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Paraoxonase 1 Q192R (A/G) Gene Polymorphism as possible risk factor for coronary heart diseases among Egyptians. Case-control study

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Keywords

- Paraoxonase 1 (PON1)
- Polymorphisms
- Coronary heart disease Egyptians

Abstract

Background: Coronary heart disease (CHD) is the leading cause of morbidity and mortality worldwide. There are many risk factors for CHD but recently the role of oxidative stress in progression of atherosclerosis has been more recognized. Paraoxonase 1 (PON1) protects against oxidation of LDL and many polymorphisms in both of exons and promoter regions of (PON1) gene have been investigated for their association with CHD. The aim of the present study was to investigate the relation between CHD suceptibility and PON1 Q192R (A/G) gene polymorphism in a cohort of Egyptian individuals**. Methods:** The study included 100 subjects, 50 patients who admitted to cardiovascular department with established diagnosis of obstructive coronary artery disease by coronary angiography and 50 healthy participants. Genotyping of PON1 Q192R (A/G) was done, and then serum concentration of PON1 was assessed by ELISA after that by spectrophotometer. **Results:** Serum PON1 enzyme was lower in patients with CHD than in control group with a statistically significant difference $p < 0.001$. A statistically significant association was observed with AG and GG genotypes of PON1 gene with CHD with P= 0.003, OR=5.02(95% CI =1.66-15.26) and P= 0.038, OR= 9.4 (95% CI =1.07-82.5); respectively. The G allele of PON1 was higher in CHD patients than controls suggesting that this allele may demonstrate a susceptibility effect to CHD in our cohort with P<0.001, OR= 5.16 (95% CI = 2.1-12.5) **Conclusion:** The Q192R polymorphism in the PON1 gene may be a susceptibility gene associated with increased risk of CHD among Egyptians.

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INTRODUCTION

Coronary heart disease (CHD) is the leading cause of morbidity and mortality all over the world. The incidence of CHD is rising, and it comes to be a true pandemic. (1), (2). The age-related death rates from CHD are declining in many developed countries and increasing in the developing countries as a result of variations in demographic, urbanization and lifestyle ***(3),(4)*** **.** In Egypt, CHD is a major health problem with the incidence of deaths about 40% of total deaths through the Egyptian population **(5)**. CHD has multiple risk factors including age, dyslipidaemia, diabetes mellitus, hypertension, sedentary lifestyle, smoking and obesity. The role of Oxidative stress in the initiation and progression of atherosclerosis and CHD has gain attention in the last few decades. ***(6)*** The newer risk factors for CHD such as the genetic factors e.g mutations at specific chromosomal locations and single nucleotide polymorphisms (SNPs) **(3)** is necessary to be included with the classical risk factors to improve the ability to expect future risk and determine the treatment plan for those patients .***(7)*** Paraoxonase 1 (PON1) is a glycoprotein synthesized in the liver and secreted in the blood where it is related to high-density lipoprotein (HDL) cholesterol in the circulation. It has antioxidant properties, through protection of low density lipoprotein cholesterol (LDL-C) and HDL-C against oxidation, it also suppress the monocytes differentiation into macrophages, so limiting the process of foam cell formation and atherosclerosis. As a result (PON1) became the focus of intensive research both at phenotypic and genetic levels**.** ***(8)*** (9).PON1 gene is situated in the long arm of chromosome 7 at q21.3. It has 9 exons and it is 26 kb in length. More than 200 single nucleotide polymorphisms (SNPs) have been defined for this gene. Multiple polymorphisms in exons and promoter regions of PON1 gene have been explored in many researches for their association with CHD. It had been proposed that low serum PON 1 concentration and PON1 Q192R (A/G) polymorphism- that is an amino acid replacement at codon 192 glutamine (Q) to arginine (R)- might be risk factor for atherosclerosis and development of CHD (10). To our knowledge, the studies that were conducted to evaluate the association between PON1 Q192R polymorphism with the CHD among Egyptian population are deficient. So, the present study was undertaken to assess the correlation between polymorphism of PON1 Q192R and CHD in Egypt.

2. Methods:

2.1. Study Population: A hospital based matched case control study was conducted during the period from August 2018 to August 2019. The study included a convenient sample of 100 subjects. 50 patients who admitted to cardiovascular department complaining of chest pain with established diagnosis of obstructive coronary artery disease by coronary angiography. Coronary angiography showed 70% or more stenosis in one or more coronary arteries are considered to have obstructive CAD *(11)***.** Patients with congenital heart disease, rheumatic heart disease, end stage renal disease, or advanced liver cirrhosis are excluded from this study. Control group included 50 healthy participants. A control was defined as age and sex matched subjects with no clinical evidence of CHD. They were recruited from other departments (such as ophthalmology, blood banks and outpatient clinics). Eligibility criteria for control include: fully conscious, co-operative, and

well-oriented with time, place, and person, who voluntary agree to participate in the study. All patients and controls were from Egypt with both Egyptian parents**.**

2.2. Study Tool: An interviewer-administered structured questionnaire was done and including socio-demographic characteristics such as age, sex, physical examination. Coronary angiography was done for all patients. Laboratory investigations were done as blood samples (5ml) were collected from antecubital vein of both patients and control subjects between 8-10 a.m after a 12-h overnight fasting. Each sample was divided as following: a) 2 ml were delivered to a test tube containing 200 µl EDTA to prevent blood coagulation and stored at -20 °C until DNA extraction for genotyping. (b) 2 ml were collected in a tube with a clot activator and serum gel separator for ELISA for estimation of serum PON 1 enzyme concentration (c) 1ml blood was collected in sterilized dry tube for lipid profile.

Typing of PON1 Q192 R (rs 662) gene polymorphisms:

Genomic DNA was extracted from whole venous EDTA blood using INTRON G-spin™ Total DNA Extraction Kit. The PON1 Q192 R (rs 662) polymorphism was genotyped by PCR based restriction fragment length polymorphism (RFLP) according to the method of Hasselwander et al. with some modifications (12). PCR amplification of the region containing A/G polymorphism in PON 1 gene was carried out with the following primers: Forward 5-TATTGTTGCTGTGGGACCTGAG-3 and Reverse 5-CACGCTAAACCCAAATACATCTC-3. Reaction volume was 25 µl: 6 µl DNA at 10 ng/µl, 12.5 µl 2X Taq PCR Tiangen master mix, Tiangen Bioline, Beijing, China, Cat. no: KT201), 0.5 µl of each primer (10 pmol/ µl) , and 5.5 µl H_2O. Reactions were carried out in thermocycler (Techne TC-312, UK) with the following cycling parameters: an initial denaturation at 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min and a final extension at 72 C for 10 min. 10 µl of PCR products were resolved in 2.5 % agarose gel to check the PCR products with 50 bp DNA ladder (INtron Biotechnology Inc, South Korea, Cat. no: 24072). The electrophoresis apparatus was adjusted at 100 volts and the run lasted for 45 minutes, the gel visualized with transilluminator. Appearance of sharp bands near 100 bp band of ladder indicates correct amplification of 99 bp PCR product.

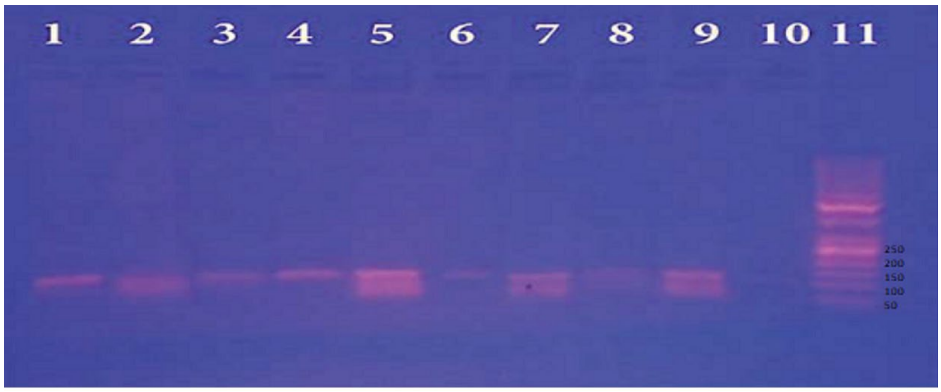

Figure (1) showing visualization of 99 bp PCR product of PON 1 gene by gel electrophoresis (2.5 % agarose gel, Lane **11** represents 50bp DNA ladder, lanes 1-10 PCR products represented by one band at 99 bp).

The PCR product was digested with specific restriction endonuclease enzyme AlwI **(Thermo Scientific BspPI AlwI Thermo Fisher scientific Inc, Eu)**. AlwI restriction enzyme had one recognition site in the G allele but no recognition site in the A allele within the amplified region generating various length fragments that were characteristic for this SNP. These fragments were separated by using gel electrophoresis.

20 µl total volume reaction was prepared by mixing: 10 µl of PCR products, 1µl of restriction enzyme, 2 µl 10X FastDigest green buffer, and 7 µl nuclease- free water. The mixture was incubated at 55 °C for 3 hours followed by heating at 80 °C for 20 minutes. The transversion of the A nucleotide into G nucleotide in the 99 bp PCR-product creates only one recognition sites for AlwI restriction endonuclease cutting PCR product into 69 and 30 bp fragments**.** After digestion, products were separated on 3% agarose gel with 50 bp ladder. The gel was run at 80 volts for 1 hour, after completion sharp bands were checked and photographed.

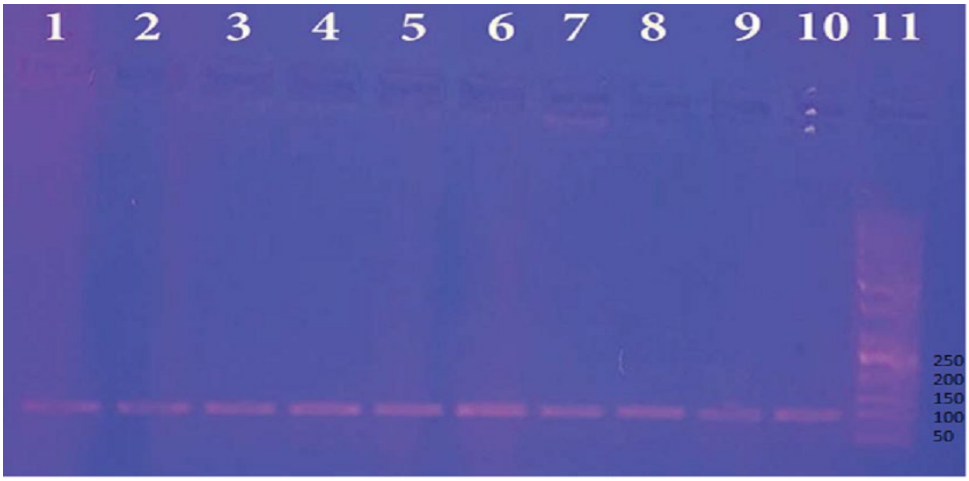

Figure (2): showing visualization of RFLP analysis of rs662 polymorphism , 3% agarose gel electrophoresis ; lane 11 represents 50bp ladder, there are two bands (69 bp and 30 bp) GG genotype in lanes 7, 8, 9 , three bands (99bp, 69bp, and 30bp) AG genotype in lane 5, and one band (99 bp) AA genotype in lanes 1,2,3,4,6.

2.3. Ethical Consideration. Subjects gave their consent to participate in the study. All the information that was obtained about the subjects was kept confidential. Study protocol was approved by Institution Research Board (IRB) of our medical college ***number: MS.16.06.48.***

2.4. Data analysis: Data were entered, cleaned to identify inconsistencies and statistically analyzed using the Statistical Package for Social Science (SPSS) version 16. The normality of data was first tested with one-sample Kolmogorov-Smirnov test. Qualitative data was described using number and percent. Association between categorical variables was tested using Chi-square test. When more than 25% of the cells have expected count less than 5, Fisher's exact test was used. Continuous variables were presented as mean ± SD (standard deviation). Independent sample t- test was used to compare means (parametric data). Deviations from Hardy–Weinberg equilibrium expectations were determined using the chi-squared test at a significance level of $P<0.05$. Polymorphisms and genotype frequencies were evaluated by gene counts. The data were tested for the goodness of fit

between the observed and expected genotype frequencies (X2 test). When the observed genotype frequencies fit to Hardy-Weinberg equilibrium, X2 tests (2-by-2 tables) were performed to calculate significantly different genotype distributions between patients and controls and also odd's ratio(OR) and confidence interval95% were calculated to detect risk ratio. A P value <0.05 was considered statistically significant in all analyses.

Results:

Table (1) showed that both cases and control groups were matched regarding all their socio-demographic characteristics. There was statistically significant association between the presence of hypertension, diabetes and current history of smoking with CHD (OR= 3.01, 4.09 and 3.3., respectively).

Serum PON1 concentration was statistically significantly higher in control group than CHD cases, p value < 0.001. Lipid profile analysis in both studied groups showed significantly higher TC, TG, LDL-C level ($p < 0.0001$).and lower HDL-C level in cases compared to controls, P= 0.04 as shown in **table (2)**

There was a significant difference between CHD patients and control in the allelic distribution of the PON1 (P<0.001*), Therefore, the G allele of PON1 was higher in CHD patients than controls suggesting that this allele may demonstrate a susceptibility effect to CHD in our cohort (OR= 5.16 (95% CI = 2.1-12.5) as shown in **tables (3)** .A statistically significant association was observed with AG and GG genotypes of PON1 gene with CHD with OR=5.02(95% CI =1.66-15.26) and 9.4 (95% CI =1.07-82.5); respectively.

Table (4) showed that in both CHD patients and control groups, there was highly significant changes between AA ,AG and GG genotypes as regard to serum PON1 concentration in each group (patients and controls) (p<0.001 for each). On the other hand, serum PON 1 level was higher in control group with AA and AG genotypes with statistically significant difference.

There was no statistically significant difference between AA, AG and GG genotypes as regard to total cholesterol, triglyceride, HDL and LDL in each group (patients and controls) with (p=0.33, 0.8), (p=0.1, 0.38), (p=0.15, 0.86) and (p=0.4,0.69); respectively.

On the other hand, cholesterol, triglyceride and LDL were higher in patient group with AA and AG genotypes than control group with statistically significant difference .HDL was found higher in control group with AA genotype than control group with statistically significant difference.

Table (5) showed that Both A &G alleles showed significant higher PON1 levels in control than CHD patients groups (P<0.001, 0.003; respectively). Both A &G alleles showed significant higher cholesterol, triglyceride and LDL levels in CHD patients than control groups (P<0.001 for each), (P<0.001 for each) and(p <0.001,0.003); respectively .Moreover A alleles showed significant higher HDL levels in control than patients groups with p= 0.002.

Table (1): Socio-demographic and clinical features of cases versus controls

Socio-demographic characteristics	Cases=50 N (%)	Controls=50 N (%)	Significance test	OR(95%CI)
Age (Mean ± SD)	**55.24** ± 8.17	**54.38**± **6.77**	t=-0.2, P=0.84	-
Sex Female (r) Male	 36 (72) 14 (28)	 36 (72) 14 (28)	Not applicable	Undefined
DM No (r) Yes	 30 (60) 20 (40)	 43 (86) 7 (14)	χ^2= 8.6 , P=0.003	4.09(1.5-10.8)
HTN No Yes	 23 (46) 27 (54)	 36 (72) 14 (28)	χ^2= 6.9 , P=0.008	3.01(1.3-6.9)
Smoking Never smoked Ex-smoker Current smoker	 16 (32) 5 (10) 29 (58)	 31 (62) 2 (4) 17 (34)	- FET ,P=0.09 χ2= 7.8 ,P=0.005	1 4.8(0.8-27.7) 3.3(1.4-7.7)

t: student t test , FET: Fisher's exact

Table (2): Serum PON 1 level and lipid profile among cases versus controls

PON 1 and lipid profile	Cases	Controls	P value
Serum PON1(ng/ml)	0.166 ± 0.059	0.228 ± 0.042	<0.001*
Cholesterol (mg/dl)	238.31 ± 40.66	5.88 ± 15.68	<0.001*
T.G (mg/dl)	199.52 ± 25.10	132.57 ± 18.01	<0.001*
HDL-C (mg/dl)	50.86 ± 10.53	55.30 ± 10.95	0.04*
LDL-C (mg/dl)	145.70 ± 40.69	95.95 ± 11.24	<0.001*

Table (3): Distribution of PON1 Q192 R (rs 662) alleles and genotypes in CHD cases versus controls.

PON1 Polymorphism	Cases (N=50) N (%)	Controls (N= 50) N (%)	Significance test	OR (95% CI)
Alleles (n=100) A (r) G	 72 (72) 28 (28)	 93(93) 7(7)	χ^2=15.3, P<0.001*	1 5.16(2.1-12.5)
Genotypes AA (r) AG GG	 28 (56) 16 (32) 6 (12)	 44 (88) 5 (10) 1 (2)	- χ^2= 9.07,P= 0.003* FET,P= 0.038*	 1 5.02(1.66-15.26) 9.4(1.07-82.5)

Table (4): Comparison of Serum PON1 and lipid profile between both patient and control groups in different genotypes

	Patient (50)			P1	Control (50)			P2	P3	P4
	AA	AG	GG		AA	AG	GG			
Serum PON1 Mean +/- SD	0.202 0.055	0.128 0.009	0.99 0.13	<0.001*	0.238 0.033	0.161 0.022	0.123 -	<0.001 *	<0.001*	<0.001*
Cholesterol Mean +/- SD	245.47 42.90	231.77 40.98	222.33 22.6	0.33	175.93 16.23	173.62 12.43	184.8 -	0.8	<0.001	0.006
TG Mean +/- SD	208.11 21.35	191.38 27.93	181.17 18.49	0.1	132.63 18.34	127.52 14.10	155 -	0.38	<0.001	<0.001
LDL Mean +/- SD	152.25 41.81	138.5 43.29	134.33 24.59	0.4	96.45 11.57	91.92 9.17	94.00 -	0.69	<0.001	0.03
HDL-c Mean +/- SD	48.43 8.02	54.75 14.11	51.83 7.73	0.15	55.00 11.41	57.22 7.78	59.00 -	0.86	0.006	0.7

*SD: standard deviation P: Probability *: significance <0.05*
P1: For comparison between genotypes in patients group
P2: for comparison between genotypes in control group
P3: For comparison between Patient & control group in AA genotype
P4: For comparison between Patient & control group in AG genotype

Table (5): Comparison of Serum PON1 and lipid profile between both patient and control groups in different alleles

	Patient		P1	Control		P2	P3	P4
	A	G		A	G			
Serum PON1 Mean +/- SD	0.185 0.057	0.116 0.018	<0.001 *	0.234 0.036	0.150 0.026	<0.001*	<0.001*	0.003*
Cholesterol Mean +/- SD	242.43 42.28	227.73 33.75	0.1	175.81 15.91	176.81 11.52	0.87	<0.001	<0.001
TG Mean +/- SD	204.39 23.68	187.00 24.22	0.001	132.36 18.01	135.37 17.67	0.67	<0.001	<0.001
LDL Mean +/- SD	149.19 41.94	136.71 35.63	0.17	96.21 11.40	92.51 7.55	0.4	<0.001	0.003
HDL-c Mean +/- SD	49.83 9.90	53.50 11.62	0.11	55.12 11.16	57.73 6.41	0.54	0.002	0.36

*SD: standard deviation P: Probability *: significance <0.05*
P1: For comparison between Alleles in patients group
P2: for comparison between Alleles in control group
P3: For comparison between Patient & control group in A Allele
P4: For comparison between Patient & control group in G Allele

Discussion:

Coronary heart disease is a global health problem with increasing magnitude over time. Many traditional risk factors for atherosclerosis are present but novel risk factors like genetic polymorphisms are recently investigated. PON1 is a glycoprotein synthesized in the liver and secreted in the blood where it is related to HDL-C in the circulation. It has antioxidant properties, through protection of LDL-C and HDL-C against oxidation, it also suppress the monocytes differentiation into macrophages, so limiting the process of atherosclerosis. Our study was carried out on 50 patients who were diagnosed formerly with CHD and admitted to Cardiology Unit in our university hospital and 50 age matched healthy non-cardiac subjects. At first genotyping of PON1 Q192R (A/G) was done, and then serum concentration of PON1 was assessed by ELISA after that by spectrophotometer. Lipid profile was done for the cases and control groups. These procedures were conducted in laboratories of Biochemistry Department. In this study, analysis of genotype frequency for *PON1* Q192R polymorphisms revealed statistically significant difference between CHD patients and controls with higher prevalence of GG genotype and the G (R) allele of PON1 Q192R (A→G) polymorphism in CHD patients. These results are in agreement with a study conducted in Saudi Arabia by Hassan et al. that showed the frequency of AG and GG genotypes in CHD patients was increased compared to controls and accordingly a significant association of the Q192R (A→G) polymorphism with CHD ***(9)*** .Also, these findings were in agreement with Randa et al. **(13)** in Egypt who mentioned that PON1 GG genotype individuals had 9 times higher risk of CHD, while PON1 AG genotype individuals had a 4 times higher risk of CHD. Similar results were obtained in studies conducted on patients suffering from ischemic strokes, in whom the PON1 192 GG genotype was correlated with the risk of stroke (14). In addition ***Gupta et al.*** (15***)*** found that the frequency of 192 G allele was significantly higher in CHD patients and that variants of 192 AG and 192 GG were accompanied with high risk of CHD. Also, a significant association between PON1 Q192R (A→G) polymorphism and Coronary artery diseases and PON1 192 G allele as an important risk factor for CHD has been also found by ***Chen et al*** in China(**16) and** ***Munshi et al.*** in India***(17).*** Logistic regression analysis of the data of ***Kaur et al.*** in India revealed that the heterozygous genotype (AG) in the PON1 gene might be associated with an about 1.6 fold augmented risk of developing CAD in an Indian population (odds ratio [OR] = 1.59, 95% CI = 1.19–2.12, $p < 0.0001$) (18).

In contrast, some studies didn't find evidences for the changes of PON1 and the association between Q192R polymorphism and CVDs risk (19)**. Mackness et al** stated that CVD was associated with the activity and concentration of paraoxonase1 but not its genotype **(20).** Furthermore, in EPIC Norfolk Prospective Population Study, neither PON1 Q192R polymorphism nor activity of PON1 predicted future risk of CHD (21). Moreover, there was no significant correlation between *PON1* L55M or Q192R (A→G) polymorphisms and susceptibility to CHD (22)**.** In addition, a study in France by ***Martínez et al.*** (23) revealed that there was no significant difference between the AA and AG/GG

genotypes in both the clinical variables and extent of CAD. These divergences between the data obtained from the different studies might be caused by methodological, environmental and life style factors. Alteration of the association between PON1 polymorphism and the risk of CHD by gene-gene interactions and/or gene-environmental factors can be the cause for such diverse results. In the present study, the serum concentration of PON1 was significantly higher in control group than patient group, and its level was significantly higher in control group with AA (QQ) and AG (QR) genotypes. These findings were in accordance with **Hampe et al.** ***(24)*** who mentioned that activity of arylesterase of PON1 which represents PON1 enzyme concentration was significantly lower in CHD patients in comparison to control group. Moreover, PON1 activity and its serum concentration in the studied individuals were lower in CHD patients than in the control subjects. This result might be elucidated by several polymorphisms that have been reported for PON1 genes in the coding and the promotor regions that affect enzyme activities and /or concentrations to a greater or lesser extent (20).A significant relationship had been found between the activity and concentration of PON1 and coronary atherosclerosis expressed as quantitative angiographic indexes of severity, extent, and overall atheroma burden of CHD. Individuals with lowest activity and concentration of PON1 had a more severe CHD than individuals with highest PON1 activity and concentration. ***(25, 26)***

Conclusion: Paraoxonase 1 levels in blood and PON 1 gene polymorphisms may promote to the susceptibility of individuals to CHD. AG and GG genotypes of PON1 gene can be considered risk factors for the development of CHD among Egyptians. Additional rigorous design, wide scale and multicenter studies, large sample of case-control, or prospective study are necessary to evaluate and investigate the relationship between gene polymorphisms -either alone or combined- and the occurrence of CHD among Egyptian population.

Study limitations: This study is a group matched case control study design and the results are restricted to the subgroup of survivors of CHD but not to the whole group of CHD patients, these observations need further confirmation using prospective study design. Also, the single center hospital based study that doesn't reflect the national situation at the community level.

Abbreviations:

CHD: Coronary Heart Disease
HDL-C: High density lipoprotein cholesterol
PON1: Paraoxonase 1
LDL-C: Low density lipoprotein cholesterol
EDTA: Ethylene di-amine tetra acetic acid
ELISA: Enzyme-linked immunosorbent assay
RFLP: Restriction fragment length polymorphism
PCR: Polymerase chain reaction
DNA: Deoxyribonucleic acid
SNP: Single-nucleotide polymorphism
IRB: Institution Research Board
SPSS: Statistical Package for Social Science
OR: Odd's ratio
TC: Total cholesterol
TG: Triglycerides
CVD: Cardiovascular disease
IHD: Ischemic heart disease

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