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Clinica Chimica Acta 352 (2005) 121–125

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# Plasma homocysteine: an independent or an interactive risk factor for coronary artery disease

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Received 24 May 2004; received in revised form 23 August 2004; accepted 24 August 2004

**Abstract**

*Background*: Coronary artery disease (CAD) is emerging as a major public health concern in most developing countries. Further conventional risk factors for CAD do not solely account for the increased mortality, particularly in Asians. Recently, increased plasma homocysteine is being considered as a risk factor, but the strength of relationship and interaction of plasma homocysteine with other risk factors is yet obscure. In this study, the association of plasma homocysteine with CAD and other risk factors was estimated.

*Methods*: In the present study, 100 patients of CAD and 50 controls of both sexes were included. Plasma total homocysteine (tHcy) concentrations were measured using reverse phase high-performance liquid chromatography.

*Results*: Plasma homocysteine concentrations were significantly raised in cases as compared to age-matched controls ($16.57 \pm 6.86$ and $11.47 \pm 5.19$ μmol/l, $p<0.001$). On calculating relative risk (RR) of each factor by univariate analysis smoking, hypertension, plasma cholesterol and homocysteine appeared to be significant risk factors. However, on applying multiple logistic regression only the latter three emerged as independent risk factors ($p<0.005$). Further, strong interactive effects were observed between homocysteine levels and increasing age, hypertension and smoking.

*Conclusion*: An increase in plasma homocysteine concentration confers an independent risk for CAD. It further increases the risk associated with increasing age, smoking and hypertension. Thus, increased homocysteine concentrations are a significant medical problem and effective strategies are urgently required to counter this challenge.


*Keywords:* Coronary artery disease; Plasma homocysteine; Risk factors; High-performance liquid chromatography

*Abbreviations:* AVD, atherosclerotic vascular disease; CAD, coronary artery disease; CI, confidence intervals; RR, relative risk; SBDF, 7 fluoro-benzo 2 oxa-1, 3 diazole-4 sulfonic acid; tHcy, total homocysteine.

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doi:10.1016/j.cccn.2004.08.005

## 1. Introduction

Coronary artery disease (CAD) is a significant problem both in terms of morbidity and mortality. This condition is now becoming more frequent in people from developing countries due to their unhealthy habits and behavior [1]. Thus, any intervention that can reduce CAD risks could have a tremendous public health impact. Several risk factors associated with CAD have been identified; which more commonly in combination than isolation interact to create a proatherogenic environment. These are advancing age, male sex, smoking, hypertension, positive family history, deranged lipid profile, diabetes and insulin resistance [2]. Despite these advances, only about half of the variation in size of atherosclerotic lesions can be attributed to known risk factors. Moreover, previous studies suggest that these risk factors are generally similar or lower among Indian Asians [3]; implying that these risk factors do not solely account for the increased CAD mortality in Asians. This observation has intensified the search for 'new non-lipid' risk factors for atherosclerotic vascular disease (AVD).

Recently, there has been considerable interest in homocysteine primarily because hyperhomocysteinemia is an important risk factor for CAD. Hyperhomocysteinemia is newly identified, independent, constitutional and/or potentially modifiable acquired risk factor associated with CAD (especially premature). A wealth of epidemiological evidence from >100 prospective cohort, cross sectional and case control studies have confined the relationship between homocysteine concentrations and vascular disease [4,5]. Plasma homocysteine concentrations are determined by both genetic and nutritional factors. The latter observation has led to various randomized control trials of supplementation with folic acid alone or in combinations with $B_6$ and/or $B_{12}$ which reduced homocysteine concentrations [6,7]. These observations may have substantial public health implications.

However, the limited data available from Indian patients with vascular disease; shows conflicting results [8,9]. Moreover, it is difficult to be certain of the strength of relationship and in particular of the independence of or interactions between elevated plasma homocysteine concentration and conventional risk factors in our population. The present study explored an association between plasma homocysteine concentrations in northwest Indian patients with CAD; along with conventional risk factors, so that some practical recommendation for screening and treatment of this modifiable risk factor could be provided.

## 2. Material and method

The study was carried on 100 adults cases of CAD, reporting to Rajindra Hospital, Patiala. To reduce bias caused by risk factor treatment, only recently diagnosed patients were evaluated. The cases either had clinical evidence of angina along with ECG changes or were case of myocardial infarction (>1 month old) who came to hospital for revaluation. Fifty normal subjects of parallel age, sex and socioeconomic status were taken as control. All the controls were free from any overt disease, i.e., had no clinical or investigative evidence of vascular, renal, hepatic or metabolic disease. Exclusion criteria for patients were recent myocardial infarction (<1 month), pregnancy, any renal, hepatic or thyroid disease, or patients on anticonvulsant therapy and recent exposure to nitrous oxide (within 3 months). Informed consent was obtained from all subjects before venepuncture. A detailed clinical history, covering dietary habits including smoking, socioeconomic status, drug history and family history was taken. Smoking status was determined at the time of vascular diagnosis. Blood pressure readings were taken in duplicate. Hypertension was deemed to be present if the mean systolic pressure was >140 mm Hg and/or diastolic pressure was >90 mm Hg or if the subject was taking antihypertensive medications. Blood was drawn from fasting individuals into EDTA vials. The samples were immediately ice packed and centrifuged within 30 min to avoid false increases of homocysteine due to release from red blood cells. Plasma samples were then refrigerated and stored at −20 °C, till analysis was done. Total plasma homocysteine was determined by using reverse phase HPLC (Waters India). The assay used measured total homocysteine, both reduced and oxidized forms [10]. The samples were thawed at room temperature (20–25 °C) and vortexed. Plasma (400 μl) was taken and mixed with dithiothreitol (100 μl). This dissociated reduced homocysteine from non-relevant proteins and other disulfides. Samples were incubated for 15 min

Table 1
Relative risk (95% CI) for various factors by univariate regression analysis

| Risk factor | RR | CI | *p* value |
|---|---|---|---|
| Age | 1.025 | 0.985–1.067 | NS |
| Sex | 1.083 | 0.549–2.137 | NS |
| Hypertension | .054 | 0.16–0.186 | <0.001 |
| Smoking | .306 | 0.087–1.119 | <0.05 |
| Cholesterol | 1.064 | 1.040–1.088 | <0.001 |
| Homocysteine | 1.202 | 1.100–1.314 | <0.001 |

Continuous variables: age, cholesterol and homocysteine.
Categorical variables: sex, hypertension and smoking.

at 37 °C. 100 μl of 0.6 mol/l perchloric acid solution was added and samples were then vortexed until proteins were thoroughly precipitated. The samples were then centrifuged at 4 °C for 10 min. The supernatant (160 μl) was taken with 320 μl of boric acid (pH 10.5). 160 μl of fluorescent reagent 7 fluoro-benzo 2 oxa-1, 3 diazole-4 sulfonic acid (SBDF) (Sigma) was added to the samples; the vials were capped and the contents vortexed. Samples were incubated for 60 min in a water bath at 60 °C. Samples turned bright yellow over the course of incubation. The samples were cooled at room temperature and then injected into the HPLC column. The column used was μBonda Pak $C_{18}$ 3.9 mm×30 cm (Waters India). Homocysteine was quantified by using fluorescence detection. The HPLC parameters were: flow rate was 1.2 ml/min, run time 8 min, fluorescence detection (excitation 385 nm, emission 515, filter 470). The injection volume was 40 μl, and the linearity of the method was 2.0–200.0 μmol/l.

## 3. Statistical analysis

For risk analysis, plasma homocysteine was taken as a continuous variable along with age and plasma cholesterol. Sex, hypertension and smoking were taken as categorical variables. Univariate regression analysis was used to calculate the relative risk (RR) of each factor with 95% confidence intervals (CI). Multiple logistic regression gave RR of each independent factor after adjusting for all other risk factors included in the study. Further to study interaction between plasma homocysteine and other risk factors as hypertension; participants were divided into four groups (no hypertension and no hyper tHcy group, hypertensive group, hyper tHcy group, and fourth group with both hypertension and hyper tHcy). Similar groups were divided for other interaction studies. All variables were taken as categorical. The cut offs were age >50 years, cholesterol > 200 mg/dl, plasma tHcy >11.5 μmol/l (mean value of our control group). The significance of interaction was tested by comparing the likelihood ratio of full regression model including cross-product of two risk factors versus the model without cross-product term. The expected joint relative risk under both the multiplicative and additive models was based on the individual relative risk for each factor on its own.

## 4. Results

Plasma homocysteine values were almost normally distributed as seen on Q–Q plot. On comparing plasma tHcy concentrations in cases and controls by unpaired *t*-test, cases were significantly higher than controls (16.57±6.86 vs. 11.47±5.19 μmol/l, $p<0.001$). On univariate analysis, RR (95% CI) for hypertension, plasma cholesterol and tHcy were highly significant ($p<0.001$) and just significant for smoking ($p<0.05$) (Table 1). However, on applying multiple logistic regression, only hypertension, plasma homocysteine and cholesterol emerged as independent risk factors, their relative risks being highly significant (Table 2). Thus, this analysis addresses the strength and independence of relationship between plasma tHcy and CAD, which is similar to that shown by hypertension or hypercholesterolemia. Table 3 shows the relative risks of various combinations of plasma tHcy concentrations and conventional risk factors. Increased fasting tHcy

Table 2
RR (with 95% CI) on multivariate logistic regression for various risk factors for CAD

| Risk factor | RR | CI | *p* value |
|---|---|---|---|
| Age | 0.998 | 0.941–1.059 | NS |
| Sex | 1.005 | 0.307–3.202 | NS |
| Hypertension | 0.033 | 0.006–0.174 | <0.001 |
| Smoking | 0.324 | 0.052–2.02 | NS |
| Cholesterol | 1.076 | 1.044–1.108 | <0.001 |
| Homocysteine | 1.234 | 1.077–1.414 | <0.005 |

Continuous variables: age, cholesterol and homocysteine.
Categorical variables: sex, hypertension and smoking.

concentrations showed greater than additive and multiplicative interaction with increasing age, hypertension and smoking. But no such interaction was observed with gender of the patient or his cholesterol concentrations.

## 5. Discussion

Thus, above data indicates that elevated plasma homocysteine concentrations are likely to be a risk factor for Indians especially northwest Indians. Similar supporting data has been provided by various studies done on Indian Asians in UK and North America [3,11]. Studies have estimated that a 5-μmol/l homocysteine increased CAD risk by as much as cholesterol increase of 20 mg/dl [4]. Also that a prolonged lowering of homocysteine by 3–4 μmol/l was associated with 30–40% reduction in risk of CAD [5]. The etiopathogenesis of hyperhomocysteinemia in CAD may be the prothrombotic and proatherogenic metabolic milieu created by homocysteine. Probable causes are endothelial cell injury due to patchy desquamation or production of reactive oxygen species, increased platelet aggregation; oxidation of LDL and/or proliferation of vascular smooth muscle cell [12,13].

A strong interaction was noticed between higher plasma tHcy concentrations with increasing age (RR=6.55). This is thought to be due to various mechanisms including age related decline in cystathionine synthase and other enzymes involved in homocysteine metabolism, progressive deterioration of kidney function and decrease in bioavailability of essential cofactors like vitamin $B_{12}$, $B_6$ and folate [14]. Similar strong interaction was seen in hypertensive patients with elevated tHcy concentrations (RR=3.06).

Table 3
Observed and expected RR in subjects with elevated plasma homocysteine and each conventional risk factor

| Risk factor | Observed RR | Expected RR of elevated tHcy and | |
|---|---|---|---|
| | | Additive model | Multiplicative model |
| Age | 6.55 | 2.5 | 2.56 |
| Sex | 1.155 | 2.08 | 1.05 |
| Hypertension | 3.06 | 1.24 | 0.48 |
| Smoking | 1.54 | 1.43 | 0.28 |
| Hypercholesterolemia | 1.29 | 2.3 | 1.3 |

Such positive correlations were observed in various other studies also [15,16].

The proposed mechanism is that hyperhomocysteinemia induces an elastolytic process on the arterial wall by increasing the synthesis and secretion of serine elastases. This loss of elastin may lead to stiffening of arterial wall resulting in hypertension [17]. Further observed RR for the disease was higher in smokers with hyperhomocysteinemia than controls with neither of the two risk factors, but the risk was just greater than expected additive value. This small increment may be because of small sample size and smoking being less common in our representative population, particularly women. The reasons for this interaction are not clearly known. Homocysteine may augment smoking-related platelet and clotting effects or exert toxic effects on endothelium in addition to reactive oxygen species produced by smoking [18].

We conclude that the relation between plasma homocysteine and CAD appears to be linear or log linear, in much the same way as increasing blood pressure and cholesterol are related to vascular disease. Hyperhomocysteinemia though is an independent risk factor for CAD, but its effect is further associated with increasing age, hypertension and smoking. These interaction studies of plasma tHcy may have implications for risk management of the disease. Control of smoking and hypertension in patients with elevated homocysteine concentrations may be particularly important. Plasma homocysteine estimates should be considered as a part of risk assessment of vascular disease. However, there is no definite threshold concentration for homocysteine that correlates with sudden increase in risk of vascular events. The normal range for homocysteine concentrations has been proposed to be between 5 and 15 μmol/l [19]. However, several studies have documented risk of vascular disease within this range. On the basis of this observation, it was proposed that homocysteine concentration of ≥14 and 11 μmol/l should be treated for primary and secondary prevention of atherosclerotic vascular disease, respectively [14]. Few investigators recommend a cut off value of 10 μmol/l to be considered as normal [20,21]. Considering the differences in dietary, genetic and ethnic factors, data published from west may not be applicable to our population. These observations are based on small group of patients and controls, so to

establish a definite cut off value beyond which homocysteine should be taken as significant and to consider the use of folic acid and pyridoxine to lower its concentrations for the prevention of cardiovascular disease, further large-scale prospective clinical trials in native Indians are needed.

## Acknowledgements

We thank Dr. S. Majumdar and his team, Department of Experimental Medicine, PGIMER, Chandigarh for providing the reagents and facility of the HPLC for homocysteine.

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