

Hyperhomocysteinemia and Low Methionine Stress Are Risk Factors for Central Retinal Venous Occlusion in an Indian Population

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PURPOSE. The underlying cause of disturbed homocysteine metabolism is incompletely understood in young persons with central retinal vein occlusion (CRVO) with mild hyperhomocysteinemia (HHcys) and no other systemic disease in India. A 2-year prospective study was undertaken to determine whether HHcys is a risk factor for CRVO in an Indian population.

METHOD. The prevalence of fasting HHcys was evaluated in a consecutive series of 29 patients with CRVO (mean age, 30 ± 6 years) along with 57 age- and sex-matched control subjects (healthy subjects, mean age 27 ± 5 years). Strict inclusion and exclusion criteria were used. Plasma levels of homocysteine (Hcys), methionine, cysteine, glutathione, B₁₂, and folate were measured. Multivariate logistic regression analysis was performed to determine the risk factors for CRVO.

RESULT. Fifteen of 29 patients with CRVO (51.72%) exhibited HHcys (>15 μM). The mean Hcys level was significantly elevated in the patients with CRVO (19.1 ± 13.1 μM) compared with that in the healthy control subjects (14.7 ± 6.2 μM) with *P* = 0.04. The increased Hcys levels in CRVO cases was associated with decreased methionine (*P* = 0.052) and decreased B₁₂ (*P* = 0.001). A multivariate logistic regression analysis revealed an odds ratio of 1.9 (95% CI = 0.50–7.16) for Hcys and 15.9 for methionine (95%CI = 1.50–169.62; *P* = 0.022).

CONCLUSION. Elevated Hcys and low methionine were risk factors for CRVO in an Indian population. (*Invest Ophthalmol Vis Sci.* 2007;48:1441–1446) DOI:10.1167/iovs.06-0905

Mild to moderate elevation of plasma homocysteine (Hcys) is reported as a risk factor for occlusive disease. Specifically, many studies demonstrate hyperhomocysteinemia (HHcys) as an independent risk factor for atherosclerosis in the coronary, cerebral, and peripheral vasculature.^{1–4} In a population-based study, Hcys was found to be associated with increased odds ratio for retinal emboli formation.⁵

The mechanisms by which Hcys damages the blood vessel wall by supporting prothrombotic effects seems to be multifactorial.⁶ The various mechanisms reported include Hcys-induced oxidative stress (redox stress),^{7,8} decreased bioavailability of nitric oxide,^{9,10} altered expression of various thrombotic factors, mitogenic effect on arterial smooth muscle cells,¹¹ and expression of acute stress-related genes.¹² Several studies have demonstrated that Hcys can also cause direct cytotoxic effects by forming disulfide protein derivatives, thereby modifying vascular cell function. Metabolic conversion of Hcys to a chemically reactive metabolite, Hcys-thiolactone is suggested to contribute to Hcys toxicity in humans (Hcys-thiolactone hypothesis).¹³ All these effects of HHcys lead to endothelial dysfunction, but until now, the cause-effect relationship was still not fully understood.¹⁴

All circulating Hcys is primarily derived from dietary methionine, which acts as a methyl group donor in the form of S-adenosyl methionine. On donating the methyl group it forms S-adenosyl Hcys and then forms Hcys. Hcys is a sulfur-containing nonprotein amino acid that is either metabolized to cystathionine by the transsulfuration pathway, requiring B₆, or it is converted back to methionine by B₁₂ and folate, requiring transmethylation¹⁵ (Fig. 1).

The cause of HHcys is varied. Severe HHcys is due to rare genetic defects resulting in deficiencies of the enzymes cystathionine β-synthase (CBS) and methyltetrahydrofolate reductase. Mild HHcys is due to impairment in the enzymes in a transmethylation pathway associated with or without nutritional deficiencies such as B₁₂ and folate.¹⁶ Geographic variation has also been reported in the methylene tetrahydrofolate reductase (MTHFR) gene, which leads to a transmethylation defect.¹⁷

Central retinal venous occlusion (CRVO) is one among the most common retinal vascular diseases. Among the six types of RVO, based on the site of occlusion and on the type of consequent vascular damage, CRVO is the most frequently occurring and clinically relevant type. It is usually seen in older adults, often associated with systemic diseases. In many cases, it also occurs in young adults with no other systemic disease. Both local and systemic risk factors have been associated with CRVO. The cause of CRVO remains multifactorial.^{16,18}

Though hypercoagulability has been reported in the pathogenesis of CRVO in young patients, laboratory tests have not accounted for this cause in most of these patients.¹⁹ Among the parameters tested, HHcys and circulating antiphospholipid antibodies are reported to be significantly more common in the patients with CRVO.²⁰ Boyd et al.²¹ have reported that there is no significant increase in factor VIII (von Wilbrand factor), apart from Hcys levels in the CRVO cases compared with the control subjects. The underlying cause of the disturbed Hcys metabolism and the molecular mechanism underlying the prothrombotic actions of Hcys are incompletely understood in cases of CRVO with mild HHcys in young adults.²²

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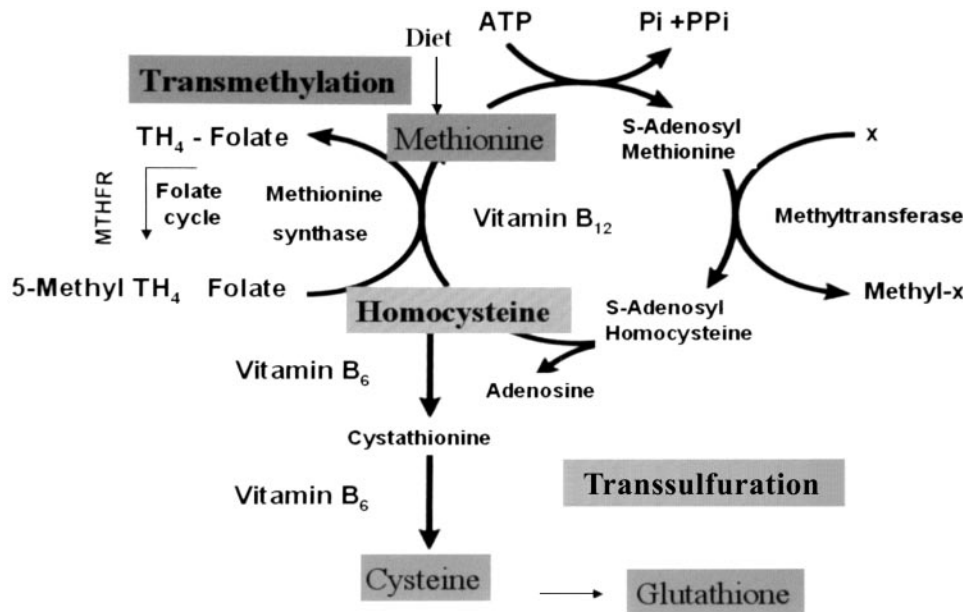


FIGURE 1. Homocysteine metabolism.

There has been no study report on mild to moderate HHcys associated with young CRVO cases in the Indian population. Therefore, a 2-year prospective study in young adult CRVO cases was performed at Sankara Nethralaya to evaluate the relationship between the plasma total Hcys (tHcys) and CRVO in which estimation of some of the key metabolites of the transmethylation and transsulfuration pathway involved in the Hcys metabolism was performed.

MATERIALS AND METHODS

Design

A 2-year prospective case-control study of consecutive, unrelated patients, aged 20 to 40 years, with a diagnosis of CRVO in the absence of any other systemic disease, was conducted at Sankara Nethralaya. The institutional research board approved the study and informed consent was obtained from all the study subjects, in accordance with the Declaration of Helsinki. Age- and sex-matched control subjects were either the staff or students at Sankara Nethralaya.

A detailed questionnaire on family history, social status, dietary habits, including other habits such as smoking, alcohol intake, history of systemic diseases, other ocular diseases and drug history was completed by all the study subjects. All potential factors that lead to HHcys, such as hypertension, cardiovascular disease, high cholesterol, renal disease, liver disease, hematologic and coagulation abnormalities were ruled out in the CRVO cases based on the biochemical and hematologic tests apart from the questionnaire.

Of the 639 patients who had CRVO diagnosed at Sankara Nethralaya, 63 (10%) were found to be in the age range of 20 to 40 years and were identified as young adult CRVO cases. Ophthalmic examination of both eyes, including visual acuity, relative afferent pupillary defect (RAPD), electroretinogram (ERG), and fundus examination, was used for the clinical diagnosis of CRVO. A total of 86 subjects participated in the study. Plasma Hcys level $>15 \mu\text{M}$ was considered to be HHcys. Based on the inclusion and exclusion criteria, 29 patients with a mean age of 31 ± 6 (22 male, 7 female) were included in the study. Of these, 19 had nonischemic CRVO, and 10 had the ischemic type; all 29 had unilateral CRVO. A total of 57 apparently healthy subjects with a mean age of 27 ± 5 (41 male, 16 female) were taken as control subjects based on the questionnaire. None of the study subjects had any history of major thromboembolic episode.

Measurement of Plasma Hcys

Venous blood samples were drawn into EDTA-containing tubes after the participants fasted overnight. Plasma was separated immediately from blood cells by centrifugation at 3000g at 25°C for 10 minutes and stored at -20°C until analysis. Total plasma Hcys was estimated with an ELISA kit (Bio-Rad Laboratories, Inc., Hercules, CA).

Measurement of Methionine, Cysteine, and Glutathione

Blood was collected in acid citrate dextrose. The plasma was separated and stored at -20°C for methionine estimation. Methionine was estimated as described by Huesgen,²³ with slight modification. The amino acid profile was obtained in the protein-free samples, followed by ortho-phthalaldehyde (OPA; Sigma-Aldrich, St. Louis, MO) derivatization and analyzed in C18 column using gradient buffer elution followed by UV detection at 338 nm. For the analysis of cysteine, the samples were collected in EDTA tubes containing 10 mM dithiothreitol (Himedia, Mumbai, India). It was estimated by the method of Arin et al.²⁴ by using dithiobis(2nitro)benzene (DTNB; Sigma-Aldrich) derivatization. Both methionine and cysteine analyses were performed using an HPLC system (model 1100; Agilent, Palo Alto, CA).

Blood samples were collected in EDTA tubes and washed in normal saline and the lysates were prepared for the analysis of glutathione in the red blood cells (RBCs). It was measured by using DTNB and read spectrophotometrically (DU 640; Beckman, Fullerton, CA) at 412 nm.²⁵

Measurement of B₁₂ and Folate

Serum samples were collected and stored at -80°C for the analysis of B₁₂ and folate by using an automated chemiluminescence system (Bayer Health Care, Leverkusen, Germany). A multivariate logistic regression analysis was performed to determine the risk factors for CRVO, based on the odds ratio (OR).

RESULTS

Of the 29 patients with CRVO in the study, 15 (51.7%) had HHcys, whereas in the control group, 20 (35%) of 57 subjects had it. The present study indicates a high prevalence of mild HHcys in CRVO cases in the study population (Fig. 2). A significant increase in the Hcys levels was seen in the patients

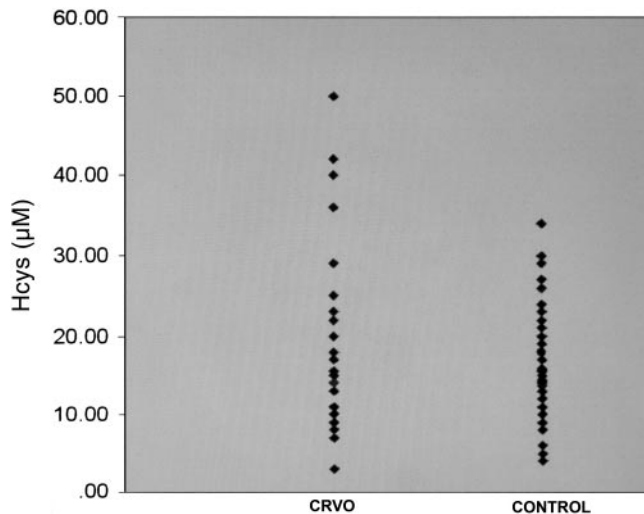


FIGURE 2. Homocysteine levels in the patients with CRVO and the healthy control subjects.

with CRVO (mean tHcys, $19.12 \pm 13.17 \mu\text{M}$) as opposed to the control subjects (mean tHcys, 14.7 ± 6.2 ; $P = 0.04$). There were no significant changes in the levels of cysteine and glutathione, but there was a significant decrease in methionine in the patients with CRVO when compared with that in the control subjects ($P = 0.001$; Table 1). Also, a negative correlation between Hcys and the methionine levels was observed in the CRVO cases ($r = -0.3640$, $P = 0.052$; Fig. 3A). There was a positive correlation between Hcys and Met ($r = 0.418$, $P = 0.002$; Fig. 3B), as expected in the control subjects. Of note, 93% of the patients with CRVO exhibited methionine deficiency as opposed to 67% in the control subjects.

Since methionine levels were significantly lower, estimation of B_{12} and folate was performed to see whether nutritional deficiency contributes to a defective transmethylation pathway. There were no significant changes in the mean levels of these vitamins between the groups, but vitamin B_{12} deficiency was found in 37% of the patients with CRVO and 63% of control subjects, whereas folate deficiency was found in 43% of the patients with CRVO and 37% of the control subjects, indicating deficiency of both vitamins in the general population correlating with the HHcys (Table 2). A significant negative correlation was seen between vitamin B_{12} ($r = -0.576$, $P = 0.032$) and Hcys levels in patients with CRVO alone (Fig. 4). There is no significant correlation between B_{12} and Hcys with $r = -0.287$ and $P = 0.069$ in control subjects.

The multivariate logistic regression analysis (Table 3) showed that, in this study population, an elevated Hcys level was a risk factor for CRVO, with an OR = 1.9 (95% CI = 0.50–7.16), and methionine with an OR = 15.9 (95% CI =

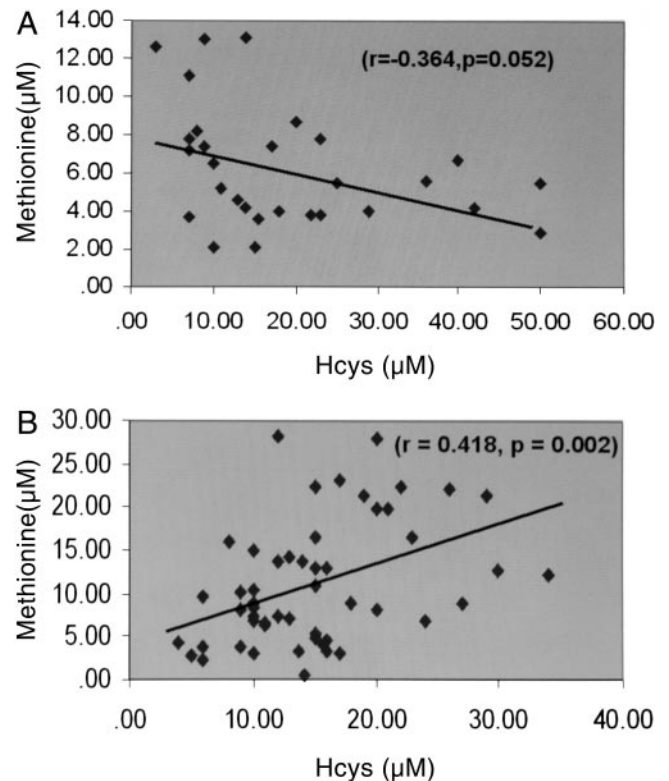


FIGURE 3. Correlation between plasma tHcys levels and plasma methionine levels in the (A) patients with CRVO and (B) control subjects.

1.50–169.62; $P = 0.022$) was also shown to be a significant risk factor for CRVO.

Thus, the patients with CRVO showed a distinct metabolic picture wherein there is significant methionine deficiency that correlates significantly with a mild to moderate increase in Hcys levels.

DISCUSSION

Although there are reports in support of the hypothesis that the plasma level of Hcys is not a primary and independent risk factor for CRVO,²⁶ but more likely a marker or a consequence of atherosclerosis,¹⁶ there are also reports strongly indicating that HHcys is an independent risk factor for CRVO.^{27,28}

In view of the fact that no data are available in the Indian population, the present study was performed to explore the association of HHcys in patients with CRVO in the Indian population. The results of the present study are consistent with many case-control, prospective studies that state that Hcys as

TABLE 1. Estimation of Homocysteine, Methionine, Cysteine, and Glutathione in CRVO Cases and Healthy Control Subjects

Subject Number	Parameter	CRVO Cases	Control Subjects	P
1	Homocysteine (μM)	19.12 ± 13.17 (n = 29)	14.7 ± 6.2 (n = 57)	0.0411
2	Methionine (μM)	6.29 ± 3.10 (n = 29)	11.0 ± 7.08 (n = 53)	0.001
3	Cysteine (μM)	64.4 ± 36.5 (n = 19)	71.52 ± 34.52 (n = 52)	0.451
4	Glutathione (mg/g Hb)	1.87 ± 0.62 (n = 16)	2.11 ± 1.07 (n = 16)	0.416

TABLE 2. Estimation of Vitamin B₁₂ and Folate in CRVO Cases and Healthy Control Subjects

Subject Number	Parameter	CRVO Cases	Control Subjects	P
1	Vitamin B ₁₂ (pg/mL)	282.44 ± 123.91 (n = 16)	220.02 ± 111.50 (n = 55)	0.059
2	Vitamin B ₁₂ deficiency	37%	63%	
3	Vitamin B ₁₂ deficiency + HHcys	100% HHcys	51.4% HHcys	
4	Folate (ng/mL)	8.19 ± 6.45 (n = 16)	7.45 ± 5.12 (n = 55)	0.632
5	Folate deficiency	43.7%	37%	
6	Folate deficiency + HHcys	50% HHcys	40% HHcys	

a risk factor for CRVO, with an OR of 1.9 (95% CI, 0.50–7.16). Lattanzio et al.¹⁶ have reported an OR of 3.0 for fasting HHcys in patients with CRVO and an OR of 1.3 was reported recently by another study in a Chinese population.²⁹ The meta-analysis by Janssen et al.³⁰ has also shown an overall OR of 8.9 (95% CI, 5.7–13.7) for Hcys.

HHcys has also been shown to be strikingly common in apparently healthy subjects. The present study also shows a high prevalence of HHcys (36%) in the control subjects. Similar reports stating a high prevalence of HHcys in the Indian population are available.^{31–35} Young Asian Indian men settled in the United States and Europe have also been reported to exhibit HHcys.^{36–39}

In our study, methionine was significantly lower ($P = 0.001$) in the patients with CRVO, with no significant change in the levels of cysteine and glutathione, showing that there is probably a defect in the transmethylation pathway rather than transsulfuration pathway. Control of Hcys metabolism involves changes in inherent kinetic properties of the enzymes in the methylation and remethylation pathways, as well as by enzymes involved in the transsulfuration pathway. A basal HHcys is said to reflect an impaired remethylation pathway, whereas transsulfuration appears to function primarily for the metabolism of excess methionine.⁴⁰

There were no significant differences in the mean levels of vitamin B₁₂ and folate between the patients with CRVO and healthy control subjects, but in general, a high prevalence of vitamin B₁₂ and folate deficiency was seen in all the study subjects. A recent study assessed whether it is vitamin B₁₂ deficiency or HHcys that is associated with cardiovascular events and concluded that high Hcys concentration is associated with cardiovascular events and not low serum vitamin B₁₂.⁴¹ But, Wilmink et al.⁴² have shown that dietary folate, apart from B₆, is an independent predictor of peripheral arterial occlusive disease in men older than 50 years. Fasting Hcys

concentrations were reported to be higher by 6% in Indian Asians than in Europeans. Lower vitamin B₁₂ and folate levels in Asians explained this difference in Hcys concentrations between the two ethnic groups. In this study, all the patients with CRVO with B₁₂ deficiency exhibited Hcys (100%; Table 2). Similarly, the combination of HHcys with folate has been shown to be more disease causing.⁴³ The meta-analysis by Cahill et al.⁴⁴ has shown that the retinal vascular occlusion is associated with elevated plasma Hcys levels and low serum folate levels and not with serum vitamin B₁₂.

Cuskelly et al.⁴⁵ have shown that there is a large reduction (37%) in the rate of methionine synthesis in folate deficiency that correlates negatively with the synthesis rate of methionine from serine, when compared with that in control subjects. In our study, 93% of the patients with CRVO exhibited a methionine deficiency compared to 67% of the control subjects.

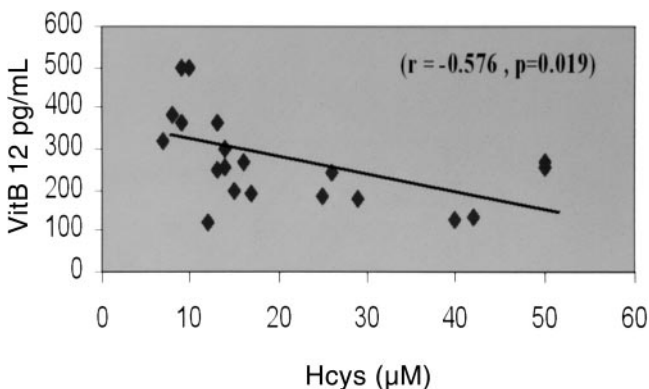
The mean methionine level was not only significantly lower in the CRVO group, but within the group, it was even lower in the patients with HHcys compared with the patients with CRVO without HHcys ($P = 0.021$), with a negative correlation between methionine and Hcys that was almost significant ($P = 0.052$). This pattern was unique to the CRVO cases, showing that lower methionine is a significant risk factor for CRVO in this study population.

The lower methionine could be due either to reduction in the relative rate of remethylation or to inadequate intake of protein (Fig. 5). The possibility that reduced methionine was due to a low-protein diet in this study is slight, because the study ensured an adequate protein diet based on the questionnaire and excluded those with inadequate intake, by stipulating the number of servings of milk, legumes, egg and other nonvegetarian foods per week.

One study has shown that defective Hcys remethylation caused by a deficiency of either methionine synthase or folate produces oxidative stress and endothelial dysfunction in the cerebral microcirculation of mice.⁴⁶ The possibility of direct cytotoxic effect of Hcys and Hcys thiolactone in the retinal vascular endothelial cells has also been reported in a case report by Poloshek et al.⁴⁷ He has reported methionine deficiency in this patient in association with microvascular damage to the retina. Moreover, the high pK_a of the sulfhydryl group (pK_a = 10.0) of Hcys is responsible for the formation of stable disulfide bonds with protein cysteine residues and, in the

TABLE 3. Results of Multivariate Logistic Regression Showing Factors Influencing CRVO

Subject Number	Multivariate Risk Factors	OR (95% CI)	P
1	Homocysteine	1.90 (0.50–7.16)	0.344
2	Methionine	15.9 (1.50–169.62)	0.022*

* $P < 0.05$.FIGURE 4. Correlation between vitamin B₁₂ and homocysteine in the patients with CRVO.

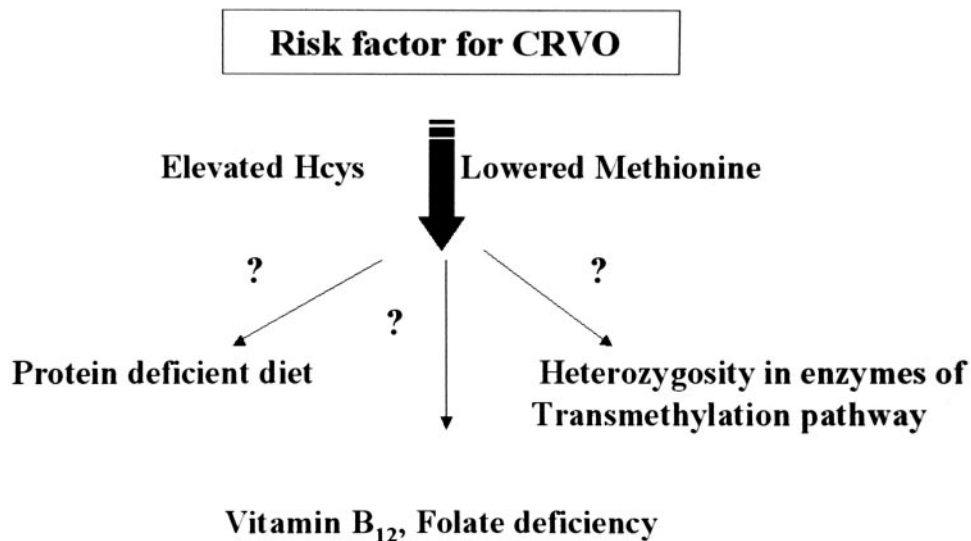


FIGURE 5. Possible factors associated with low methionine and HHcys.

process, alters or impairs the function of many proteins. Albumin, fibronectin, transthyretin, annexin II, and factor V have now been identified as molecular targets for Hcys.⁴⁸ Therefore, with a similar picture, the patients with CRVO show a significant methionine deficiency along with elevated Hcys. There is a possibility of a direct cytotoxic effect of Hcys on the retinal vascular cells, apart from its prothrombotic effects. Exposure of vascular smooth muscle cells to HHcys can lead to upregulation of the inflammatory response that characterizes early atherogenesis and may, in part, account for the adverse vascular effects of HHcys.⁴⁹

A low methionine level seemed to be a significant factor in CRVO as well as HHcys in our study population. Therefore, in addition to the B₁₂ and folate supplementation suggested to correct the HHcys, monitored methionine supplementation in patients with CRVO would be worth considering. This treatment would shift the enzyme kinetics in favor of the transsulfuration pathway, to clear the excess Hcys, because methionine supplementation would alter the S-adenosylmethionine (SAM)/S-adenosylhomocysteine (SAHC) ratio in favor of an augmented transsulfuration pathway. SAM is said to act as a switch between remethylation and transsulfuration through its allosteric inhibition of methylenetetrahydrofolate reductase and activation of cystathionine β -synthase.⁵⁰

In the absence of any systemic disease or abnormal hematologic and coagulation parameters, including hereditary thrombophilic defects, as ruled out by the exclusion criteria, no major thromboembolic activity or coagulation disorders were recorded in the young adult patients with CRVO recruited in the study. However, a microvascular thrombus cannot be ruled out in the CRVO cases. Hcys-induced thrombosis may be a crucial factor triggering vascular occlusion.⁵¹ A primary thrombogenic effect of Hcys concentration may explain why it is consistently associated with an increased risk of coronary heart disease in high-risk subjects.⁵² Regarding thrombophilic risk factors and retinal venous occlusion, as per the meta-analysis by Janssen et al.,³⁰ there is evidence of association between HHcys venous thrombosis. However, more randomized controlled trials of Hcys-lowering therapy are needed, to verify the causal relationship of Hcys with cardiovascular disease.

A limitation of this study is that, except for the questionnaire, there are no concrete grounds on which to eliminate low protein intake to account for the dietary deficiency of methionine. Genetic variation in the key enzymes of transmethylation and remethylation pathways of Hcys metabolism can give

a further insight into the underlying cause of the mild HHcys present in the patients with CRVO.

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