Preliminary Analysis of *PON3* rs2375003 Polymorphism in Pakistani Patients with Coronary Artery Disease

Maryam SAMEEM and Muhammad ARSHAD

Original article

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Coronary artery disease (CAD) is an inflammatory heart disease characterized by the narrowing of coronary arteries. Paraoxonase 3 (*PON3*) is a candidate gene for protection against CAD development as it reduces oxidative stress and lipid peroxidation. The present study aimed to explore the association of *PON3* rs2375003 polymorphism with CAD development and serum lipid levels in the Pakistani population. Study subjects included 300 CAD patients and 300 age and sex matched healthy individuals. The genotyping of rs2375003 polymorphism was done using an allele specific polymerase chain reaction and serum lipid levels were determined. In this study, the genotype frequencies of rs2375003 polymorphismin in CAD patients were TT (2%), CT (8%), CC (90%) as compared to TT (3%), CT (6%), CC (91%) in the healthy subjects. No association was observed between rs2375003 polymorphism and CAD risk (p>0.05). The CT genotype of rs2375003 polymorphism marginally increased the risk for CAD development (OR: 1.36; 95% CI 0.72-2.56) by causing a marginal rise in total cholesterol, low density lipoprotein cholesterol and triglyceride levels, and a marginal drop in high density lipoprotein cholesterol levels. The CT genotype of rs2375003 polymorphism and altered lipid levels might act as potential risk factors in the etiology of CAD in the Pakistani population.

Key words: Coronary artery disease, cholesterol, genotype, paraoxonase 3, polymerase chain reaction, rs2375003, Pakistan.

Maryam SAMEEM✉, Department of Zoology, Faculty of Sciences, University of Sargodha, Sargodha, Punjab, Pakistan
E-mail: maryamsameem@yahoo.com
Muhammad ARSHAD, Department of Zoology, Division of Science and Technology, University of Education, Lower Mall Campus, Lahore, Punjab, Pakistan

Coronary artery disease (CAD) is a complex disease in which coronary arteries that supply blood to the heart get hardened, inflamed and narrow. This is due to a buildup of plaque made up of immune cells, endothelial cells, smooth muscle cells, and lipids on the inner walls of arteries. Later on, the formation of a fibrous cap over the plaque converts it into an atherosclerotic lesion (ROBERTS 2014; SAKAKURA *et al.* 2013). The disease continues to progress as more atherosclerotic lesions are formed one after the other. Stable plaques interrupt the blood flow to the heart causing heart attacks, while the plaques that migrate to other sites cause thrombosis (SAYOLS-BAIXERAS *et al.* 2014).

Normal physiological processes such as the electron transport chain or oxidative bursts result in the production of free radicals, reactive oxygen species (ROS), and reactive nitrogen species in our body. These reactive species induce a number of responses which trigger cellular signaling, gene transcription, and immune responses. But the overproduction of ROS leads to harmful redox reactions, developing oxidative stress. This affects macromolecules, cell membranes, DNA, enzymes, and receptors, impairing the functioning of cells, followed by cell death (VALKO *et al.* 2007). In vascular cells, excessive ROS contribute to vascular inflammation, atherosclerosis, the accumulation of oxidized low density lipoprotein (oxLDL), and an increased risk of CAD. Thus, reducing oxidative stress may have a preventive role in CAD development (GRUZDEVA *et al.* 2014).

Various exogenous and endogenous antioxidants reduce oxidative stress by preventing the un-

necessary production and buildup of reactive species in cells. Exogenous antioxidants include vitamin C, vitamin E, and carotenoids which we obtain from our diet. Endogenous antioxidants present in the body are various enzymes such as superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase, and paraoxonases (*PONs*). Variations in the genes encoding these antioxidants may affect the levels of reactive species leading to cardiovascular diseases (DA COSTA *et al.* 2012).

The Paraoxonase (PON) gene family is comprised of three members, namely Paraoxonase 1 (*PON1*), Paraoxonase 2 (*PON2*), and Paraoxonase 3 (*PON3*), located on the long arm of chromosome 7q21-22 (BADTKE 2014). Their structure is 70% similar at nucleotide level and 60% identical at the amino acid level. This similarity in structure is why their functions are so similar (HAREL *et al.* 2004).

The *PON3* gene plays a principal role in the inhibition of oxidative stress, the reduction of inflammation, the metabolism of lipids, and the reversal of atherosclerosis (NG *et al.* 2005; SHIH *et al.* 2015). *PON3* is associated with high density lipoprotein cholesterol (HDL-C) in serum and shows enzymatic activity in preventing serum lipids from oxidation, hydrolyzing oxidized lipids, promoting cholesterol efflux from macrophages, and normalizing vascular endothelium function (HAFEZ *et al.* 2014; ZHANG *et al.* 2013; SHE *et al.* 2012).

Polymorphisms in the *PON3* gene may increase the risk of CAD by enhancing oxidative stress, causing inflammation in vascular cells, lowering HDL-C, and elevating low density lipoprotein cholesterol (LDL-C) and triglyceride (TG) levels (MARSILLACH *et al.* 2015; SARKAR & RAUTARAY 2008).

The polymorphism, rs2375003 in the *PON3* gene, is a guanine to adenine (G/A) transition at genomic position 29115 (G29155A) (RIEDMAIER *et al.* 2011). The result is an amino acid change from aspartic acid to asparagine at position 107 in protein (D107N) (ROBERTSON *et al.* 2003), which may influence normal gene function and affect CAD risk. The present study aimed to find out the genotype and allele frequencies of the rs2375003 polymorphism in the *PON3* gene; its association with CAD and lipid levels in the Pakistani population was also determined. This study was of its first kind, and so would help in the investigation of the genetic attributes of CAD in detail.

Materials and Methods

The Declaration of Helsinki was followed during all procedures of this study. The protocol of the present study was approved by the Advanced Studies and Research Board (ASRB). Permission for the start of research work was granted by institutional Ethical Committee (No. UOS/ZOL/871).

The study included 600 participants in total, out of which 300 were CAD patients (CAD group) and 300 were age and sex matched healthy individuals (Healthy group). The study sample included adult patients of both genders > 35 years of age, showing symptoms of CAD (pain, tightness or burning in chest, and heart palpitations) with a documented diagnosis (resting ECG, 50% stenosis in angiography) while asymptomatic patients, patients having psychiatric or cognitive disorders, patients with any chronic or acute illness (malignancy, bacterial, or viral infections), patients using medications which interfere with CAD development, and patients who had undergone bypass, angioplasty, or stenting recently were excluded from the study. Regarding the healthy group, for appropriate selection and successful recruitment of controls, friends and family members of the authors were asked to nominate someone who would fit age and gender requirements of the control group. Case patients were also requested to provide information on their friends and families who could serve as a source of controls. Most of the selected controls provided consent to participate in the research and completed the questionnaire. The limitations of this approach included the unwillingness of various potential controls to participate and the lack of an age and gender match for some of the consented controls. The persons who were unwilling to participate were mostly uneducated or elderly and could not understand the importance of health research. Some feared to share their personal information with strangers or they had a previous bad research experience, but the percentage of such people was as low as 20% of the total people contacted.

A record of the following data was kept for each research participant: age, sex, research group, lipid levels (total cholesterol (TC), LDL-C, HDL-C, TG) in milligrams per deciliter (mg/dl), and genotype of rs2375003 polymorphism.

Prior to sample collection, informed consent was taken from all research participants. Blood samples were collected using sterilized syringes (BD, USA) and were stored in ethylene diamine tetraacetic acid (EDTA) coated vials (BD, USA) at -20 °C for further study. A general blood test determined levels of TC, HDL-C, LDL-C and TG in the CAD and healthy group. Optimal values for TC, LDL-C and TG were considered to be <200 mg/dl, <100 mg/dl and <150 mg/dl respectively. For HDL-C, optimal values for men were 40-50 mg/dl and between 50-59 mg/dl for women.

For genetic analysis, isolation of genomic DNA was done using a DNA extraction kit (Vivantis, USA), followed by agarose gel electrophoresis and a PCR amplification of rs2375003 polymorphism using one forward and two reverse primers (Macrogen, USA). Primers having the following sequences were used in the study.

Forward (F) 5' ATTCAAACAGGTTGTGACAG3'

Reverse 1 (R$_1$) 5'TAGAAATCAGTGGTGGATTTG3'

Reverse 2 (R$_2$) 5'TAGAAATCAGTGGTGGATTTA3'

Each 50 µl allele specific PCR reaction was set up using 25 µl of PCR Master Mix (Bio Basic, Canada), 15 µl of graded water, 4 µl of genomic DNA, and 3 µl of F primer mixed with 3 µl of either R1 or R2 primers in separate PCR tubes. During the thermal amplification program, the heat activation of DNA polymerase was done at 94°C for 2 min, then thirty two cycles of denaturation at 94°C for 30 s, followed by primer annealing at 53.7°C for 1 min, extension at 68°C for 1 min, and a final extension at 68°C for 12 min.

Following amplification, PCR products were separated on 2% agarose gel (Bio Basic, Canada) stained with ethidium bromide (Invitrogen, USA) in an electrophoresis chamber (Thermo Scientific, China) and bands of PCR product were visualized under a UV transilluminator (Biotop, China). In the case of the CC homozygous genotype, bands (330bp) appeared only with the R1 primer. The TT homozygous genotype caused the bands (330bp) to appear only with the R2 primer, while the CT heterozygous genotype showed bands (330bp) with both R1 and R2 primers.

The demographical characteristics and lipid levels of the individuals of the CAD and healthy groups were compared using a 2-sample t-test. The genotype/allele frequencies and the difference in genetic and allelic frequencies considering the Hardy-Weinberg equilibrium were determined using a chi-square analysis. An odds ratio (OR) with a 95% confidence interval (95% CI) was calculated using an online calculator (BLAND & ALTMAN 2000), thus determining the association of rs2375003 polymorphism and incidence of CAD. While a one-way analysis of variance (ANOVA) test was conducted to compare the effect of different genotypes (TT, CT and CC) of rs2375003 polymorphism on lipid levels in the CAD and healthy groups. SPSS 16.0 (SPSS Inc., USA) was used to apply the chi-square test, 2-sample t-test and one-way ANOVA.

Results

Table 1 compares the age and gender of study participants. Based on this table, the difference in values for age and gender was not statistically significant in both groups (p>0.05).

Table 2 compares the levels of TC (mg/dl), HDL-C (mg/dl), LDL-C (mg/dl), and TG (mg/dl) among individuals of the CAD and healthy groups. The table shows that the mean values of TC, LDL-C and TG, among CAD group were higher than that of healthy group while the mean value of HDL-C levels was higher in healthy individuals than in CAD patients. Based on this table, values for LDL-C and TG were significantly different in the CAD and healthy groups (p<0.05) while the difference in values for TC and HDL-C was not statistically significant in both groups (p>0.05).

Table 3 presents the genotype frequencies of rs2375003 polymorphism in the CAD group, TT 7 (2%), CT 24 (8%), CC 269 (90%) as compared with TT 9 (3%), CT 18 (6%), CC 273 (91%) in the healthy subjects. The chi-square analysis showed no association between rs2375003 polymorphism and CAD (p>0.05). TT and CC genotypes of rs2375003 polymorphism exhibited no association with CAD development (OR: 0.77; 95% CI 0.28-2.10 and OR: 0.85; 95% CI 0.49-1.47 respectively). The CT genotype showed a marginal association with development of CAD (OR: 1.36; 95% CI 0.72-2.56).

Table 4 presents the allele frequencies of rs2375003 polymorphism in the CAD group as compared to the healthy subjects. C allele fre-

Table 1

Demographical data of the study participants

Demographical Data	CAD Group (N = 300)	Healthy Group (N = 300)	Total (N = 600)	p value
Age (Years) [a]	48.10 ± 9.70	46.64 ± 9.54	47.37 ± 9.64	0.064
Gender (Male) [b]	194 (65%)	178 (59%)	372 (62%)	0.404

[a] Data are shown as mean ± SD. 2-sample t-test was used for comparison of the CAD and healthy groups.

[b] Data are shown as number of individuals. The chi-square test of difference between the CAD and healthy groups defined in terms of disease presence.

Table 2

Lipid Levels of Study Participants

Lipid Levels (mg/dl)	CAD Group (N = 300)	Healthy Group (N = 300)	Total (N = 600)	p value
TC	182.44 ± 26.30	177.35 ± 25.58	179.90 ± 26.04	0.664
HDL-C	56.82 ± 16.97	59.66 ± 16.34	58.24 ± 16.71	0.387
LDL-C	90.98 ± 28.99	85.43 ± 23.69	88.20 ± 26.60	0.000*
TG	125.04 ± 57.56	120.20 ± 50.17	122.62 ± 54.00	0.004*

TC: Total cholesterol, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol, TG: Triglycerides, asterisk* shows significance at <0.05 level in mean lipid levels among individuals of both study groups; data are shown as mean ± SD. 2-sample t-test was used for comparison of CAD and healthy groups.

Table 3

Genotype frequencies of *PON3* rs2375003 polymorphism and its' association with CAD

Study Group	Genotype frequencies		
	TT	CT	CC
CAD Group N (%)	7 (2%)	24 (8%)	269 (90%)
Healthy Group N (%)	9 (3%)	18 (6%)	273 (91%)
Total N (%)	16 (3%)	42 (7%)	542 (90%)
Odds Ratio	0.77	1.36	0.85
95% CI	0.28-2.10	0.72-2.56	0.49-1.47
X^2 (p value)	1.137 (0.88)		

95% CI: 95% Confidence interval, X^2: Chi square value

Table 4

Allele frequencies of *PON3* rs2375003 polymorphism and its' association with CAD

Study Group	Allele frequencies		HWE (p value)
	T	C	
CAD Group	0.06	0.94	31.83 (0.000)***
Healthy Group	0.06	0.94	65.73 (0.000)***
Total	0.06	0.94	93.68 (0.000)***

HWE: Hardy-Weinberg equilibrium; asterisk*** shows significance at <0.001 level of difference in allele frequencies among individuals of each study group

quency (0.94) was higher than that of T allele (0.06) in CAD patients and healthy subjects. The allele frequencies deviated significantly from the Hardy-Weinberg equilibrium (HWE) in the healthy group and in the CAD group (p<0.001 for all).

Table 5 shows the association of rs2375003 polymorphism genotypes with lipid profile in the CAD and healthy groups determined by OR and 95% CI. The TT genotype caused a marginal rise in TG levels, a marginal fall in HDL-C levels, and showed no association with HDL-C and LDL-C levels. The CT genotype caused a marginal rise in TC, LDL-C and TG levels and a marginal drop in HDL-C levels. The CC genotype showed no association with any of the lipid classes.

Table 6 shows the comparison of mean lipid levels among the three genotypes (TT, CT and CC) of *PON3* rs2375003 polymorphism between the CAD and healthy groups. The results of the one-way ANOVA test showed that there were significant differences in the levels of serum TC, HDL-C, LDL-C, and TG (p<0.05 for all) among the three genotypes in both groups. TT genotype

Table 5

Association of *PON3* rs2375003 polymorphism and lipid profile

	Genotype	TT		CT		CC	
	Group	CAD	Healthy	CAD	Healthy	CAD	Healthy
	Total (N)	7	9	24	18	269	273
TC≥200mg/dl	N	5	4	15	11	57	43
	Odds ratio 95% CI	0.93 0.24-3.65		1.03 0.43-2.45		0.99 0.45-2.16	
HDL-C≤40mg/dl	N	3	2	14	7	43	33
	Odds ratio 95% CI	1.05 0.16-6.59		1.52 0.55-4.17		0.68 0.27-1.74	
LDL-C≥100mg/dl	N	4	3	10	4	65	41
	Odds ratio 95% CI	0.80 0.17-3.73		1.59 0.47-5.39		0.79 0.29-2.12	
TG≥150mg/dl	N	6	4	17	10	51	36
	Odds ratio 95% CI	1.01 0.27-3.79		1.19 0.49-2.87		0.86 0.39-1.89	

TC: Total cholesterol, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol, TG: Triglycerides, 95% CI: 95% Confidence interval

Data are shown as number of individuals of each genotype having lipid levels ≥ or ≤ optimal values in both study groups; data were analyzed using odds ratio and 95% CI.

Table 6

Association of *PON3* rs2375003 polymorphism and mean lipid levels

Lipid Levels	CAD Group			Healthy Group		
	TT	CT	CC	TT	CT	CC
TC	193.75 ±46.46	205.75 ±20.55	180.07 ±25.09	185.78 ±38.09	202.78 ±19.78	175.36 ±24.56
F value	11.95			10.87		
p value	0.000*			0.000*		
HDL-C	45.85 ±17.50	38.54 ±10.09	58.74 ±16.39	49.55 ±12.50	39.94 ±10.93	61.29 ±15.81
F value	19.20			18.00		
p value	0.000*			0.000*		
LDL-C	98.71 ±30.76	115.88 ±42.95	88.59 ±26.40	88.44 ±24.13	103.89 ±29.73	84.11 ±22.81
F value	10.63			06.15		
p value	0.000*			0.002*		
TG	185.57 ±64.86	192.12 ±74.80	117.48 ±50.66	162.11 ±40.48	180.22 ±82.76	114.86 ±44.33
F value	26.29			19.77		
p value	0.000*			0.000*		

TC: Total cholesterol, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol, TG: Triglycerides

Data are shown as mean±SD values for lipid levels in mg/dl; asterisk* shows significance at <0.05 level of difference in lipid levels among individuals of each genotype in both study groups using a one-way ANOVA test.

was only associated with higher mean TG levels, having no effect on TC, HDL-C, and LDL-C levels at all. CT genotype carriers showed the highest mean TC, LDL-C, TG levels and the lowest HDL-C levels compared to the TT and CC genotypes. CC genotype carriers had the highest HDL-C levels, while having normal levels for all other lipid classes. The trend for lipid levels were similar in both the CAD and healthy groups with the difference being that TC, HDL-C and LDL-C levels were lower while HDL-C levels were higher in the healthy group when compared with their counterparts in the CAD group.

Table 7

Post hoc comparison of differences in mean lipid levels among *PON3* rs2375003 polymorphism genotypes

Serum lipids	Genotype	CAD Group			Healthy Group		
		TT	CT	CC	TT	CT	CC
TC	TT	–	12.178	13.500	–	17.000	10.422
	CT	–	–	25.679*	–	–	27.422*
	CC	–	–	–	–	–	–
HDL-C	TT	–	07.315	06.882	–	09.6111	11.737
	CT	–	–	20.205*	–	–	21.348*
	CC	–	–	–	–	–	–
LDL-C	TT	–	17.160	10.115	–	15.444	04.327
	CT	–	–	27.276*	–	–	19.771*
	CC	–	–	–	–	–	–
TG	TT	–	06.553	68.091*	–	18.111	47.250*
	CT	–	–	74.645*	–	–	65.361*
	CC	–	–	–	–	–	–

Data are shown as mean difference in mean lipid levels; asterisk* shows significance at <0.05 level of difference among individuals of each genotype in both study groups using a post hoc Tuckey's HSD test.

Table 7 presents the results of multiple comparisons of differences in mean lipid levels among *PON3* rs2375003 polymorphism genotypes in both groups by a post hoc Tuckey's HSD test. Results suggested that the mean differences in serum TC, HDL-C, and LDL-C levels in the CT genotype carriers were significantly different than that of subjects having the CC genotype in both the CAD and healthy groups. For TG levels, a significant difference in mean lipid levels was observed in the case of the TT and CC genotype carriers as well as the CT and TT genotype carriers. No significant difference was detected in levels of any lipid class between the TT and CT genotype subjects.

Discussion

Our study reports *PON3* rs2375003 polymorphism minor allele frequency to be 0.06 in the Pakistani population. This finding is contrary to ERLICH *et al.* (2012) who reported the minor allele frequency of *PON3* rs2375003 polymorphism to be 0.00 in Americans and Caucasians. This difference may be due to unrelated ethnic backgrounds, diverse environmental exposures, and different rates of mutations in Americans and Asians.

Numerous epidemiological studies have reported an association of various *PON3* polymorphisms with atherosclerosis derived diseases, but no study has clearly reported the role of *PON3*

rs2375003 polymorphism in the development of CAD. ZHANG *et al.* (2013) did not find any positive association of *PON3* gene polymorphisms rs2074353 and rs1053275 with stroke risk. RANADE *et al.* (2005) reported that polymorphisms Glu146Lys, Ala179Asp, Tyr233Cys and Ala99Ala in the *PON3* gene were not associated with stroke. Similar studies by SU *et al.* (2005) and WANG *et al.* (2003) found that *PON3* gene – 133C>A polymorphism in the promoter region showed no significant effect on coronary heart disease (CHD) risk. KIM *et al.* (2012) found no contribution of *PON3* polymorphisms rs17884000, rs9640632, rs468, rs11768074, rs10487132, rs740264 in the development of vascular disease. SANGHERA *et al.* (2008) found that *PON3* polymorphisms A2115T, T10340G, C30588T, A40512G, C45486A, G55146A were not associated with cardiovascular disease. Our results are consistent with the above studies as no association was found between *PON3* rs2375003 polymorphism and CAD risk (p>0.05). TT and CC genotypes of rs2375003 polymorphism showed no association with CAD development (OR: 0.77; 95% CI 0.28-2.10 and OR: 0.85; 95% CI 0.49-1.47 respectively).

SHIH *et al.* (2015) reported that elevated TC, LDL-C and TG, with decreased HDL-C levels due to reduced PON3 gene activity contributed to a 60% increased risk of atherosclerosis. Our findings are in line with SHIH *et al.* (2015), as higher mean TC, LDL-C and TG levels with lower

HDL-C levels in the CT genotype of rs2375003 polymorphism showed a marginal association of this genotype with CAD risk (OR: 1.36; 95% CI 0.72-2.56).

PASDAR *et al.* (2006) found that the GG genotype of *PON3* rs1053275 polymorphism contributed only to higher TG levels in Caucasians. The results of our study are similar to PASDAR *et al.* (2006), as subjects with the TT genotype of *PON3* rs2375003 polymorphism exhibited normal lipid levels with the exception that our subjects exhibited higher mean TG levels.

RANADE *et al.* (2005) reported that polymorphisms Glu146Lys, Ala179Asp, Tyr233Cys and Ala99Ala in the *PON3* gene were not associated with lipid levels. This finding is in accordance with the above study, as the CC genotype of rs2375003 polymorphism showed no association with any of the lipid classes.

Conclusions

This study was of its first kind in finding the role of *PON3* rs2375003 polymorphism in predicting CAD risk as well as in determining its association with serum lipid levels in the Pakistani population. We found that the TT and CC genotypes of *PON3* rs2375003 polymorphism exhibited no association with CAD development. The CT genotype of *PON3* rs2375003 polymorphism might contribute to CAD susceptibility due to its role in pathophysiological consequences regarding lipid metabolism in its carriers. Additional studies involving a larger sample size, more detailed data of CAD risk factors, employing many techniques for single SNP detection, and consideration of other SNPs in the *PON3* gene are required to explore a more precise association of rs2375003 polymorphism with CAD risk and lipid profile.

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Author Contributions

Research concept and design: M.S.; Collection and/or assembly of data: M.S.; Data analysis and interpretation: M.S.; Writing the article: M.S.; Critical revision of the article: M.A.; Final approval of article: M.A.

Conflict of Interest

The authors declare no conflict of interest.

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