Homocysteine in occlusive vascular disease: A risk marker or risk factor

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Homocysteine has emerged as a significant marker for occlusive vascular disease, but there has been some debate as to whether it is just an association (risk marker) or actually a causative factor (risk factor). To elucidate this, a retrospective statistical analysis was done of data generated in the course of our study on homocysteine and vascular disease. Homocysteine, lipid profile components and lipoprotein(a) were estimated in fasting blood samples drawn from 252 controls and 536 patients of occlusive vascular disease. The data were analyzed by SPSS version 17. Mean homocysteine levels were significantly higher (p<0.001) in all patients categories, as compared to controls. In fact, homocysteine level was the most significant biochemical risk factor for vascular disease. The odds ratios due to hyperhomocysteinemia varied from 3.170-4.153. When the cut-off was increased by 5 µmol/L, the odds ratio became almost three-fold. The prevalence of hyperhomocysteinemia increased by ≅20%, when the cut-off was reduced by 5 µmol/L. Statistical analysis of our data revealed that homocysteine conformed to Hill’s criteria of causation. Moreover, hyperhomocysteinemia was treatable by the administration of B-vitamins, even if the cause was genetic. Hence morbidity due to vascular disease could be reduced by identification and treatment of hyperhomocysteinemia.

Keywords: Homocysteine, Hill’s criteria, Risk marker, Risk factor, Occlusive vascular disease.

It is well-documented that the pathogenesis of vascular disease is multi-factorial. Many risk factors have traditionally been associated with atherosclerosis and atherothrombosis. Apart from disease states like hypertension, diabetes mellitus and chronic renal failure, the major biochemical risk factors implicated are cholesterols and triglycerides, which have been coined “conventional risk factors”. Recently, the levels of these “conventional risk factors” in many cases of vascular disease have been within the biological reference interval (BRI). Hence it has become necessary to identify new risk factors.

The lesions of vascular disease represent the result of a complex, multi-cellular, inflammatory-healing response in the vessel wall which involves all the layers of these walls. These lesions are heralded by an altered vascular endothelium. Hence any substance contributing to such an alteration becomes a risk marker/factor for vascular disease. One such substance is homocysteine, a naturally occurring sulphur-containing amino acid derived primarily from the breakdown of dietary methionine in the activated methylation cycle. Homocysteine either re-enters the cycle to form methionine or gets trans-sulfurated to cysteine and α-ketoglutarate. These reactions are dependent on vitamins of the B group (B6, B12 and folate) and the enzymes methylenetetrahydrofolate reductase (MTHFR) and cystathionine-beta-synthetase (CBS). It follows that deficiency of any of these vitamins or polymorphisms of the genes for either of these enzymes would lead to accumulation of homocysteine in the blood stream. Interestingly, homocysteine concentrations in blood are known to decrease by the administration of the B vitamins, specifically B6, B12 and folate, irrespective of the cause of hyperhomocysteinemia.

Although homocysteine was initially discovered by du Vigneaud in 1931, it was only in 1969 that McKully first described its association with atherosclerosis while discussing the autopsy findings of two children with homocysteinuria. In the last four decades, many cross-sectional and case-control studies have demonstrated the association between homocysteine and vascular disease. Yet there has been a lot of debate as to whether homocysteine is a risk marker or a risk factor. The data generated in
the course of our study on the prevalence of hyperhomocysteinemia in Indian patients of vascular disease was, therefore, retrospectively analyzed to evaluate whether homocysteine conforms to Hill’s criteria defining risk factors.

**Materials and Methods**

**Subjects**

The subjects included 536 patients of occlusive vascular disease and 252 healthy controls. The subjects were 18-75 yrs of age and did not have any past history or family history of vascular disease or any other chronic disease like hypertension, chronic renal disease and diabetes mellitus. The 536 patients were selected from amongst those attending the cardiology, neurology or the vascular and endovascular surgery clinics for the first time and were diagnosed as patients of occlusive vascular disease as confirmed by ultrasound, CT scan, angiography or Doppler studies. These patients were further categorized as per the organ system involved into coronary artery disease (CAD), cerebrovascular disease (CVD) and peripheral vascular disease (PVD). Thus, there were 226 patients of CAD, 189 patients of CVD and 121 patients of PVD.

**Biochemical analysis**

Homocysteine, components of the lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides) and lipoprotein(a) were estimated in the fasting blood samples of all subjects. All the biochemical investigations were performed on fully automated analyzers. Homocysteine was estimated by chemiluminescent immunoassay\(^\text{10}\) which was performed on Immulite (Siemens). Total cholesterol was estimated by the cholesterol oxidase peroxidase method (CHOD-POD)\(^\text{11}\). HDL and LDL cholesterol were estimated by the elimination method followed by CHOD-POD\(^\text{12}\). For triglycerides, GPO-PAP method was employed\(^\text{13}\). Lipoprotein(a) was quantitated by latex agglutination turbidimetry\(^\text{14}\). These parameters [lipid profile and Lp(a)] were estimated on the Beckman Coulter DX-C 800.

**Follow-up**

In addition, 54 patients with hyperhomocysteinemia were followed up for at least six months after reduction of plasma homocysteine levels to within the BRI to assess incidence of recurrence of vascular events. Repeat estimation of homocysteine was done every 3 months till the levels achieved were within the BRI.

**Statistical analysis**

The data generated were subjected to analysis by the SPSS version 17 statistical package (Chicago IL) and expressed as mean ± standard error of mean. Univariate as well as multi-variate analysis was done by applying student’s t test, chi square and Fisher’s exact test. Correlation was analyzed by Pearson correlation coefficient and a p value of <0.05 was considered significant.

**Results and Discussion**

Several risk markers for vascular disease have been identified and demonstrated by a number of epidemiological studies\(^\text{8,15-21}\). These markers have been classified into two categories — those that are proven to be causal have been termed as ‘risk factors’ and those that show an association with vascular disease, but for whom a cause and effect association is yet to be proven have been termed as ‘risk markers’. These risk markers have been described as predisposing factors which work, at least in part, through an impact on other risk factors. To justify preventive measures against these “risk markers”, they would have to be proved as “risk factors”, i.e. that they actually cause disease and are not just associated with the disease process. Whether or not these associations are causal is decided by applying Hill’s Criteria of Causality\(^\text{22}\). These criteria state that a marker may be defined as ‘causal’, if it shows: (i) Biological plausibility and coherence, i.e. in keeping with current knowledge various theories for its effect are biologically plausible, (ii) consistency, i.e. repeated studies in different settings demonstrate an association, (iii) strength, i.e. size of association as measured by correlation and significance, (iv) consideration of alternate explanations, i.e. other possible explanations have been taken into account and ruled out, (v) dose-response relationship, i.e. an increasing amount of exposure increases the risk, (vi) experimental evidence that removal of the exposure reduces risk, and (vii) temporal relationship, i.e. exposure to the marker precedes the outcome.

In the present study, the data generated in the course of our study on the prevalence of hyperhomocysteinemia in Indian patients of vascular disease were retrospectively analyzed to evaluate whether homocysteine conforms to Hill’s criteria defining risk factors. It may be pointed here that the major disadvantages of a retrospective study are the bias due to ignorance of the method of record-keeping, and the difference in the study population
and controls. However, these did not hold true for our retrospective analysis, since the original study was conducted by the current authors, the raw data were available with us and the original study included controls from the same population. Also, since the data for the original study were collected for evaluation of association of hyperhomocysteinemia in vascular disease, it was relevant to the retrospective statistical analysis performed. At the same time, it was not biased as the hypothesis being tested was not considered at the time of data collection.23

Our data analysis with respect to Hill’s criteria of causality is presented here in a step-wise manner.

(i) Biological plausibility and coherence

Several mechanisms of action have been postulated by which homocysteine causes vascular endothelial damage and thereby results in atherosclerosis or thrombosis. These mechanisms involve platelet function abnormalities in arterial thrombosis (Fig. 1) and abnormalities of coagulation and/or fibrinolysis in venous thromboembolism.18 For example, (i) direct toxicity to the endothelium, (ii) increased thromboxane A2 and hence increased platelet adhesion, (iii) suppressed expression of heparin sulphate, protein C and antithrombin III, (iv) stimulation of smooth muscle cell proliferation in substantia propria of vessel walls, hence decreased luminal diameter, increased turbulence and deposition of plaque/thrombus, (v) impaired regeneration of endothelial cells, (vi) impaired regulation of endothelium-derived relaxing factor and related oxides, and (vii) generation of superoxides and hydrogen peroxide leading to oxidative endothelial damage.24-27 All these mechanisms are biologically plausible and coherent as per current knowledge. Hence homocysteine conforms to the first criteria of causality.

(ii) Consistency of association

The mean concentrations of all parameters [homocysteine, lipid profile and Lp(a)] analyzed in 252 controls and 536 patients are tabulated in Table 1. Their significance for each vascular disease category as calculated by univariate analysis and the chi square test are also shown in the table. This revealed that plasma homocysteine concentration was strongly associated with all types of vascular disease. In fact,
the significance of homocysteine was even more than that of the conventional biochemical risk markers. A review of literature has revealed that several cross-sectional and retrospective studies have linked premature vascular disorders with hyperhomocysteinemia. Homocysteine has been implicated even in cases with milder hyperhomocysteinemia and without enzyme defects or deficiencies. A meta-analysis of 27 case-control studies has also related hyperhomocysteinemia to atherosclerotic vascular disease. These indicate that homocysteine fulfills the Hill’s criterion of consistency as a risk “factor” for vascular disease.

(iii) Strength and size of association

Table 1 shows that the mean homocysteine in patients of vascular disease was higher than in the controls by a factor of 2.26. This was highly significant with p<0.001. Hence it fulfilled Hill’s criterion of strength of association.

The odds ratio for CAD conferred by the conventional risk factors i.e. the parameters of the lipid profile and lipoprotein(a) ranged from 1.134 due to elevated total cholesterol (Table 2) to 1.855 due to low HDL-cholesterol. But the odds ratio for CAD conferred by elevated homocysteine levels was 4.153, almost 4-times as much as that conferred by total cholesterol. Similarly, for CVD elevated homocysteine levels conferred an odds ratio of 3.336, which was twice as much as that conferred by low levels of HDL-cholesterol, 2.5-times as much as that conferred by elevated LDL-cholesterol levels or high triglycerides and thrice as much that conferred by elevated levels of total cholesterol or lipoprotein(a). For PVD also, the odds ratio conferred by an elevated homocysteine was 3.170 (Table 2), which far exceeded the corresponding odds ratios conferred by altered levels of total cholesterol, HDL-cholesterol and LDL-cholesterol – 1.034, 1.389 and 1.235, respectively.

The high odds ratio conferred by elevated levels of homocysteine for all categories of vascular disease again implied homocysteine a highly significant “risk
factor” for occlusive vascular disease with a high strength and size of association with vascular disease – one of Hill’s criteria for causality. Several studies have elucidated the odds ratios conferred by homocysteine. For CAD, odds ratios of 1.24, 1.7 and 1.8 for ischemic stroke odds ratios of 1.56, 2.5, 4.2 and 4.6 and for peripheral arterial disease, odds ratios of 1.4, 1.92 and 2.5 have been reported. The data reported from our study corroborated the high odds ratio for vascular disease conferred by hyperhomocysteinemia, as reported in these above-mentioned studies. Hence once again, Hill’s criterion of consistency was fulfilled.

(iv) Consideration of alternate explanations

Univariate analysis of the data with simple logistic regression, comparing the mean value of each parameter in each category of vascular disease patients with the mean in corresponding controls revealed that (i) homocysteine was significant in all three disease categories with a p value of <0.001 (Table 1), (ii) HDL-cholesterol levels were found to be significantly lower in all three vascular disease categories – p<0.001 in CAD, p = 0.008 in CVD and p<0.010 in PVD, (iii) LDL-cholesterol levels were significantly higher only in CAD (p<0.01) and so were lipoprotein(a) levels (p<0.005), and (iv) triglycerides levels were significantly higher in CAD (p<0.001) and CVD (p<0.05).

Thus, on the basis of Hill’s criterion of association, homocysteine was comparable to several “conventional” risk factors like HDL-cholesterol, LDL-cholesterol and triglycerides in its association with vascular disease. Since the p value was least in case of the association of homocysteine with vascular disease as compared to that of HDL-cholesterol, LDL-cholesterol and triglycerides, it was observed to be the most significant biochemical risk factor for vascular disease. In fact, only homocysteine and HDL-cholesterol fulfilled this criterion in all three categories of vascular disease.

But, simple logistic regression analysis is prone to the ‘omitted variable bias’, since it is a standalone calculation for each parameter. Hence to enhance the derivations, a multiple regression analysis or multivariate analysis (Table 3) was performed, where each significant parameter was added to the calculation model one at a time, so that the individual effect of that parameter may be calculated and interdependence of variables may be minimized. This statistical analysis revealed that homocysteine was the only variable that showed high significance in all three categories of vascular disease. Thus, it conformed to Hill’s criterion of significance despite consideration of alternate explanations (i.e. other significant risk factors). Hence it might be inferred that homocysteine was the one risk factor that was highly significant (p<0.001) in all types of vascular disease.

(v) Dose-response relationship

Homocysteine was found to be pathogenic even when its levels were within the BRI, as indicated by the high odds ratio that it conferred to vascular disease even at the level of the population mean. To demonstrate its absence of threshold and dose-response relationship, the odds ratio of elevated levels of homocysteine above the upper limit of the BRI was also calculated for each category of vascular disease (i.e. CAD, CVD and PVD). It was observed that with an increase in homocysteine levels of almost 5 µmol/L (from population mean to upper limit of the reference interval), the odds ratio for CAD, CVD and PVD increased 3-fold as depicted in Table 4. This demonstrated the dose-response relationship between homocysteine levels and vascular disease, thus

<table>
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<th>Parameter</th>
<th>CAD</th>
<th>CVD</th>
<th>PVD</th>
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<td>0.000**</td>
<td>0.000**</td>
</tr>
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<td>HDL-Cholesterol</td>
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<td>-</td>
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<tr>
<td>Triglyceride</td>
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<td>0.002**</td>
<td>-</td>
</tr>
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<td>Lipoprotein(a)</td>
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<td>-</td>
</tr>
<tr>
<td>Age</td>
<td>0.000**</td>
<td>0.000**</td>
<td>-</td>
</tr>
</tbody>
</table>

*p<0.05 was considered significant; **p<0.005 was considered highly significant

<table>
<thead>
<tr>
<th>Category</th>
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<th>Odds ratio due to Hcy &gt;15.0 µmol/L</th>
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fulfilling yet another of Hill’s criteria of causality, which states that an increasing amount of exposure should increase the risk of disease. In fact, these findings go one step further and indicate a multiplicative increase in pathogenecity by increasing levels of homocysteine.

The dose-response criterion of causality was also demonstrated by calculating the percentage of patients with homocysteine levels >15 µmol/L, as well as >10.0 µmol/L, in each vascular disease category. The percentage of patients who had homocysteine levels >15.0 µmol/L (the upper limit of BRI) was highest (64.6%) in patients of CAD. In CVD and PVD categories, it was 59.79% and 62.81%, respectively (Table 5). These values were similar, i.e. ≈60%. When the percentage of patients with homocysteine >10.0 µmol/L was derived, the increase in percentage was similar in all categories of vascular disease patients, i.e. ≈20% for all three categories, again suggesting its dose-response relationship with vascular disease and an absence of a threshold.

(vi) Experimental evidence that removal of the exposure reduces risk

In 54 patients of occlusive vascular disease, biochemical investigations revealed the only abnormal parameter to be a raised plasma homocysteine. Of these, 36 patients were diagnosed as recurrent deep vein thrombosis and 18 as stroke. To elucidate that homocysteine was the risk factor, one would have to ‘remove’ homocysteine and observe the patient for recurrence. So, therapy with vitamins B6, B12 and folate was instituted and estimation of homocysteine levels was repeated every 3 months till the levels were within the BRI. All these patients came for follow-up for at least one year, though only 9 continued to follow-up for 5 years. Amongst the patients with DVT who came for follow-up, none had a recurrence of DVT or any other vascular occlusive episode. Two of the 18 patients of CVD had one repeat episode of stroke, while the remaining 16 patients were free of any vascular events for the period of follow-up (Table 6). Hence reducing homocysteine reduced recurrence, suggesting that removal of exposure reduced risk – another of Hill’s criteria of causality.

Thus, the conformance of homocysteine to 6 of the 7 Hill’s criteria of causality was demonstrated. However, as temporal relationship would require a prospective epidemiological study of a normal population to demonstrate that exposure precedes the outcome, this was the only criterion that was out of the purview of this study.

Conclusion

Our data demonstrate that homocysteine is a highly significant risk marker for vascular disease in the Indian population. Our retrospective analysis reveals
that in accordance with Hill’s criteria of causality, it emerges as a significant ‘risk factor’ rather than just a ‘risk marker’ for occlusive vascular disease amongst Indians. It has been experimentally demonstrated that homocysteine can be lowered by the administration of the B group vitamins (specifically folate, B6 and B12), even if the cause of hyperhomocysteinemia is a genetic polymorphism of its metabolizing enzymes33. Hence homocysteine-lowering measures may be instituted for the population as a whole, either in the form of vitamin supplements or as fortified foods/grains to decrease the incidence and morbidity of vascular disease.

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References