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Original Work

Evaluation of Homocysteine, Lipoprotein(a) and Endothelin as diagnostic markers of Coronary Artery Disease in Indian population

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ABSTRACT: Indians have been reported to have high prevalence rates of coronary artery disease (CAD) even in the absence of traditional risk factors. The objective of this study was to assess the role of endothelin, lipoprotein(a), homocysteine and lipid profile as markers of CAD in Indian population. It was a hospital based observational case-control study, which included 60 documented patients of CAD, and 50 age and sex matched controls. Routine biochemical parameters were performed. Lipoprotein(a), homocysteine and endothelin levels were estimated by enzyme linked immunosorbent assay. The levels of endothelin (9.78±0.40 pg/ml vs. 7.86±0.31 pg/ml), lipoprotein(a) (51.42±1.71 mg/dl vs. 36.26±1.21 mg/dl), homocysteine (21.31±1.22 µmol/L vs. 10.41±0.844 µmol/L) and LDL/HDL cholesterol ratio (4.23±0.32 vs. 2.60±0.10) were significantly higher whereas that of HDL (29.82±1.39 mg/dl vs. 40.82±6.24 mg/dl) was significantly lower in patients of CAD as compared to the controls ($p<0.001$). Area under receiver operating characteristic curve was >0.7 for all the markers. Higher levels of homocysteine, endothelin, and lipoprotein(a) were independently associated with increased risk of CAD. Thus, they may be helpful in risk assessment in premature cardiovascular disease and in individuals where traditional risk factors are not present.
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KEY WORDS: ***Atherosclerosis; CAD; Endothelin; Homocysteine; Lipoprotein(a)***

INTRODUCTION

Cardiovascular disease is the leading cause of death, accounting for 29% of all deaths in 2005, according to the WHO[1]. The reported prevalence of coronary heart disease (CAD) in adult surveys has risen 4-fold over the last 40 years (to a present level of around 10%), and even in rural areas, the prevalence has doubled over the past 30 years (to a present level of around 4%)[1]. CAD strikes Indian population at younger age and kills many in their productive mid-life years[2]. Deaths due to CAD, in the age group of 35 to 64 years, resulted in 9.2 million potentially productive years of life being lost in 2000[2]. Cardiovascular disease is the result of interaction between polygenic, lifestyle and environmental factors. Studies suggest that most CAD events are noted in individuals with one or more elevated risk factors[3]. However, at least 25 percent of patients have myocardial infarction or sudden death without prior symptoms[4].

Endothelial dysfunction may play a key role in the pathogenesis of atherosclerosis[5]. The endothelium plays an important role in the maintenance of vascular homeostasis by modulating vascular tone with the help of vasoconstrictors and vasodilators. Endothelin(ET-1) is a potent vasoconstrictor peptide which has also been implicated in cytokine and vascular cell adhesion molecule expression, smooth muscle cell proliferation and synthesis of extracellular matrix substances[6,7].

Homocysteine (hcy) is a biomarker, which causes direct toxic damage to endothelial cells via increased oxidative stress and also contributes towards reduced nitric oxide bioavailability[8]. Lipoprotein(a)(Lp(a)) consists of a low density lipoprotein (LDL) particle with its apoB-100 component linked to a variable length protein,

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apo(a). Lp(a) levels may prove to be a better discriminator of CAD risk at an early age since its levels are said to be genetically determined. However, some studies reported no correlation between plasma Lp(a) levels and atherosclerosis[9]. Thus, the aim of the study was to evaluate Hcy, Lp(a), ET-1, and lipid profile as markers of CAD in Indian population.

METHODOLOGY

This case-control study was conducted on 60 randomly selected patients of CAD attending the Medicine outpatient clinic of Lady Hardinge medical college and associated hospitals, New Delhi and 50 healthy controls. The CAD patients were documented (on the basis of angiography, ECG, cardiac markers and past records). The control population comprised of age and sex-matched healthy volunteers with no clinical or ECG evidence of CAD and negative history of major CAD risk factors (diabetes mellitus, hypertension, smoking, and family history). Institutional ethical committee approved the study and the samples were collected after informed consent. All subjects were examined and information on medical histories, age, weight, height, BMI, systolic and diastolic pressure, cigarette smoking and medications were obtained via questionnaire and patients medical records. Patients with recent history of acute coronary syndrome or cerebrovascular event (<8 weeks), chronic liver and kidney disease and malignancy were excluded from the study (**figure 1**).

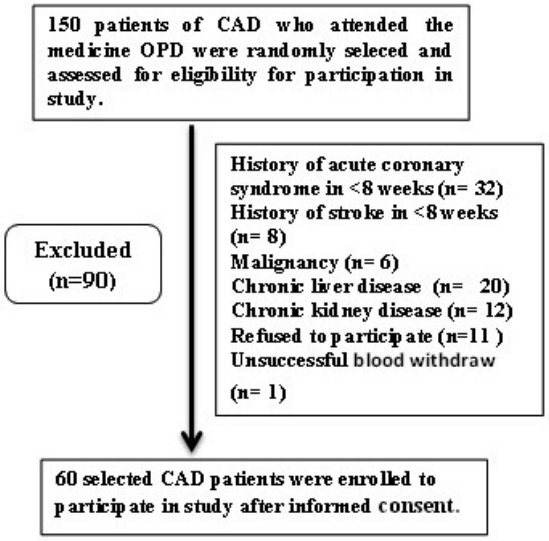

Figure 1: Flow chart of participants

Blood collection and storage

Approximately 8 mL of fasting venous blood sample (12-14 hours) was collected from each study participant using aseptic precautions. 2 mL blood was collected in sodium fluoride- oxalate vial for estimation of sugar. 6 mL blood sample was collected for estimation of routine biochemical parameters and special parameters-ET-1, Hcy, Lp(a) in a plain vacutainer. Blood was allowed to clot and serum was separated by centrifugation (15 min at 2500 x g). Serum lipid profile, liver and renal function tests were carried out the same day. Remaining serum was divided into aliquots for evaluation of Hcy, Lp(a) and ET-1 and stored at -40^{0}C until batch analyzed.

Estimation of serum lipids

Serum total cholesterol (TC) and triglycerides (TG) were estimated by enzymatic method on Beckmann Coulter CX9 autoanalyzer using standard kits and reagents from Randox (UK). Estimation of HDL – C was done using magnesium chloride and sodium phosphotungstate to precipitate low density and very low-density lipoproteins, which were then removed by centrifugation at 3000rpm. The HDL-C left in the supernatant was estimated by enzymatic method as for total TC. Serum low-density lipoprotein (LDL) cholesterol was calculated using Friedewald and Fredrickson's formula[10].

Estimation of endothelin

Plasma endothelin was estimated by ELISA using DRG's human ET-1 enzyme immunometric assay kit. The color generated was read at 450 nm. The measured optical density was directly proportional to the concentration of endothelin.

Estimation of homocysteine

The total Hcy in the blood was estimated using diazyme homocysteine microtitre plate assay (EIA). The assay employs a genetically engineered Homocysteine Binding Protein (HBP) as the capturing agent. The measured optical density was inversely correlated to the concentration of Hcy in the sample.

Estimation of lipoprotein(a)

The plasma Lp(a) was determined using DRG Elitest assay kit. The amount of color produced is proportional to the amount of Lp(a) present in the sample.

Statistical Analysis

Results were expressed as mean ± standard error of mean (S.E.M). We used unpaired 't' test to compare individual CAD marker between patients and controls. P value <0.05 was taken as significant. Logistic regression analysis was done

and Exp (B)(odd's ratio) was calculated to predict the occurence of CAD from novel risk markers- Lp(a), ET-1 and Hcy using the forward stepwise model and adjusting all other variables. Area under receiver operating characteristic curve (ROC) was calculated. Sensitivity and specificity were calculated at a particular cut off point to predict the usefulness of each marker in CAD patients. Correlation between different markers was determined by Pearson's correlation test. All statistical analysis was performed using SPSS version 17.0 (SPSS, Inc., Chicago, Illinois).

RESULT

The study population included 60 patients of CAD in the age group of 40 to 79 years. Among these, 42% were men and 58% were women. The control group included 44% men and 56% women in the age group 42-80 years. The distribution of risk factors in the cases is shown in **figure 2**.

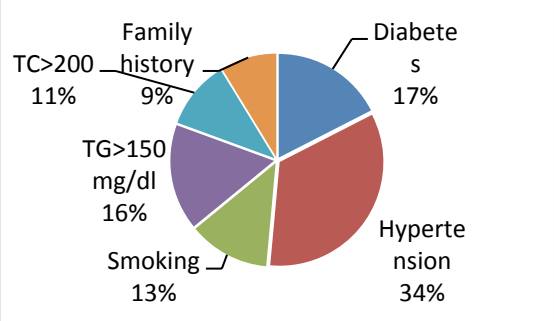

Figure 2: Distribution of Risk factors in patients of CAD

The demographic and biochemical characteristics of the study population are shown in **table 1**. The levels of blood glucose, serum creatinine and LDL/HDL cholesterol ratio (104.26±7.24 mg/dl vs. 82.28±1.19 mg/dl, 0.98±0.04 mg/dl vs. 0.82±0.03 mg/dl, 0.98±0.04 vs. 0.82±0.03) were significantly higher whereas that of HDL cholesterol (29.82±1.39 mg/dl vs. 40.82±6.24 mg/dl) was significantly lower in CAD patients as compared to the controls.

A significant difference was observed between the Hcy, Lp(a) and ET-1 levels of the CAD patients and controls ($p<0.01$) as shown in **table 2**. The CAD patients had significantly higher levels of Hcy, Lp(a) and ET-1 as compared to the control group. No correlation was observed among any of the risk markers in the CAD patients.

Logistic regression analysis is shown in **table 3**. Odd's ratio was calculated to predict the CAD event from the independent variables. All values were highly significant suggesting that a unit change in these biomarkers had significant impact on occurrence of CAD.

Table 1: Demographic and biochemical characteristics of CAD patients and controls

	Cases (n=60)	Controls (n=50)	p value
Age (years)	59.46± 1.603	58.32± 1.667	0.623
BMI (kg/m^2)	23.672± 0.643	22.836± 0.637	0.340
Total Cholesterol	167.98± 5.98	169.30± 3.00	0.844
Triglycerides	134.02± 8.68	129.12± 3.85	0.607
HDL-C	29.82± 1.39	40.82± 6.24	0.000
LDL-C	110.26± 5.36	102.99± 20.81	0.238
LDL-C/ HDL-C	4.23± 0.32	2.60± 0.10	0.000
FBS	104.26± 7.24	82.28± 1.19	0.003
Urea	33.80± 1.68	30.10± 1.06	0.066
Creatinine	0.98± 0.04	0.82± 0.03	0.001
Uric Acid	4.59± 0.16	4.86± 0.19	0.274
Total protein (g/dl)	6.89± 0.12	6.99± .09	0.482
Albumin (g/dl)	3.72± .08	3.81± 0.12	0.531

All values in mg/dl unless specified otherwise. Values are represented as mean±standard error of mean. P<0.05 is significant

Table 2: Plasma homocysteine, lipoprotein(a) and endothelin levels in CAD patients and controls

Risk parameter	CAD	Control	P value
Homocysteine (µmol/L)	21.31± 1.228	10.41± 0.844	0.000
Lipoprotein(a) (mg/dl)	51.42± 1.710	36.26± 1.216	0.000
Endothelin (pg/ml)	9.78± 0.401	7.86± 0.312	0.000

Values are represented as mean±standard error of mean. P<0.05 is significant

Table 3: Association between CAD and biomarkers assessed by stepwise logistic regression analysis

	Odd's ratio Exp(B)	**P value**
Hcy	19.8	0.001
Lp(a)	4.88	0.048
ET-1	4.09	0.005

Table 4 shows the variation in the levels of Hcy, Lp(a) and endothelin according to demographic and biochemical characteristics in CAD patients. The levels of hcy were affected by age, male gender and diabetes mellitus whereas lp(a) levels were affected by sex.

AUROC was >0.7 for all the parameters and maximum was for Lp(a). This suggests that each of these parameters can be used to evaluate risk of CAD in Indian population and best parameter to use is Lp(a) (**table 5**).The ROC curve is shown in **figure 3**. Sensitivity and specificity of each parameter at a particular cut off point has been shown in **table 5**.

Table 4: Variation in the levels of Hcy, Lp(a) and endothelin according to demographic and laboratory characteristics in CAD patients

		Hcy	**Lp(a)**	**endothelin**
Age	< 50 years	26.40±1.54*	57.20±2.55*	9.70±0.62
	> 50 years	20.04±1.42	49.98±1.99	9.80±0.48
Sex	Male	24.66±2.13*	53.30±2.26	9.70±0.53
	Female	19.07±1.35	50.17±2.42	9.83±0.58
BMI	<25 kg/m^2	21.25±1.74	53.03±2.16	9.93±0.57
	>25 kg/m^2	21.38±1.71	49.19±2.76	9.57±0.55
HT	Present	23.01±2.27	51.29±2.22	9.94±0.47
	Absent	20.58±1.47	51.69±2.60	9.44±0.76
DM	Present	19.52±1.52*	51.06±3.27	9.56±0.64
	Absent	25.11±1.79	51.63±1.98	9.93±0.52
Smoking	Present	22.66±2.68	53.77±2.86	10.31±0.64
	Absent	20.83±1.38	50.59±2.08	9.59±0.49
Total Cholesterol	<200 mg/dl	21.57±1.42	52.25±4.15	10.0±0.46
	>200 mg/dl	20.49±2.56	51.16±1.86	9.08±0.79
Triglycerides	<150 mg/dl	22.20±1.55	52.40±2.02	9.45±0.49
	>150 mg/dl	19.58±2.01	49.53±3.18	10.41±0.90
HDL-C	<40 mg/dl	20.67±1.45	51.68±1.97	9.66±0.47
	>40 mg/dl	24.22±1.55	50.22±3.33	10.33±0.71
LDL-C	<100 mg/dl	23.06±1.90	49.50±2.21	10.15±0.65
	>100 mg/dl	20.14±1.60	52.70±2.44	9.53±0.52

Table 5: Homocysteine, lipoprotein(a) and endothelin as a screening tests in CAD patients

	AUROC	Sensitivity	Specificity	cut off value
Homocysteine	0.832	80%	72%	15.5µmol/L
Lipoprotein(a)	0.842	70%	82%	45mg/dl
Endothelin	0.706	76%	56%	8.5pg/dl

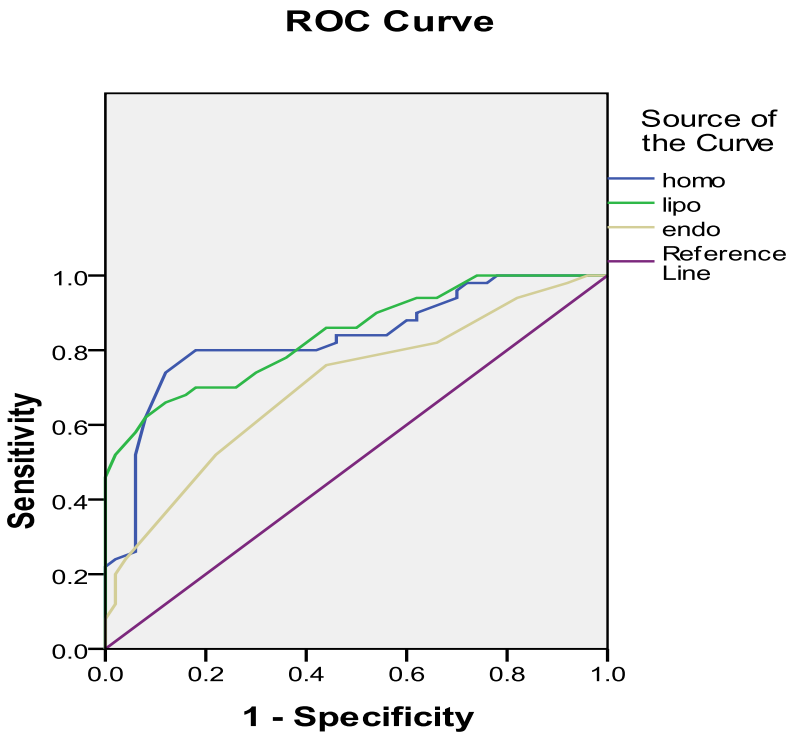

Figure 3: ROC curves for homocysteine, lipoprotein(a) and endothelin in CAD patients

DISCUSSION

The present study evaluated the significance of the novel biomarkers- hcy, Lp(a) and ET-1 in CAD patients. These biomarkers were found to be significantly higher in the CAD patients as compared to the controls. There are several traditional risk factors for cardiovascular disease; however, they have been ineffective in completely predicting the development of atherosclerotic process. In the present study, highest occurrence (34%) of CAD events was found in 61-70 years of age, followed by 40-50 years age group (32%). This shows that, though the occurrence of a CAD event is still more common in elderly population, however the incidence is rapidly increasing at an earlier age. 82% of women in the CAD group were post-menopausal. This may be related to estrogen deprivation, accelerating the risk of atherosclerotic process[11].

The HDL cholesterol in CAD patients was significantly lower whereas LDL/HDL cholesterol ratio was significantly higher as compared to the control group. Low levels of HDL-C are reported to increase the risk of cardiovascular disease even when total cholesterol is not elevated[12,13]. The levels of serum creatinine were significantly higher in the cases. According to Pereg et al, reduced renal function in the normal to mildly impaired range was independently associated with increased risk for CAD among young healthy males[14].

The mean plasma endothelin level in CAD patients was significantly higher than controls. Further to its vasoconstrictor and mitogenic properties, endothelin appears to be involved in the inflammatory process that underlies active atheromatous plaques[15]. Increased plasma endothelin levels may indicate early disturbances of endothelial function. Lerman et al reported significantly elevated levels of endothelin in atherosclerotic patients and also found significant

correlation between plasma endothelin and number of sites of disease involvement[16]. These findings were consistent with the studies of Salmone et al[17] and Borries et al[18]. Thus, endothelin may play a significant role in cardiovascular disease.

Lp(a) levels were significantly higher in CAD patients as compared to controls showing a correlation between Lp(a) and cardiovascular disease. Lp(a) is emerging as a strong biomarker for CAD. In previous studies, it was found that subjects with Lp(a) levels above 30 mg/dl had risk of CAD increased by about three-fold[19,20]. In our study, cut off value was 45 mg/dl with 70% sensitivity and 82% specificity. It was interesting to observe that control population had Lp(a) levels (36.26±1.216 mg/dl) higher than those reported in other populations. Few studies done in Indian population have also shown higher Lp(a) levels compared to other ethnic groups[21,22] suggesting that inherently high Lp(a) levels are present in this genetic pool. Thus, these patients should be followed long term. Recently, elevated levels of Lp(a) have been associated with premature coronary artery disease in migrant Asian Indians[23]. Other studies also suggest that the atherogenecity of Lp(a) is more marked in the presence of a concomitant decrease in HDL cholesterol level[24] which was also seen in our study.

There are very few studies in India to show the association of hcy levels with CAD. Chambers et al[25] reported that elevated hcy levels were independently associated with cardiovascular risk in UK Indians. In the general Indian population, modest elevations of hcy are common and may reflect poor dietary intake of folate and vitamin B12[26]. In the present study, hcy levels in the CAD patients were significantly higher as compared to the controls. There is evidence that hcy might disturb the bioavailability of nitric oxide (NO), which would, at least in part, contribute to the pathophysiology of circulatory disorders. Stühlinger et al[27] examined the relationship among hcy, NO, and endothelial function in patients with peripheral arterial disease and demonstrated that experimentally induced hyperhomocysteinemia increased plasma asymmetric dimethylarginine, an endogenous NO synthase inhibitor, that may be related to a decline in endothelial vasodilator function. This might be an important mechanism for the endothelial dysfunction that occurs in subjects with hyperhomocysteinemia[28].

Multivariate logistic regression analysis showed that each of these markers could independently predict a CAD event irrespective of traditional risk factors with relatively good sensitivity and specificity. The cut off values of hcy, Lp(a) and ET-1 came out to be15.5µmol/L,45mg/dl and 8.5 mg/dl respectively in Indian population.

It is recommended that studies from different areas involving larger sample size are carried out to confirm the findings of the present study. Moreover, interventional studies are needed to determine whether decreasing the levels of these biomarkers indeed reduces the risk of CAD.

CONCLUSION

The levels of hcy, Lp(a) and ET-1 are elevated in CAD patients as compared to the controls. Thus, they may serve as potential markers of CAD in Indian population. These biomarkers may be helpful in risk assessment in premature cardiovascular disease and in individuals where traditional risk factors are not present.

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