

# Homocysteine, lipid indices and antioxidants in patients with ischaemic heart disease from Maharashtra, India

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## ABSTRACT

**Introduction:** Geographical and ethnic factors have recently been shown to have a significant role to play in cardiovascular diseases. The exact relationship between nutritional and geographical factors in cardiovascular diseases is not very clear. This study examined the relationship of hyperhomocysteinaemia with lipid profile and antioxidants in patients with ischaemic heart disease from rural areas in Maharashtra, India.

**Methods:** Blood cholesterol (total, high- and low-density lipoproteins cholesterol), triglycerides along with thiobarbituric acid reactive substances (TBARS), superoxide dismutase, glutathione peroxidase and catalase activities were measured in acute coronary syndrome (ACS) and chronic stable angina (CSA) patients from rural areas and in normal healthy controls from the same area. Plasma total homocysteine was measured by high pressure liquid chromatography with fluorescence detection. Folic acid and vitamin B<sub>12</sub> were measured by chemiluminescence immunoassay.

**Results:** The relative lipid ratios were higher in the patients and had a poor correlation with antioxidants. Total homocysteine levels were significantly higher by almost three times more than the controls. TBARS levels also showed a similar pattern, whereas antioxidant enzymes showed a significantly greater fall in ACS than CSA. There was a definite inverse relationship between total homocysteine, TBARS and antioxidants in the patients. The levels of folic acid and vitamin B<sub>12</sub> were 3–4 times higher in the patients compared to the controls. There was a poor correlation between the total homocysteine and vitamin levels in the patients.

**Conclusion:** Blood homocysteine is a very important biomarker of cardiovascular diseases

and must be evaluated along with other risk factors. There is a higher prevalence of hyperhomocysteinaemia in rural Indian patients. There appears to be a strong association of genetic factors in the development of ischaemic heart disease in Indian patients.

**Keywords:** antioxidants, folic acid, homocysteine, ischaemic heart disease, lipids, vitamin B<sub>12</sub>

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## INTRODUCTION

India is in epidemiological transition. In addition to the burden of endemic infections, the emerging threat of non-communicable diseases is a matter of concern. The epidemiology of non-communicable diseases in South Asians is characterised by a higher burden of cardiovascular diseases (CVD) and diabetes mellitus.<sup>(1,2)</sup> CVD is the number one cause of mortality in India today. Previously, CVD was considered to be a result of an urban lifestyle; however, many recently published studies have indicated that CVD is also on the rise in rural areas.<sup>(3,4)</sup> The prevalence of CVD is four-fold higher in urban India and two-fold higher in rural India than in the United States.<sup>(4)</sup> Apart from current lifestyle factors, there is an additional 3–4-fold risk from genetic and ethnic factors for heart disease. Certain ethnic groups in India, like the Jains, Marwaris, Baniyas and other communities belonging to the states of Rajasthan, Gujarat, Haryana and Punjab, are genetically predisposed to central obesity.<sup>(5)</sup> They are at a higher risk of developing CVD. The picture is even gloomier for migrant Indians.<sup>(6)</sup>

Raised blood homocysteine (Hcy) has been recognised recently as an independent risk factor for the presence of atherosclerotic disease.<sup>(6-9)</sup> A blood level of total Hcy (tHcy) exceeding 15 µmol/L is now labelled as hyperhomocysteinaemia (HHcy).<sup>(7)</sup> Oxidative stress is a putative cause of atherosclerotic disease and plays an important role in the development as well as progression of ischaemic heart disease (IHD).<sup>(10)</sup> Raised low-density lipoprotein cholesterol (LDLc) and decreased high-density

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**Table I. The classic lipid profile parameters in the ischaemic heart disease and control groups.**

Group	No. of subjects	TG (mg/dL)	TC (mg/dL)	HDLc (mg/dL)	LDLc (mg/dL)	VLDLc (mg/dL)
Controls	50	100.88 ± 18.26	172.08 ± 26.80	47.51 ± 14.53	110.29 ± 24.79	19.74 ± 3.87
ACS	42	152.28 ± 66.36*	215.73 ± 38.56*	41.78 ± 4.63 <sup>†</sup>	119.75 ± 37.04	33.79 ± 16.40*
CSA	50	132.07 ± 49.16*	184.23 ± 47.12	45.09 ± 16.07	95.60 ± 40.46	26.36 ± 8.21*

Data is expressed as mean ± SD. \*p < 0.001; <sup>†</sup>p < 0.01, compared to controls.

TG: triglycerides; TC: total cholesterol; HDLc: HDL cholesterol; LDLc: LDL cholesterol; VLDLc: VLDL cholesterol; ACS: acute coronary syndrome; CSA: chronic stable angina.

**Table II. The relative lipid ratios in the ischaemic heart disease and control groups.**

Group	No. of subjects	TC:HDLc	LDLc:HDLc	TG:HDLc	Atherogenic index
Control	50	3.98 ± 0.80	2.68 ± 0.70	2.46 ± 0.61	0.36 ± 0.09
ACS	42	5.07 ± 2.06 <sup>†</sup>	3.18 ± 1.41 <sup>‡</sup>	4.07 ± 1.96*	0.56 ± 0.25*
CSA	50	4.49 ± 1.63 <sup>‡</sup>	2.91 ± 1.44	3.30 ± 1.80 <sup>†</sup>	0.45 ± 0.22 <sup>§</sup>

Data is expressed as mean ± SD. \*p < 0.001; <sup>†</sup>p < 0.005; <sup>‡</sup>p < 0.05, <sup>§</sup>p < 0.01, compared to controls.

Atherogenic index = Log (TG/HDLc).

TG: triglycerides; TC: total cholesterol; HDLc: HDL cholesterol; LDLc: LDL cholesterol; VLDLc: VLDL cholesterol; ACS: acute coronary syndrome; CSA: chronic stable angina.

lipoprotein cholesterol (HDLc) are the well-established major indicators for impending IHD. However, some patients develop premature IHD with normal values of lipid profile.<sup>(11)</sup> Therefore, it becomes necessary to review and revise the criteria for risk assessment in the context of new data and the information available. The present paper reports the status of homocysteine, lipid profile ratios and antioxidants in IHD patients from rural areas of Maharashtra state in India.

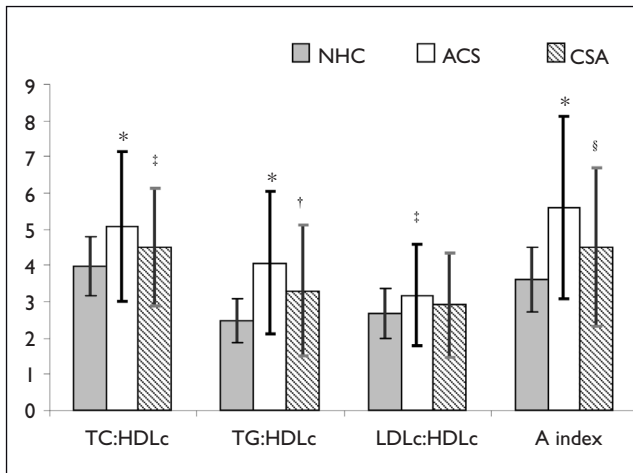
## METHODS

The present study was a cross-sectional control study of 100 patients from rural areas of Maharashtra, India, and comprised 64 males and 36 females. The patients were diagnosed based on history, clinical presentation, electrocardiogram, angiography, laboratory investigations, stress and other tests. 50 patients who showed positive findings of IHD (S-T elevated myocardial infarction and non-S-T elevated myocardial infarction) as well as those who had a recent history of myocardial ischaemia / angina not exceeding a month (unstable angina) were labelled as acute coronary syndrome (ACS). 50 other asymptomatic known IHD patients with no history of any abnormal cardiac activity / angina for a period exceeding a month were labelled as chronic stable angina (CSA). Most of the patients from this group had their last abnormal cardiac event over 10–24 months previously. The exclusion criteria were the presence of concomitant disorders such as diabetes mellitus, malignancies, thyroid, liver, kidney diseases and any recent (> 3 months) history of taking vitamins.

The 50 normal healthy subjects (30 males and 20 females) recruited as controls were also residents of the same villages as the patients. These were aged between 20 and 50 years, and were age- and gender-matched with

the patients. The controls were clinically symptomless and had no past history of any cardiovascular disorder. The exclusion criteria for the controls were the same as those for the patients. The patients and controls were selected by a random-clustered sampling method from 14 small villages within a 20–25 km radial distance from Latur city in Maharashtra. They had lived in their respective villages since their childhood and hardly travelled (besides occasional visits of 2–4 days to nearby cities). None of the subjects were recent migrants from urban to rural areas; their residential status was truly rural. Both the patients and the controls consumed a normal diet composed of 600–1,500 g carbohydrates, 70–180 g fats, 30–50 g proteins, and their total caloric intake was 12,600–33,600 kJ/d. The daily diet included chapattis, dahl, rice, green/ other vegetables, etc. The entire study period spanned 28 months, from October 2001 to January 2004. The present study was conducted under ethical guidelines.

Fasting venous blood samples were collected from the study subjects in ethylene diaminetetraacetic acid (EDTA) vacutainers and plain bulbs using disposable syringes. The blood collected in the EDTA vacutainer was centrifuged and plasma was separated without further delay. The plasma was used for the estimation of tHcy by the high pressure liquid chromatography (HPLC) system consisting of the Varian Prostar Model 210, solvent delivery module and with fluorescence detection using cysteamine as an internal standard.<sup>(12)</sup> The reagents from RECIPE Chemicals & Instruments GmbH, Munich, Germany were used for the Hcy assay. The serum levels of total cholesterol, HDLc and triglyceride (TG) levels<sup>(13–15)</sup> were measured using reagents from Bayer Diagnostics India Ltd, Baroda, India. The values of very LDLc (VLDLc) and LDLc were calculated using Friedewald's equation.<sup>(16)</sup> Only patients with TG



**Fig. 1** Bar chart shows the relative lipid ratios in the patients (ACS and CSA) in comparison to the normal healthy controls (NHC).

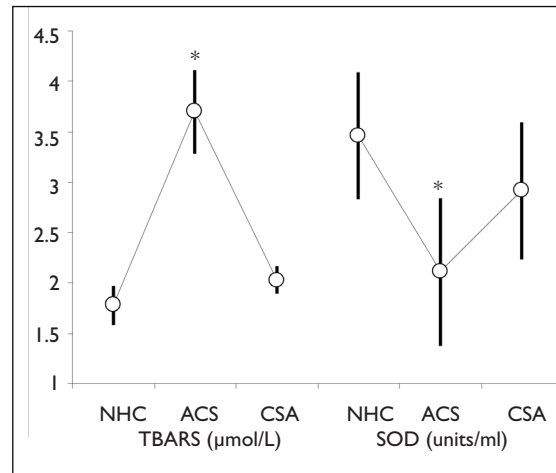
TC: total cholesterol; TG: triglycerides; HDLc: high-density lipoprotein cholesterol; LDLc: low-density lipoprotein cholesterol; A index: atherogenic index.

\*  $p < 0.001$ ; †  $p < 0.005$ ; ‡  $p < 0.05$ ; §  $p < 0.01$ , when compared to NHC.

levels of  $< 350$  mg/dL were included in the study. The serum levels of folic acid and vitamin B<sub>12</sub> were measured by direct chemiluminescence immunoassay,<sup>(17,18)</sup> using the ADVIA Centaur system (Bayer Corporation, Health Care Division, Tarrytown, New York, USA). The serum lipid peroxides level was measured as thiobarbituric acid reactive substances (TBARS) by the colorimetric method<sup>(19)</sup> using malondialdehyde as a standard. Superoxide dismutase (SOD) activity in serum was determined by measuring the inhibition of auto-oxidation of the pyrogallol method,<sup>(20)</sup> erythrocyte glutathione peroxidase (GPx) activity was measured using t-butyl hydroperoxide as a substrate,<sup>(21)</sup> catalase activity in erythrocyte was determined by the colorimetric method<sup>(22)</sup> and expressed in terms of “katalase faehigkeit” (Kat.f) units. All the high purity chemicals used for manual assays of TBARS, SOD, GPx and catalase were from Sigma Chemical Company, St Louis, MO, USA and the Qualigen fine chemicals were from GlaxoSmithKline Pharmaceuticals Ltd, Mumbai, India.

## RESULTS

The results of the individual classic lipid profile parameters are shown in Table I. Simple ratios were calculated using primary data on the classical lipid profile. The lipid ratios calculated were TC:HDLc, TG:HDLc, LDLc:HDLc and the atherogenic index, Log (TG:HDLc). The biochemical data was subjected to an unpaired Z-test with a p-value level range fixed at 0.01–0.001. The statistical correlation between the two sets of biochemical variables was done using a linear regression method. The mean values for



**Fig. 2** Graph shows the serum levels of thiobarbituric acid reactive substances (TBARS) and superoxide dismutase (SOD) activity in the patients (ACS and CSA) as compared to the normal healthy controls (NHC).

Vertical bars indicate the standard deviation. Mean values of TBARS are higher in ACS than the healthy controls and CSA, while SOD values for ACS and CSA are significantly lower than the controls.

\*  $p < 0.001$ , compared to NHC.

any of the ratios were highest in ACS (Table II). The mean values were lower in CSA compared to ACS on the one hand and were higher than the controls on the other (Fig. 1). The values of the lipid profile in the controls were agreeable and within the acceptable range as per the national cholesterol education programme.<sup>(23)</sup>

The mean value of the pro-oxidant factor like TBARS in ACS was significantly higher ( $p < 0.001$ ) by almost two times than that of the controls; however, serum SOD levels were significantly lower in ACS as well as in CSA (Fig. 2). These values in CSA did show minor variations compared with the controls but were statistically not significant (Table III). Thus, there was a clear inverse relationship between TBARS and SOD in the patients (Fig. 2). The mean values of other antioxidant enzymes had a similar pattern as shown for SOD. These were significantly lower ( $p < 0.005$ ) in ACS than in controls, while there was no significant change in the CSA group. (Table III, Fig. 3). The plasma tHcy levels in ACS as well as in CSA patients were higher by almost three times the levels in the controls, and these differences were statistically significant ( $p < 0.001$ ) (Figs. 3 & 4, Table III).

The statistical correlations of the lipid ratio values with those of the pro- and antioxidants did not show any significant relationship. Most of the indices displayed unsatisfactory correlation coefficient values (range  $-0.246$  to  $0.172$ ), showing no association with any of the individual biochemical parameters of oxidative metabolism. Serum folic acid levels were highest in CSA and almost four times the level seen in the controls, while in ACS, the levels were

**Table III. Plasma homocysteine, pro- and antioxidants in the ischaemic heart disease and control groups.**

Group	No. of subjects	Plasma tHcy (µmol/L)	Serum TBARS (nmole/L)	Serum SOD (Units/ml)	Erythrocyte GPx (Units/ml)	Erythrocyte catalase (Kat.f units)
Controls	50	10.76 ± 02.77	1.78 ± 0.19	3.46 ± 0.63	546.62 ± 43.18	100.82 ± 12.29
ACS	42	32.48 ± 18.99*	3.70 ± 0.41*	2.11 ± 0.73*	337.56 ± 28.18*	74.95 ± 9.62*
CSA	50	30.30 ± 17.72*	2.03 ± 0.14	2.92 ± 0.68	503.13 ± 84.45	96.90 ± 16.13

Data is expressed as mean ± SD. \*p < 0.001, compared to controls.

tHcy: total homocysteine; TBARS: thiobarbituric acid reactive substances; SOD: superoxide dismutase; GPx: glutathione peroxidase; ACS: acute coronary syndrome; CSA: chronic stable angina.

**Table IV. Plasma homocysteine and vitamins in the ischaemic heart disease and control groups.**

Group	No. of subjects	Plasma tHcy (µmol/L)	Serum folic acid (ng/ml)	Serum vitamin B <sub>12</sub> (pg/ml)
Controls	24	10.76 ± 02.77	6.53 ± 5.23	259.3 ± 81.1
ACS	23	32.48 ± 18.99*	18.49 ± 8.41*	1007.2 ± 89.2*
CSA	16	30.30 ± 17.72*	23.69 ± 6.84*	978.8 ± 85.3*

Data is expressed as mean ± SD. \*p < 0.001, compared to controls.

tHcy: total homocysteine; ACS: acute coronary syndrome; CSA: chronic stable angina.

almost three times higher (Table IV). The values for serum B<sub>12</sub> in both groups were also significantly higher by four-fold in ACS and by three-fold in CSA (p < 0.001) than that of the controls (Fig. 5). There was no significant correlation between tHcy and vitamin levels in the patients. The 'r' values fluctuated between -0.593 and +0.353.

## DISCUSSION

Although the relationship between classical risk factors and cardiovascular mortality is widely known, recent studies have shown that only < 50 % of cardiac patients present with classical risk factors.<sup>(9,11)</sup> Thus, the current information on classical risk factors only partly explains the risk factors in CVD. The present results on classical lipids show elevated values in all the patients. The mean levels were significantly higher in ACS than in CSA compared to the controls. High plasma cholesterol is known to alter the cholesterol: phospholipids ratio in the plasma membrane, which leads to increased membrane viscosity. These changes facilitate monocyte adhesion and chemotaxis that precedes the development of fatty streaks.

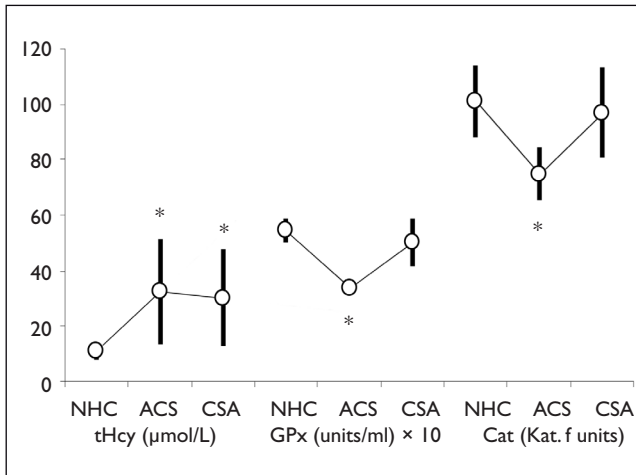
Hypertriglyceridaemia can occur either by the overproduction of VLDL-TG or lipoprotein particles or impaired catabolism of TG-rich lipoproteins or a combined defect in TG metabolism. It is also likely that the observed raised TG and low HDLc in the patients could be related to a highly carbohydrate rich diet. The recommended range is 10,500–12,600 kJ/d, in contrast to the subjects consuming 12,600–33,600 kJ/d; or it may be due to a relative insulin resistance in the patients. It is known physiologically that insulin resistance leads to enhanced hepatic VLDL, TG

secretion rates and therefore hypertriglyceridaemia.<sup>(24)</sup>

It has been well-established that individual classic lipids are a predictor of IHD risk; however, the sensitivity of prediction increases further if the ratios of these lipids are considered. The ratios such as TC:HDLc, TG:HDLc and LDLc:HDLc are all indicators of atherogenicity, since the common denominator in these ratios is an anti-atherogenic lipid, HDLc, while the numerators are atherogenic lipids. The results, when analysed in terms of these ratios, show a similar pattern to the individual lipids. TC:HDLc and TG:HDLc ratios are indicators of the HDL sub-class distribution.<sup>(25)</sup> The parallel increased ratios show small HDL size and the maturation of HDL is impeded, therefore indicating a weakened reverse cholesterol transport in the patients.

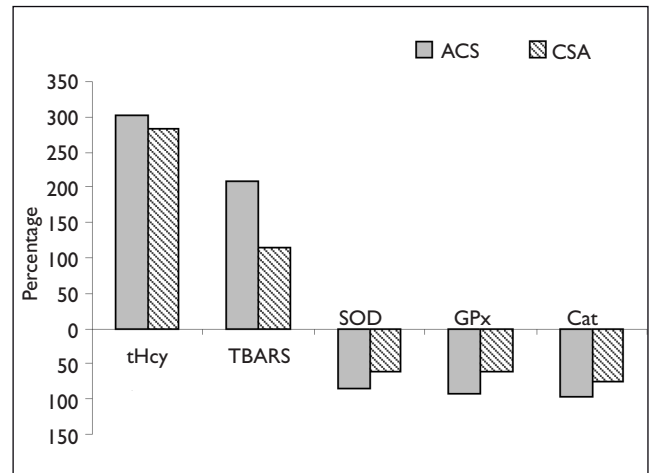
There are numerous reports that indicate some role for geographical or ethnic factors in IHD.<sup>(2,6,8,9)</sup> In a case-control study, Asian Indians with CAD had a 40% higher mortality rate as compared to Europeans.<sup>(9)</sup> In a recent report, it was pointed out that Southeastern Asians are more prone to develop ACS at a younger age compared to individuals from other countries.<sup>(2)</sup> Precise aetiological factors for these observations remain elusive. Many studies have demonstrated the role of Hcy as an independent risk factor.<sup>(7-9,26,27)</sup> A national representative survey study from the USA reported that a two-fold increase in Hcy increases the likelihood of myocardial infarction.<sup>(26)</sup> The present results clearly show that HHcy in the patients with a mean tHcy are almost three times higher than that of the controls. These results indicate a higher prevalence of HHcy in rural Indian patients.

It is now increasingly being realised that prolonged



**Fig. 3** Graph shows the serum total homocysteine (tHcy) and antioxidant enzymes (GPx: glutathione peroxidase; Cat: catalase) activities in erythrocytes from the patients (ACS and CSA) and in the normal healthy controls (NHC).

Vertical bars indicate the standard deviation. Mean values of GPx and Cat in ACS are significantly lower than in CSA and controls. \*  $p < 0.001$ , as compared to NHC.



**Fig. 4** Bar chart shows the percentage changes in the mean values of the pro- and antioxidant parameters in the ACS and CSA patients with reference to the mean values in the normal healthy controls.

tHcy: total homocysteine; TBARS: thiobarbituric acid reactive substances; SOD: superoxide dismutase; GPx: glutathione peroxidase; Cat: catalase.

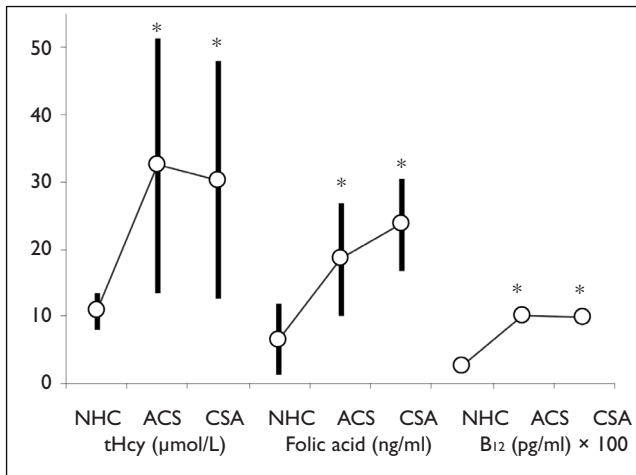
high-grade oxidative stress plays an important role in the pathogenesis of CVD.<sup>(10)</sup> HHcy should be viewed as the major risk factor believed to exert its effect through a mechanism involving oxidative stress. Several hypotheses have been proposed to explain the cellular mechanism by which HHcy promotes CVD. Hcy has been shown to activate endothelial cells through upregulation of components of the inflammatory cascade. Hcy most likely activates the NFκB transcription factor, through the creation of oxidative stress by altering the redox thiol status of the cells.<sup>(28)</sup> As a consequence, there is increased production of pro-inflammatory cytokines and expression of adhesion molecules and chemotactic factors in HHcy.

Elevation of plasma Hcy is multifactorial. These factors include dietary intake, metabolic clearance, vitamin deficiencies, enzyme blocks, age, gender, serum creatinine and multivitamin usage.<sup>(29)</sup> The patients and the controls were consuming a normal diet. There was no qualitative or quantitative difference in the diet consumed by both groups in this study. The diet consisted of typical Maharashtrian food with chapattis (prepared from wheat/millet/sorghum flour), rice, dahl and vegetables (dry/with gravy). This accounted for an average daily intake of 600–1,500 g carbohydrates, 70–180 g fats and 30–50 g proteins. Therefore, the possibility of dietary factors affecting the results is ruled out as the patients had identical dietary habits and history as the controls.

*In vivo*, Hcy is normally metabolised by two principal pathways. One route is via the trans-sulphuration via cystathionine to cysteine, and the other route is via the trans-

methylation by methionine synthase involving B<sub>12</sub> and folate coenzymes.<sup>(30)</sup> A block in the former route is unlikely as the renal clearance of Hcy or cysteine was quite normal in the patients.<sup>(31)</sup> There was no evidence of any abnormal renal functions in the patients. It appears that the re-methylation route of Hcy could be blocked due to vitamin deficiencies. In this context, it has been shown that high plasma Hcy could be reversed with vitamin supplements.<sup>(26)</sup> However, correlative studies on Hcy, folates and B<sub>12</sub> in Asian Indians are sparse and controversial.<sup>(32-36)</sup> Some studies reported increased levels,<sup>(35,36)</sup> while others did not show any difference.<sup>(32,34)</sup> Our results demonstrated that blood folates and B<sub>12</sub> levels were in fact three-fold higher than the controls. As the patients and the controls were consuming a normal diet which was qualitatively and quantitatively similar, the dietary factor was excluded as a possible cause. Similarly, none of the subjects recruited for the study were on prophylactic multivitamins (including folates and/or vitamin B<sub>12</sub>). Thus, the cause of raised blood levels due to an exogenous therapeutic factor was ruled out. As the age and gender were matched between the patients and the controls, this also could not be a contributing factor. The only remaining possibility was an impaired metabolic clearance and/or enzyme block.

It seems that vitamin levels were saturated in the patients. However, these are relatively unavailable for the metabolism of Hcy.<sup>(27)</sup> The simple logical reason that presents itself is that the conversion of these vitamins to their bioactive form of coenzymes is blocked. Recent reports strongly suggest that abnormalities in HHcy have genetic



**Fig. 5** Graph shows the serum levels of vitamins (folic acid and vitamin B<sub>12</sub>) and total homocysteine (tHcy) in the patients (ACS and CSA) as compared to the healthy controls (NHC). Vertical bars indicate the standard deviation. The mean value of folic acid is almost three times higher in ACS compared to NHC, while B<sub>12</sub> levels in the patients are almost four times higher in the patients compared to NHC.  
\*  $p < 0.001$ , as compared to NHC.

links.<sup>(37,38)</sup> It has been demonstrated that there is significant association of HHcy with methyltetrahydrofolate reductase and methionine synthase genotypes.<sup>(39)</sup> This provides strong support to the most likely case that remethylation of Hcy is blocked in these patients from rural India, causing HHcy. An alternative route of Hcy metabolism in HHcy is the formation of Hcy-thiolactone which then reacts with LDL to form LDL-Hcy-thiolactone aggregates. These are taken up by macrophages and are subsequently incorporated into foam cells in early atherosclerotic plaques. Within these plaques, Hcy-thiolactone acylates proteins and modifies oxidative processes of the vessels, thereby promoting atherothrombosis. In addition, auto-oxidation of Hcy results in the formation of superoxide and hydrogen peroxide.<sup>(10)</sup> These reactive oxygen derived molecules may contribute to the oxidation of LDL and endothelial dysfunction and promote the proliferation of smooth muscle cells.

The present results reflect and support the oxidative hypothesis of HHcy in IHD. The pro-oxidants, such as TBARS and Hcy, were significantly raised while the activities of antioxidant enzymes, such as SOD, GPx and catalase, were all significantly lowered in ACS. The changes in CSA patients were mild, demonstrating that oxidative stress was reduced as the influences of pro-oxidants was weaned out during the post-ischaemic recovery phase. Therefore, the available current information and the present results taken together suggest that it is very essential to consider the contribution of Hcy as the principle biomarker in risk stratification for ischaemic heart diseases which must be evaluated with other conventional risk factors

of CVDs. This biomarker has proven to be a major risk indicator for CVDs in general, and specifically for Indian patients with strong genetic links.

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