II diabetes mellitus.

The mean age of the 63 patients was 60.3 years (SD 16.2) and controls 60.8 years (16.1). Three patients with CRVO were excluded from the study, two because of previous central and branch retinal artery occlusion and one because of recent onset of type II diabetes mellitus.

Twenty patients presented with an ischaemic and 43 with a non-ischaemic CRVO.

Plasma homocysteine was slightly higher in patients, median = 12.4  $\mu\text{mol/l}$  (IQR 9.4,15.7) *v* median = 11.6  $\mu\text{mol/l}$  (IQR 9.8,13.9) in controls but this was insignificant ( $p=0.20$ ). Five patients were homozygous for the MTHFR C677T mutation versus seven controls, and 30 patients versus 29 controls were heterozygous. No patients were homozygous for the factor II G20210A mutation, and only one control and no patient was heterozygous. Factor VIII levels were slightly higher in patients, median 115 (IQR 98,145) than controls, median 113 (IQR 90,140) ( $p=0.20$ ). vWF levels were marginally higher in patients, median 115 (IQR 93,141) than controls, median 108 (IQR 87,157) but this was again insignificant ( $p=0.32$ ) (see Table 2). Logistic regression analysis for plasma homocysteine, MTHFR homozygosity, factor VIII, and vWF levels showed no significant additive effect for variables considered together and subgroup analysis revealed no correlation between these variables and ischaemic and non-ischaemic phenotypes. There were no significant differences between CRVO patients and controls in any other variables (data not shown).

## Discussion

Plasma homocysteine was not elevated in CRVO patients and showed no correlation with homozygosity for the MTHFR C677T polymorphism. Of previous studies, three have suggested an association between plasma homocysteine and CRVO, although two were retrospective<sup>5,7</sup>; in one a significant number of cases was not included and in both, cases and controls were not fully matched. In the third, cases were significantly older than controls and

homocysteine is known to increase with age.<sup>8</sup> Two further studies found no correlation between CRVO and homocysteine.<sup>4,9</sup> One prospectively examined 116 patients and age matched controls and found no difference in serum homocysteine levels.<sup>9</sup> Furthermore, this study detected no difference in the prevalence of the heterozygous and homozygous states for the C677T MTHFR mutation, although an association was found for the homozygous state in a previous study. Our study therefore lends support to the view that there is no association between plasma homocysteine, the C677T polymorphism, and CRVO. It is recognised that controls in this study were patients with other eye conditions and may have introduced bias if their prevalence of vascular disease and therefore elevated plasma homocysteine was above normal.

The factor II (prothrombin) gene G20210A polymorphism has recently been described as a risk factor for venous thrombosis in familial cases.<sup>10</sup> The prothrombin gene lies on chromosome 11 at position 11p11-q12. The G20210A polymorphism, which lies in the 3' untranslated region, that may have a regulatory role in gene expression, probably causes elevated prothrombin levels. How raised prothrombin levels stimulate the formation of venous thrombi is unclear, but higher concentrations of prothrombin may lead to increased rates of thrombin generation, excessive growth of fibrin clots, and possibly increased activation of platelets. One small study to date has reported an association between factor II G20210A and CRVO, although a statistically significant result was obtained only when it was considered in conjunction with APCr due to factor V Leiden.<sup>11</sup> A more comprehensive report found a similar prevalence of the G20210A mutation in CRVO patients and controls<sup>6</sup> and our study supports such a finding.

Increased factor VIII and vWF levels have been reported in a small series of CRVO

patients, but our study was unable to confirm any difference between patient and control groups. In summary, this relatively small study does not suggest hyperhomocysteinaemia, the MTHFR C677T or factor II G20210A polymorphisms, factor VIII or vWF are independent risk factors for CRVO and would not support the routine evaluation of these factors in CRVO patients.

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