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Paraoxonase gene *Q192R* & *L55M* polymorphisms in Indians with acute myocardial infarction & association with oxidized low density lipoprotein

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***Background & objectives*: Paraoxonase (PON) is an HDL associated ester hydrolase with an ability to retard LDL oxidation *in vitro* by preventing lipid peroxide generation. The population variability in enzyme activity is attributed to polymorphisms in paraoxonase gene. For example, polymorphism at codon 192 and 55 of the paraoxonase gene has been reported to be associated with coronary heart disease (CAD) and diabetes among different ethnic groups. The present study looks at *PON192* and *55* polymorphism among hospitalized Asian Indian patients with myocardial infarction (MI) and their association with circulating oxidized LDL and antioxidant status.**

***Methods*: One hundred and twenty four consecutive patients of acute myocardial infarction and 221 age-matched controls were recruited for the study. Oxidized LDL was measured in serum by ELISA and total antioxidant levels by the 2,2'-azino-bis-(3 ethyl benzothiozoline-6-sulfonate) (ABTS) method. Other known cardiovascular risk factors, apolipoprotein B, apolipoproteinA1, lipoprotein(a), hsCRP and homocysteine were also measured. Paraoxonase gene polymorphism at codon 192 and 55 were analyzed by PCR-RFLP.**

***Results*: Patients with MI had significantly higher oxidized LDL ($P<0.05$) and lower total antioxidant capacity ($P<0.001$) as compared to controls. Oxidized LDL correlated with total cholesterol, LDL and Apo B in patients. B allele frequency of the codon 192 polymorphism in paraoxonase gene was higher in cases as compared to controls and odds ratio of developing the MI with BB genotype versus AA genotype was 2.37, ($P=0.044$). Codon 55 polymorphism in paraoxonase gene was not associated with CAD. There was no difference in oxidized LDL between the different genotypes of *PON192* and *PON55*.**

***Interpretation & conclusions*: Although *PON192* polymorphism was associated with CAD, no correlation of *PON192* or *55* polymorphism was found with oxidized LDL suggesting that presence of other antioxidant factors may be of equal importance in preventing LDL oxidation.**

Key words Antioxidant status - Indians - oxidized LDL - paraoxonase gene polymorphism

Oxidative modification of low density lipoprotein (LDL) in the arterial wall is central to the pathogenesis of atherosclerosis[1]. LDL oxidation has been shown to be inhibited by HDL *in vitro*[2-4]. In co-culture HDL prevents the production of mildly oxidized LDL by artery wall cells[5]. This protective effect of HDL relates to enzymes associated with it. Paraoxonase[6], platelet activating factor acetyl hydrolase[7] and lecithin: cholesterol acyl transferase[8] are the HDL associated enzymes that retard the oxidation of LDL by preventing the generation of lipid peroxides; of these, paraoxonase being most extensively studied. Human paraoxonase (PON1) is a calcium dependent HDL associated ester hydrolase that catalyses the hydrolysis of organophosphates, aromatic carboxylic acid esters and carbamates. Purified human HDL associated PON1 retards the oxidation of LDL by preventing the generation of lipid peroxides.

While the serum level of PON1 in a given individual is relatively stable over time, the enzymatic activity of PON1 varies among individuals by 10-40 fold[9]. The genetic basis of the inter-individual variability of PON1 activity has been attributed to the presence of an A-G polymorphism in the coding region of the gene coding for this enzyme[10-11]. The A/G polymorphism corresponds to glutamine/arginine polymorphism at amino acid position 192. Individuals homozygous for arginine at position 192 (B genotype) show a significantly higher serum PON1 activity than those homozygous for Gln (A genotype)[12]. Another polymorphism at amino acid 55, a leucine to methionine substitution also affects PON1 activity. Case control studies investigating the relationship between PON1 genetic polymorphisms and the presence of coronary artery disease (CAD) have been reported from many laboratories. Some studies have reported no relationship[13,14] while others have reported association between PON1 genetic polymorphism and CAD[15-18]. Wheelers *et al*[19] reported a weak association between *Q192R* polymorphism of PON1 and CAD in a meta-analysis of 43 studies.

The relative contribution of *PON192* and *PON 55* to development of myocardial infarction (MI) in resident Indian population and their association with oxidized LDL and total antioxidant have not been adequately addressed. Since association between PON1 polymorphism and CAD varies among population with different ethnic background the objective of the present study was to look at the genotype frequencies of *PON 192* and *PON55* in Indians with and without myocardial infarction and further to see the association of the genotypes with oxidized LDL and total antioxidant status.

Material & Methods

Study design: Consecutive patients (n=124) of incident acute myocardial infarction (AMI) and 221 age-matched controls were recruited. Written consent was obtained from all the subjects. Ethical clearance for the conduct of the study was obtained from institutional ethics committee. Patients aged 25 yr and above but less than 75 yr of either sex admitted to All India Institute of Medical Sciences (AIIMS), New Delhi, within 24 h of onset of first AMI were eligible for recruitment into the study. The study was conducted from March 1999 - June 2003. AMI was diagnosed if the patient had characteristic symptoms plus electrocardiogram changes indicative of a new MI (new pathologic Q waves, at least 1 mm ST elevation in any 2 or more contiguous limb leads or a new left bundle branch block, or new persistent ST-T wave changes diagnostic of a non-Q wave MI) or a elevated plasma level of creatine kinase-MB isofrom (CK-MB). Those with history of heart disease diagnosed more than 30 days prior to enrolment in the study, presence of cardiogenic shock, with known liver, thyroid or renal diseases, malignancy or pregnancy or with acute viral infection within the previous 4 wk were excluded. Controls were group matched to cases by age and gender and were individuals who had no previous diagnosis of heart disease or history of exertional chest pain. Controls were recruited from attendants or relatives of non cardiac patients from non cardiac wards or unrelated attendants of cardiac patients or subjects attending hospital out patient clinics for unrelated events. Subjects with any history of heart disease at any time in the past were excluded. The study was carried out over a period of two years.

Within 24 h of onset of chest pain, five ml blood sample was drawn with subject in the fasting state into a tube without anticoagulant. Serum samples were stored at -70^{0}C before analysis. The samples were used for the measurement of oxidized LDL, total antioxidant and other cardiovascular risk factors. DNA was isolated from the buffy coat on the day of sample collection.

Serum cholesterol and triglycerides were estimated by CHOD-PAP and GPO-PAP method respectively using kits from RANDOX Labs Ltd (Antrim, UK). HDL was isolated by precipitating the low density lipoproteins with phosphotungstate/magnesium. LDL was calculated by friedwald formula[20]. Total antioxidant measurement was by 2-2'-azino-di-(3-ethylbenzthiazoline sulphonate (ABTS) method

using kits from RANDOX (UK). Oxidized LDL was measured by ELISA using kits from Mercodia (Uppsala, Sweden). Lipoprotein (a), apolipoprotein A1 and B were determined by immunoturbidimetric method using kits from RANDOX Lab Ltd (UK). HsCRP and homocysteine were measured by ELISA kits from BioCheck Inc (CA, USA) and Axis Shield (Scotland) respectively. Paraoxonase activity was measured using procedure described by Eckerson *et al*[21]. Briefly for basal paraoxonase activity measurement, the method entailed addition of 5 µl of serum to 0.8 ml of mixture of 1.0 mM paraoxon and 1.0 mM $CaCl_2$ in 0.05 M glycine buffer, *p*H 10.5, lacking added NaCl. The liberation of p-nitrophenol was measured at 412 nm spectrophotometrically. Salt stimulated paraoxonase activity was measured with 1 M NaCl added.

DNA was extracted by standard phenol-chloroform method[22]. The Gln192Arg polymorphism was identified in the extracted DNA by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis[23]. Amplification was performed using the following primers from Sigma Aldrich, UK: 5'-TATTGTTGCTGTGGGACCTGAG-3'(forward) and 5'-CACGCTAAACCCAAATACATCTC-3' (reverse) and Jumpstart Taq Ready mix Kit (Sigma-Aldrich, UK) with 34 cycles of 30 sec at 94^0C, 45 sec at 59^0C and 30 sec at 72^0C. After amplifying a 99 bp PCR product was digested with 1U of *BspPI* enzyme for 5 h at 37^0C.

For genotyping the Leu55Met polymorphism, PCR was carried out using the same PCR ready-mix used above and primers for amplification of 170-bp DNA containing a sequence encoding codon 55 were 5'-GAAGAGTGATGTATAGCCCCAG-3'(forward) and 5'-TTTAATCCAGAGCTAATGAAAGCC-3' (reverse). Amplification was performed with 34 cycles of 30 sec at 94^0C, 45 sec at 60^0C and 30 sec at 72^0C. PCR products were digested with 4U of *NlaIII* enzyme in the presence of BSA for 2 h at 37^0C. Genotyping for PON55 was done in only 154 controls.

Statistical analysis: Statistical analysis of data was carried out using SPSS (version 11.5) software. Normality of the sampling distribution of each dependent variable was tested with the Lilliefors test for normality (a modified Kolmogorov-Smirnov test) using SPSS statistical package. Baseline characteristics and biochemical estimates in cases and controls were compared using student's t-test for continuous variables and chi-square test for categorical variables. The distribution of glucose, HDL cholesterol, apo (a), total antioxidant, oxidized LDL, lipoprotein (a) and hsCRP were not normal and therefore log transformed for analysis. Allele frequencies of PON192 and PON 55 were calculated by allele counting. Concordance of genotype frequencies with Hardy Weinberg equilibrium was tested with χ^2 goodness of fit. Distribution of genotype frequency between cases and controls was compared with χ^2 test. Odds ratio was computed to calculate the contribution of PON polymorphisms to MI. Distribution of oxidized LDL, total antioxidant status and HDL in different *PON192* and *PON55* genotpyes were compared by Kruskal Wallis tests as the numbers in some genotypes were small.

Results

The mean age and the ratio of males and females in the two groups were not different because the controls were age and sex matched to cases. Patients with MI were also more likely to have family history of hypertension and diabetes. Mean BMI and Waist-hip ratio were higher among patients as compared to controls (Table I).

There were significant differences in mean plasma glucose, total cholesterol and LDL cholesterol with higher levels in patients as compared to controls. Mean Apo B which is considered as a better predictor of CAD, was significantly (P<0.001) higher in cases and so was Apo B/Apo A1 (P<0.01). Among the other risk factors significant differences were observed in hsCRP (P<0.001) and homocysteine levels (P<0.05) which were higher in cases. Total antioxidant capacity was significantly (P<0.001) lower in cases compared to controls and this was accompanied by a high circulating oxidized LDL levels in cases with MI compared to healthy controls (Table II). The paraoxonase activity both basal and salt stimulated were comparable between cases and controls.

Table I. Baseline characteristics of cases and controls

	Cases (n=124)	Controls (n=221)
Age (yr)	52.23 ±11.49	52.00 ± 11.32
Sex (M/F) (%)	108/16 (87.1/12.9)	169/25 (86.7/13.3)
Smokers (%)	48.0	45.1
Alcohol users (%)	46.3**	29.7
History of hypertension (%)	22.0***	6.7
History of diabetes (%)	12.2**	3.1
BMI (kg/m^2)	24.8 ± 3.6**	23.5 ± 3.8
Waist/hip	0.95 ± 0.065*	0.93 ± 0.077
SBP (mmHg)	127.31 ±15.59	124.70 ± 11.55
DBP (mmHg)	80.98 ± 10.43	80.76 ± 7.06

Values are mean ± SD

*P<0.05; **<0.01; ***<0.001 compared to control

Table II. Biochemical variables in cases and controls

	Cases (n=124)	Controls (n=221)
Glucose (mmol/l)$^{+}$	8.71 ± 4.79***	5.38 ± 2.10
Total cholesterol(mmol/l)	4.91 ± 1.54*	4.54 ± 1.23
Triglycerides (mmol/l)	1.85 ± 1.09	1.73 ± 0.93
HDL cholesterol (mmol/l)$^{+}$	1.03 ± 0.27	1.02 ± 0.26
LDL cholesterol (mmol/l)	3.04 ± 1.32*	2.73 ± 1.06
Apo A1 (mg/dl)$^{+}$	125.97 ± 42.27	125.78 ± 41.17
Apo B (mg/dl)	118.34 ± 38.49***	90.66 ± 25.57
ApoB/ApoA1 (mg/dl)	1.048 ± 0.524**	0.826 ± 0.335
Lipoprotein (a) (mg/dl)$^{+}$	19.15 ± 20.48	14.64 ± 15.04
HsCRP (mg/dl)$^{+}$	8.76 ± 5.03***	3.40 ± 4.52
Homocysteine (µmol/l)$^{+}$	22.00 ± 12.43*	17.88 ± 8.55
Oxidized LDL (mg/dl)$^{+}$	34.4 ± 16.9*	30.6 ± 12.1
Total antioxidant (mmol/l)$^{+}$	1.13 ± 0.43***	1.32 ± 0.49
PON activity (U/l) (Basal)$^{+}$	93.52 ± 59.14	90.37 ± 65.38
(stimulated) (U/l)$^{+}$	165.89 ± 147.8	146.85 ± 134.9

Values are mean ± SD; HsCRP, human serum C reactive protein
$^{+}$Values were log transformed for analysis
P^{*}<0.05; **<0.01; ***<0.001 compared to control

Oxidized LDL correlated positively with total cholesterol (r=0.294, P<0.005), LDL (r=0.265, P<0.005) and apolipoprotein B (r=0.373, P<0.001) in patients. There was no correlation with any other parameter studied.

Fig. (a) depicts the paraoxonase activity in cases and controls with different *PON192* genotypes. In both cases and controls, subjects with AA genotype had the least paraoxonase activity. The mean paraoxonase activites were significantly higher in cases and controls with BB genotype. Although the mean paraoxonase levels were lower in cases with AA genotype as compared to controls the difference was not statistically significant. A correlation of 0.305 (spearman correlation coefficient) and 0.473 was found between *PON192* genotypes and PON basal and stimulated activity respectively. The Fig. (b) gives the PON1 activity in subjects with different *PON55* genotypes. Both cases and controls with MM genotype tended to have lowest PON1 activity. No differences were apparent in PON activity between cases and controls with different genotypes.

The genotypic distribution of *PON1192* and *55* was in Hardy Weinberg equilibrium. Patients had a higher frequency of AB and BB genotype as compared to controls (Table III). The odds ratio of developing the MI with BB genotype versus AA genotype was 2.37 (95% CI 1.00 to 5.62) and 1.8 (95% CI 1.13 to 2.88) with AB genotype versus AA genotype. The genotype frequency of *PON 55* as well as the allelic frequency of L and M allele was not different statistically among patients and controls. The odds ratio of getting MI with L allele was also below one (data not shown). Although cases with MI had higher circulating oxidized LDL as compared to controls (Table II), the levels were not significantly different among subjects with different *PON 192* genotypes in cases or controls although

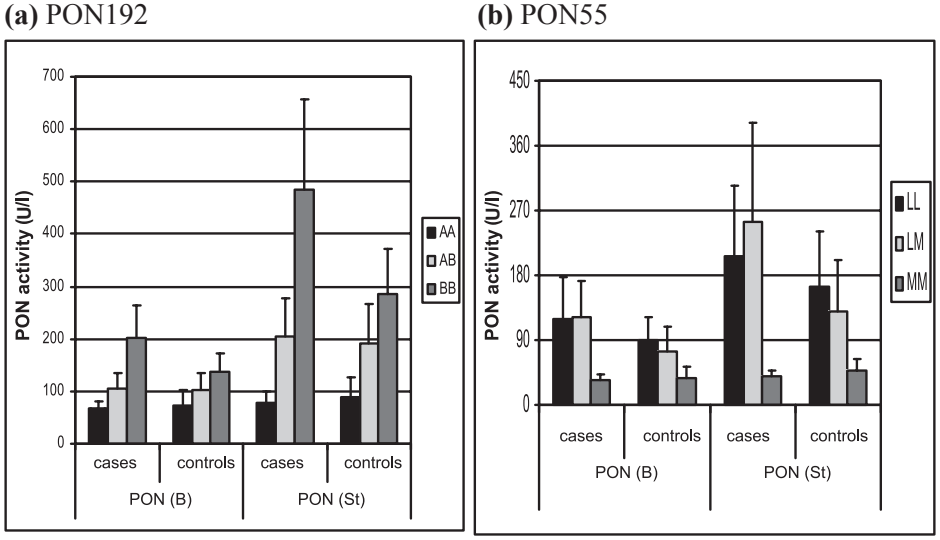

Fig. Paraoxonase activity (U/l) with different PON 192 **(a)** and PON 55 **(b)** genotypes in cases and controls. Values are mean ± SD. PON (B) indicates basal paraoxonase activity and PON (st) denotes stimulated paraoxonase activity. Differences in PON1 activity between different genotypes were assessed by one way ANOVA. Values in AA genotypes in cases and controls were significantly different from values in BB genotype at P<0.001 for both basal and stimulated activity.

Table III. Genotype and allele frequencies in cases and controls

	PON1 codon 192 polymorphism			Allele frequency	
Groups	AA, n (%)	AB, n (%)	BB, n (%)	A	B
Cases	42 (33.9)	70 (56.4)	12 (9.7)	0.62	0.38
Controls	108 (48.9)	100 (45.2)	13 (5.9)	0.71	0.29
Chi square test: *P*=0.021					
	PON1 codon 55 polymorphism			Allele frequency	
Groups	LL, n (%)	LM, n (%)	MM, n (%)	L	M
Cases	80 (64.5)	41 (33.1)	3 (2.4)	0.81	0.19
Controls	88 (57.1)	63 (40.9)	3 (1.9)	0.78	0.22
Chi square test: *P*=0.054					

a trend was apparent. Cases tended to have a lower total antioxidant status in all genotypes; however the difference was statistically significant ($P<0.05$) with patients with AB genotype. Both cases and controls with BB genotype tended to have the least mean total antioxidant level. MI patients with A allele showed significantly higher total antioxidant status as compared to subjects with B allele. MI patients showed lower total antioxidant compared to controls irrespective of whether they belonged to A or B allele. Cases with AA genotype showed higher oxidized LDL levels as compared to controls with AA genotype ($P<0.05$). Differences between genotype or different alleles were not so apparent in oxidized LDL levels. Similarly HDL levels were similar in patients with different genotypes and alleles and differences were also not apparent between cases and controls (Table IV). Table V shows the total antioxidant status, oxidized LDL and HDL with different *PON 55* genotype. There were no significant differences in total antioxidant levels with different genotypes or alleles in cases and controls. Oxidized LDL was significantly higher ($P<0.05$) in cases with LM genotype as compared to controls. MI patients with both L and M allele tended to have higher oxidized LDL as compared to controls with the same allele. There were no differences apparent between different alleles in cases and controls.

Discussion

The present study was undertaken to assess circulating oxidized LDL and the codon 192 and codon 55 PON polymorphism in incident acute MI patients. Since total antioxidant status is likely to influence oxidized LDL and PON1 may contribute to total antioxidant capacity, levels of total antioxidant was also evaluated in MI patients and controls. Although there are considerable accumulated data on effect of PON1 on LDL oxidation *in vitro*, data showing direct correlation between PON1 levels or PON1 genetic polymorphisms and oxidized LDL or antioxidant capacity *in vivo* are limited. Our results showed higher circulating oxidized LDL levels in patients with MI as compared to healthy controls and this paralleled with a lower antioxidant capacity in patients. Both increased oxidative stress and decreased antioxidant capacity can promote lipid peroxidation. Total antioxidant has been shown to be inversely related to the levels of circulating oxidized LDL[24] and further lipid-lowering therapy has been shown to contribute to the reduction in levels of AuAb-Ox-LDL and the increase in the antioxidant capacity of plasma LDL and total antioxidant status[25].

Although levels of oxidized LDL were higher in cases, we found similar PON1 activity in MI patients and controls. Similar finding have been described in Iranian subjects[26]. One of the main reasons for similar PON1 activity in our study could be because we did not exclude subjects on lipid lowering drugs which positively influence PON1 activity and possibly had more MI patients on lipid lowering therapy as compared to controls. It is reported in a recent study that statins more effectively increase PON1 activity in patients with AB+BB phenotype as compared to those with AA genotype[27]. Another explanation for the similar PON1 activity could be due to the fact that subjects with AB and BB genotype who had a higher PON1 activity were substantially more in number in cases as compared to controls. Similar PON1 activity in our study could also be attributed to the fact that the cases were age and sex matched to controls unlike in most other studies, where cases were older than controls and age is known to be a determinant of PON1 activity[28]. Low PON1 activity has been reported in CAD patients in a recent study from India[29]. In this study also the mean age of controls was lower than cases. We also found similar HDL cholesterol levels in MI patients and controls.

Distribution of *PON 192* polymorphism was significantly different between MI patients and

Table IV. Comparison of oxidized LDL, total antioxidant status and HDL levels between different PON192 genotype and alleles

	AA		AB		BB		A allele		B allele	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Total antioxidant	1.18 ± 0.50	1.34^{a} ± 0.47	1.11^{a,b} ± 0.39	1.33^{b} ± 0.54	0.97 ± 0.29	1.05 ± 0.18	1.14^{a} ± 0.44	1.33^{b} ± 0.50	1.09^{a,b,c} ± 0.39	1.29^{a,c} ± 0.52
Oxidized LDL	38.30^{a} ± 19.73	30.70^{a} ± 12.67	31.00 ± 14.49	30.32^{a} ± 11.16	42.33 ± 13.87	32.23 ± 13.86	33.92 ± 17.07	30.51 ± 12.04	31.98 ± 14.69	30.55 ± 11.46
HDL	41.33 ± 9.94	39.80 ± 10.15	38.47 ± 10.53	39.06 ± 9.40	41.00 ± 8.45	39.39 ± 11.79	39.61 ± 10.35	39.45 ± 9.78	38.69 ± 10.34	39.10 ± 9.65

Values are mean ± SD

^{a,b,c}indicate differences from each other at $P<0.05$ (Kruskal Wallis test)

Table V. Comparison of oxidized LDL, total antioxidant status and HDL levels between different PON55 genotype and alleles

	LL		LM		MM		L allele		M allele	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
Total antioxidant	1.11 ± 0.40	1.17 ± 0.37	1.12 ± 0.31	1.07 ± 0.33	0.83 ± 0.17	1.21 ± 0.43	1.11 ± 0.37	1.13 ± 0.36	1.09 ± 0.31	1.08 ± 0.35
Oxidized LDL	32.29 ± 16.29	27.63 ± 11.65	33.94^{a} ± 12.31	26.33^{a} ± 12.30	54.35 ± 40.00	26.67 ± 11.15	32.95^{a,b} ± 14.94	27.10^{a,c} ± 11.90	34.98^{c,d} ± 15.08	26.35^{b,d} ± 12.16
HDL	39.34 ± 9.93	38.02 ± 9.88	40.10 ± 10.73	37.30 ± 8.46	38.67 ± 2.52	35.67 ± 9.61	39.33 ± 10.18	37.72 ± 9.28	40.00 ± 10.37	37.23 ± 8.29

Values are mean ± SD

^{a,b,c,d}indicates differences from each other at $P<0.05$ (Kruskal Wallis test)

controls with patients having significantly higher frequency of B allele than controls. In a study on ethnic populations in Singapore[18], Indians were reported to have a significantly lower frequency of the B allele than Chinese. The authors reported that the genotypic distribution of the PON polymorphism was comparable between Chinese case patients and control subjects, but it varied significantly between Indian case patients and control subjects. Indian patients had a significantly higher frequency of the AB genotype (59 versus 40%) and lower frequency of the AA genotype (27% versus 47%). The only study from India[30] reported a B allele frequency of 0.45 in CAD patients from India as against 0.17 in controls. Pitfalls of selecting younger controls are that some of the younger controls may develop CAD later. No difference in PON55 M allele frequency was found in the present study. Similar results have been reported in Indians residing in Singapore[31]. Several studies in Caucasians and other ethnic groups have implicated PON1 codon 192 polymorphism with CAD risk[15-18]. However, several other studies on Caucasians and non Caucasians have not confirmed this association[13,14]. Polymorphism at codon 55 has similarly been shown to be associated with CAD in some populations but not in others[31-33].

A higher total antioxidant capacity in MI patients with A allele as compared to patients with B allele suggest that PON1 may be an important antioxidant contributing to the total antioxidant pool. Although oxidized LDL was highest in cases with BB genotype the difference was not staticstically significant probably to due to small number of subjects. It can also be deduced that contribution of other antioxidants in conjunction with paraoxonase may be of importance in inhibition of LDL oxidation. Our finding is similar to that by Sampson *et al*[34] who found circulating oxLDL concentrations *in vivo* to be unrelated to PON-1 genotypes or activity, except in male type II diabetics where there was a direct association between PON activity and oxLDL levels. In another study on smokers van Himbergen *et al*[35] found lack of association of PON-1 genotype with oxidative status parameters. Antioxidants such as flavonoids, glabridin or quercitin when present during LDL oxidation in presence of PON *in vitro* reduce the amount of lipid peroxides and preserve PON activities[36] suggesting that other antioxidants present in circulation may be important determinant of oxidized LDL levels.

One of the limitations of our study was that subjects on statins were not excluded and probably there were

more cases on statins as compared to controls which may have favourably influenced lipid variables. However, in spite of this oxidized LDL was found to be high in cases.

In conclusion, although *PON 192* polymorphism was significantly associated with MI, the oxidized LDL levels did not significantly differ with different *PON192* or *55* genotype implying that interaction with other antioxidant factors present in HDL and/or in circulation may be of importance in the inhibition of LDL oxidation in addition to PON1 in the population studied.

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