Lowering homocysteine and modifying nutritional status with folic acid and vitamin B₁₂ in Indian patients of vascular disease

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Hyperhomocysteinemia is more commonly associated with vascular disease in Indians than in the western populations. It is caused by genetic polymorphisms or dietary deficiencies of the B vitamins. We attempted to identify the association of hyperhomocysteinemia with vitamin B₁₂ and folate in Indian patients of vascular disease. Homocysteine, vitamin B₁₂ and folate levels were estimated in 100 controls and 100 patients of vascular disease. Homocysteine estimation was repeated in 73 patients on different vitamin supplements for 6 months. Homocysteine exhibited a significant negative correlation with B₁₂ only in cerebrovascular disease and peripheral vascular disease patients, and with folate in coronary artery disease and cerebrovascular disease patients as well as controls. Single daily dose of folate was as effective as a combination of folate and cobalamin in reducing plasma homocysteine concentrations. Low levels of B₁₂ contribute to the higher incidence of cerebrovascular disease and peripheral vascular disease, and low folate levels account for higher prevalence of hyperhomocysteinemia in coronary artery disease and cerebrovascular disease. Moreover, irrespective of the cause of hyperhomocysteinemia, folate is known to ameliorate it. Hence, large-scale corrective measures like food fortification or dietary supplementation with folate might benefit the Indian population and reduce the incidence and morbidity of vascular disease.

Key Words: vitamin B₁₂, folate, food fortification, hyperhomocysteinemia, vascular disease

Epidemiological transition has resulted in an increase in morbidity and mortality due to degenerative diseases such as vascular disease. This has predominantly been seen in developing countries, that now account for 80% of the global burden of vascular disease.¹ In such countries, conventional markers are often within the biological reference interval and, therefore, cannot account for the higher incidence of vascular disease. This has led to a search for newer markers, of which homocysteine has emerged as a significant marker of vascular disease, especially in patients of Asian origin.² Several studies have elucidated an association of hyperhomocysteinemia with vascular disease independent of the conventional factors.³–⁶

The association of homocysteine with vascular disease is demonstrated to be inter-related with its role in the methionine metabolism. In the remethylation cycle, homocysteine is irreversibly integrated to the folate cycle (Fig. 1), where circulating folate is converted to tetrahydrofolate (THF). This, on methylation, yields 5,10 methylene THF, which is then reduced to 5-methyl THF by the action of the enzyme methylene tetrahydrofolate reductase (MTHFR). The remethylation cycle is so named as homocysteine gets remethylated to methionine by acquiring a methyl group from 5-methyl THF from the folate cycle through a reaction catalysed by the enzyme methionine synthase that requires vitamin B₁₂ as a cofactor. Hence, folate and vitamin B₁₂ are both necessary for the remethylation of homocysteine.

The methionine, thus yielded, is further converted to S-adenosyl methionine (SAM), the universal methyl donor of the human metabolism. If SAM production is deficient, there results a hypomethylation of several proteins and DNA causing a destabilisation of these molecules. Ultimately, this translates into synthesis of defective proteins and collagen resulting in vascular structures and neurons that are more susceptible to damage and apoptosis.⁷

Thus, it follows that homocysteine metabolism is dependent on folate and vitamin B₁₂. Deficiencies of these vitamins would result in hyperhomocysteinemia.⁸⁻⁹ Several studies have demonstrated an association of deficiency of B₁₂ and folate with raised plasma homocysteine concentration.¹⁰ This may acquire more importance in developing countries, like India, where the burden of vascular disease is compounded by the continuing burden of nutritional deficiencies—the double burden.¹¹ In such countries, it would, therefore, be pertinent to institute measures to reduce both nutritional deficiencies as well as risk of vascular disease. This study was therefore conducted to assess the role of vitamin B₁₂ and folate deficiencies as a cause of hyperhomocysteinemia in North Indian patients of vascular disease and, thereby, assess the justification of food fortification for preventing vascular disease.

Materials and Methods

The project was approved by the Hospital Ethics Committee as conforming to the Declaration of Helsinki. One hundred patients were selected amongst those who had been admitted to Sir Ganga Ram Hospital, New Delhi, India, in the departments of neurology, cardiology/cardiac surgery, or amongst those who were being treated for peripheral vascular disease on an out-patient or in-patient basis. Informed consent was obtained from all subjects. They were then categorised as per the type of disease and the diagnoses confirmed by ultrasonography, angiography, computerised axial tomography (CAT) scan and Doppler studies (by Duplex Sonography). Of these, thirty-five patients were diagnosed as coronary artery disease (CAD), 35 as cerebrovascular disease (CVD), and 30 as peripheral vascular disease (PVD). One hundred healthy controls were selected who had no personal or family history of any vascular disease or chronic illness. All subjects were then allocated to the control group or to one of the four treatment groups: 1), 2), or 3).

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were above 18 years of age. Blood samples were drawn from all subjects and vitamin B₁₂, folic acid and homocysteine levels were estimated by chemiluminescent immunoassay. Seventy three of these hundred patients of vascular disease were followed up for six months during which time they were administered various homocysteine-lowering vitamin regimen. At the end of six months, their blood samples were drawn and a repeat homocysteine estimation done. Statistical analysis of the data was done by the SPSS 17 statistical package (SPSS, Chicago, IL). Data was expressed as mean ± SEM. Significance of association was determined by regression analysis, Student’s t test and correlation studies.

Results

As shown in Table 1, vitamin B₁₂ levels were found to be similar in controls and patients, as a whole, and even when taken as individual categories (CAD, CVD and PVD). Folate levels were significantly reduced (though well within the Biological Reference Interval-BRI) in the patients as compared to the controls. Amongst the different categories of patients, it was significantly reduced in patients of CAD and CVD, but not in patients of PVD. Homocysteine levels were significantly higher in all patient categories as compared to the controls. As seen in Tables 2 and 3, homocysteine levels bore a negative correlation with vitamin B₁₂ levels as well as with folic acid levels. Table 2 depicts the correlation of homocysteine levels with those of vitamin B₁₂ in controls as well as patients of vascular disease. In controls, homocysteine levels bore an insignificant negative correlation with vitamin B₁₂ levels. When patients of vascular disease were taken as a whole, homocysteine levels bore a significant negative correlation with vitamin B₁₂ levels. This significance was due to the significant negative correlation between homocysteine levels and vitamin B₁₂ levels in patients of CVD and PVD. In CAD, too, homocysteine levels bore a negative correlation with vitamin B₁₂ levels, but this was not significant.

Table 3 depicts the correlation of homocysteine levels with concentration of folic acid levels in controls as well as patients. In controls, homocysteine levels bore a significant negative correlation with vitamin B₁₂ levels. In patients, too, the negative correlation between homocysteine levels and folic acid levels was highly significant. In patients of CAD and CVD, this negative correlation was significant, but in patients of PVD, it was insignificant.

Fig. 1. Homocysteine metabolism and its relation with folate cycle.

DNA Deoxyribonucleic Acid dTMP Thymidine monophosphate dUMP Deoxyuridine monophosphate
DHF Dihydrofolate THF Tetrahydrofolate DMG Dimethyl Glycine
SAM S-Adenosyl Methionine SAH S-Adenosyl Homocysteine MS Methionine Synthase
MTHFR Methylene Tetrahydrofolate Reductase CBS Cystathionine-β-Synthase

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In the developing countries, India being one of them, the increased incidence of vascular disease adds to the continuing burden of infectious, nutritional and perinatal diseases, which has been termed the double burden. (1997–1999 World Health Statistics). Also, Yusuf et al. reported that the predominant further categorized into those with folate deficiency (i.e. folate <3.0 ng/mL), those with normal folate levels but below the mean of the BRI (folate 3.0 to 10.0 ng/mL), and those with normal folate with values above the BRI mean (folate >10.0 ng/mL). The reduction in plasma homocysteine concentrations achieved in 6 months was similar in all three categories (p value ranging from 0.9987 to 0.3266). Similarly these patients were again segregated into three categories on basis of their vitamin B12 levels (B12 <3.0 pg/mL; B12 3.0 to 590 pg/mL and B12 >590 pg/mL). Again the reduction in plasma homocysteine concentrations achieved was not significantly different in the three categories (Tables 4 and 5). A similar exercise of segregation of patients receiving a combination of 500 mg of B12 and 1.5 mg of folate, on basis of their folate levels or their B12 levels revealed that the reduction in plasma homocysteine concentrations achieved in all the categories was similar.

### Table 1. Serum levels of vitamin B12, folic acid and homocysteine in controls and patients

| Category   | Vitamin B12 in pg/mL | Folic Acid in ng/mL | Homocysteine in μmol/L |
|------------|----------------------|---------------------|------------------------|
|            | Mean ± SEM           | Mean ± SEM          | Mean ± SEM             |
| Controls   | 505.69 ± 29.98       | 13.04 ± 0.71        | 12.42 ± 0.85           |
| [n = 100]  | p = 0.991            |                     |                        |
| All Patients| 506.18 ± 30.47       | 9.51 ± 0.61         | 22.33 ± 2.06           |
| [n = 100]  |                      | p = 0.000**         |                        |
| CAD        | 516.74 ± 50.89       | 8.40 ± 0.71         | 27.17 ± 4.16           |
| [n = 35]   |                      | p = 0.001**         |                        |
| CVD        | 588.86 ± 58.27       | 9.49 ± 1.28         | 19.30 ± 2.41           |
| [n = 35]   |                      | p = 0.004**         |                        |
| PVD        | 397.40 ± 41.06       | 10.84 ± 1.11        | 20.21 ± 3.71           |
| [n = 30]   |                      | p = 0.078           |                        |

p values here are comparison of each group with controls. *p<0.05 significant. **p<0.005 highly significant. Homocysteine concentrations were significantly lower in all patient categories as compared to controls. However, only folic acid levels were significantly lower in patients of CAD and CVD than in controls. The levels of both vitamin B12 and folic acid were within the BRI in controls as well as patients.

### Table 2. Correlation between plasma homocysteine and serum vitamin B12 levels

| Category   | Correlation | p value |
|------------|-------------|---------|
| Controls   | -0.165      | 0.102   |
| [n = 100]  |             |         |
| All Patients| -0.263      | 0.008*  |
| [n = 100]  |             |         |
| CAD        | -0.111      | 0.526   |
| [n = 35]   |             |         |
| CVD        | -0.426      | 0.011*  |
| [n = 35]   |             |         |
| PVD        | -0.46       | 0.010*  |
| [n = 30]   |             |         |

*p<0.05 significant. **p<0.005 highly significant. Despite vitamin B12 levels being within the BRI in all patients of disease, plasma homocysteine bore a significant negative correlation with vitamin B12 in patients of CVD and PVD.

### Table 3. Correlation between plasma homocysteine and serum folic acid levels

| Category   | Correlation | p value |
|------------|-------------|---------|
| Controls   | -0.241      | 0.016*  |
| [n = 100]  |             |         |
| All Patients| -0.294      | 0.003** |
| [n = 100]  |             |         |
| CAD        | -0.349      | 0.040*  |
| [n = 35]   |             |         |
| CVD        | -0.377      | 0.025** |
| [n = 35]   |             |         |
| PVD        | -0.208      | 0.271   |
| [n = 30]   |             |         |

*p<0.05 significant. **p<0.005 highly significant. Plasma homocysteine levels bore a significant negative correlation with folate levels in controls as well as patients of CAD and CVD.

Thus, it is observed that -folic acid levels were significantly less in CAD and CVD patients as compared to controls, -vitamin B12 levels were not significantly reduced in any patient category as compared to controls, -in CAD, homocysteine levels bore a significant negative correlation with folic acid levels, but not with vitamin B12 levels, -in CVD, homocysteine levels bore a significant negative correlation with vitamin B12 levels as well as folic acid levels, -in PVD, homocysteine levels bore a significant correlation only with vitamin B12 levels but not with folic acid levels.

This indicates that the vascular morbidity due to hyperhomocysteinemia could be due, at least partly, to relative deficiencies of folate and vitamin B12. Estimation of homocysteine levels was repeated in the plasma of seventy three patients on different regimen of vitamin therapy to ascertain their response. Forty one patients received a single daily dose of 5 mg of folic acid, and 33 received a combination of 500 mg of cobalamine (vitamin B12) and 1.5 mg of folate (folic acid). Repeat estimation of homocysteine was done at the end of 6 months and the average decrease in plasma homocysteine levels calculated. The response to therapy (in terms of percentage reduction of homocysteine over a period of six months) was similar in the patients receiving a single daily dose of only 5 mg folate (39.17%) and those receiving a combination of 1.5 mg of folate and 500 mg cobalamine (37.02%).

The patients receiving a single daily dose of 5 mg folate were
diseases are now atherosclerotic vascular disease, 80% of such cases being found in the developing countries. Thus, we are faced with a double burden as well as 80% of the global burden of vascular disease. Hence, it is imperative that our efforts should be continuous and unslacked towards identifying all risk factors, for vascular disease especially, and taking appropriate action to minimize their pathogenicity and, thereby, reduce the morbidity and mortality due to vascular disease the world over.

Many cross-sectional and case-control studies have demonstrated the association of homocysteine with occlusive vascular disease.(3–10) Most of these studies have been conducted in a variety of populations, but did not include subjects from the Indian subcontinent. Since 80% of the global burden of vascular disease is in the developing countries (India being one of them), it follows that there is a need to gather data from the Indian subcontinent and subsequently institute appropriate preventive measures. The role of vitamin B12 and folate in the metabolism of homocysteine is shown in Fig. 1. When either folate or B12 are deficient, the remethylation of homocysteine slows down, homocysteine accumulates and its level in the circulation rises, predisposing to atherothrombotic plaque formation.

As revealed in Table 1, the mean levels of the vitamins B12 and folate in controls as well as patients were well within the BRI, but the mean folate level in vascular disease patients was significantly less (p = 0.000) than that in the controls (even though it was within the BRI). This significance was due to the significantly lower levels of folate in the patients of CAD and CVD as compared to those in controls (Table 3). This would confer an association of folate with CAD and CVD categories of vascular disease, even within its BRI.

In many Western and European populations, it has been reported by at least 10 scientists that homocysteine decreases as folate levels increase. (8,11–20) The results of our study corroborate these observations. We found that serum folate levels correlated negatively with the plasma homocysteine concentrations in patients of vascular disease and healthy controls. Also, Sauberlich et al.(21) demonstrated that dietary intake of folate reflects biochemically measured folate status. Hence, it would follow that increasing dietary folate would increase serum folate and, thereby, reduce plasma homocysteine concentrations. The end result would be reduction of risk of vascular disease. This has also been demonstrated by several scientists in Western and European populations even in subjects with normal levels of folate. (9,13,22–27)

On the other hand, vitamin B12 supplements have been found to be effective in lowering homocysteine only in those with overt cobalamin deficiency. (9,23,25,29) Since most of our subjects (controls as well as patients) had normal B12 levels, there should have been a lack of correlation between homocysteine levels and vitamin B12 levels; but this was not observed to be so in our vascular disease patients group in whom there was a significant negative correlation between plasma homocysteine concentrations and serum vitamin B12 levels (Table 2). This may indicate the need to revise the BRI of vitamin B12 in the developing countries.

It has been demonstrated that the risk of vascular disease is 40% higher in Indians as compared to Europeans,(30) and that Indians are prone to vascular disease 5–10 years earlier than other ethnicities, and the disease progression is more severe. (31) In addition, the Western and European countries are amongst the more developed countries as compared to India (which is a developing country), and nutritional deficiencies are part of our double burden of disease. This would indicate that dietary supplements would be even more effective in our population. As evidenced by our current data, dietary supplements with folate as well as vitamin B12 should benefit us. In keeping with this line of thought, we followed up 73 patients for 6 months and did a repeat estimation of homocysteine thereafter. These patients were on different regimen of homocysteine-lowering vitamin therapy: forty-one patients received a single daily dose of 5 mg of folic acid, thirty-three patients received a combination of 500 mg of B12 and 1.5 mg of folic acid everyday.

The administration of a single daily dose of 5 mg of folic acid achieved as much reduction in homocysteine levels as the combination of folate (1.5 mg) and vitamin B12 (500 mg). These are higher doses of folate than suggested by previous reports. (32) The reduction in homocysteine levels achieved (37.02%) by the daily administration of a combination of 500 μg of B12 and 1.5 mg of folic acid was similar to that achieved by the administration of a single daily dose of 5 mg of folate (39.17%). This was irrespective of the presence or absence of individual deficiencies of folate or vitamin B12, or even the concurrent presence of deficiencies of both these vitamins.

Thus, we see that in Indians, vascular disease due to hyperhomocysteinemia is correlated to the serum folate and vitamin B12 concentrations. Also, a single daily dose of 5 mg of folic acid is as effective in reducing homocysteine, as a combination of 500 μg of B12 and 1.5 mg of folic acid, even when the serum vitamin levels are within the BRI.

The Food and Drug Administration (FDA), USA, has discussed food fortification of flours and cereal products at 350 mg of folate per 100 gram of flour/cereal as the best means of population folate supplementation for the prevention of neural tube defects in the American population. Boushey et al.(32) projected that such a fortification would annually prevent 50,000 deaths due to CAD, alone, in the Western population. There is, therefore, a need to extrapolate these data to North Indian urban population, keeping in mind that as per epidemiological transition, India is weighed down by a double burden of disease nutritional deficiencies as well as degenerative diseases (e.g. vascular disease). It would follow that not only is there a greater need for folate supplementation, but also that probably a higher dose (equivalent of

| Table 4. Response to single daily dose of 5 mg folic acid in terms of reduction of homocysteine levels over a period of 6 months |
|--------------------------------------------------|
| Patient group | No. of patients | Average % reduction in plasma homocysteine levels |
|----------------|-----------------|-----------------------------------------------|
| Folate <3 ng/mL | 6               | 51.3                                          |
| Folate 3–10 ng/mL | 28              | 42.59 [p = 0.865]                             |
| Folate >10 ng/mL | 7               | 24.31 [p = 0.999]                             |
| B12 <220 pg/mL  | 16              | 42.18 [p = 0.964]                             |
| B12 = 220–590 pg/mL | 17            | 37.84 [p = 0.886]                             |
| B12 >590 pg/mL  | 8               | 34.78 [p = 0.996]                             |

Patients of vascular disease who had received a single daily dose of 5 mg folate for 6 months were segregated first on basis of their folate levels and then on the basis of their B12 levels. | Table 5. Response to combined daily dose of 1.5 mg folate and 500 μg cobalamin in terms of reduction of homocysteine levels over a period of 6 months |
|--------------------------------------------------|
| Patient group | No. of patients | Average % reduction in plasma homocysteine levels |
|----------------|-----------------|-----------------------------------------------|
| Folate <3 ng/mL | 4               | 37.76                                          |
| Folate 3–10 ng/mL | 19              | 32.89 [p = 0.329]                             |
| Folate >10 ng/mL | 8               | 41.27 [p = 0.408]                             |
| B12 <220 pg/mL  | 9               | 46.00 [p = 0.515]                             |
| B12 = 220–590 pg/mL | 16            | 36.22 [p = 0.356]                             |
| B12 >590 pg/mL  | 7               | 33.12 [p = 0.374]                             |

Patients of vascular disease who had received combination of 1.5 mg folate and 500 μg cobalamin were segregated first on basis of their folate levels and then on basis of their vitamin B12 levels. The average percentage reduction in homocysteine levels was not significantly different in any group.
daily dietary intake of 1.5 mg of folate) would be required to be equally effective.

Hankey and Eikelboom\(^\text{226}\) described a potential hazard of therapy with folate alone. Progressive neurological damage (subacute combined degeneration of the spinal cord) has been seen in subjects with subclinical vitamin B\(_{12}\) deficiency in whom folic acid therapy may mask the development of the haematological manifestations of the B\(_{12}\) deficiency.

In view of the above-mentioned hazard of folate therapy, and the fact that the homocysteine lowering effect of 5 mg of folate alone as well as a combination of folate (1.5 mg) and cobalamin (500 mg) are similar, food fortification with both these cheap, easily available, water-soluble vitamins would best benefit the North Indian population. It would, hence, be pertinent to suggest that scientists, clinicians and policy-makers in our country should further evaluate the relevance of food fortification with folate and vitamin B\(_{12}\) so as to correct nutritional deficiencies as well as reduce risk for vascular disease—the double benefit for the double burden of disease in the developing countries.

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**Abbreviations**

BRI biological reference interval
CAD coronary artery disease
CVD cerebrovascular disease
MTHFR methylene tetrahydro folate reductase
PVD peripheral vascular disease
SAM s-adenosyl methionine
THF tetrahydrofolate

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