Distribution of Pon L/M55 and Q/R192 Genotypes in Turkish Patients with Angiographically-Defined Coronary Artery Disease: Effects on Serum Lipids

Anjiyografik Olarak Koroner Arter Hastalığı Tanısı Konulmuş Türk Hastalarda Pon L/M55 ve Q/R192 Genotiplerinin Dağılımı: Serum Lipitlerine Etkileri

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ABSTRACT Objective: Paraoxonase 1 (PON1) was suggested to lower the risk of coronary artery disease (CAD) by preventing high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein-cholesterol (LDL-C) oxidation during atherosclerotic process. The relation between PON1 activity and CAD has been attributed to polymorphisms in the gene coding for this enzyme. The PON1 gene has two common polymorphisms, which lead to a leucine→methionine substitution at position 55 (PON55) and a glutamine→arginine substitution at position 192 (PON192), have been defined as the molecular basis of interindividual variability in PON activity. The aim of this study was to investigate the frequency of PON55 and PON192 polymorphisms in Turkish patients with CAD and to determine whether this polymorphism was effective on plasma lipid levels. **Material and Methods:** One hundred and thirty-five angiographically-defined CAD patients and 110 healthy controls were screened for the PON55 and PON192 genotypes and plasma lipids. **Results:** The frequency of PON55 MM genotype was significantly higher in the CAD patients than in the controls. The frequencies of PON192 genotypes were similar among CAD patients and controls. Both for PON55 and PON192 genotypes the HDL-C levels of CAD patients were significantly lower than in the corresponding controls. The severity of CAD was not associated with either the PON55 or the PON192 genotype. **Conclusion:** PON55 and PON192 genotypes are not useful markers for the estimation of either cardiovascular risk or the severity of coronary artery disease in the Turkish population.

Key Words: Coronary disease; cholesterol, HDL; pon1 protein, human; polymorphism, genetic; aryldialkylphosphatase

ÖZET Amaç: Paraoksonaz 1'in (PON1) yüksek yoğunluklu lipoprotein-kolesterol (HDL-K) ve düşük yoğunluklu lipoprotein kolesterol (LDL-K)'leri oksidasyondan koruyarak koroner arter hastalığı (KAH) görülme sıklığını azalttığı ileri sürülmektedir. PON1 aktivitesi ile KAH arasındaki ilişki, bu enzimi kodlayan genin polimorfizmlerine atfedilmektedir. Bireyler arasındaki PON aktivitesi farklığına moleküler temel oluşturan, genin 55. pozisyonundaki lösin→metiyonin değişimi (PON55) ile 192. pozisiyonundaki glutamin→arginin değişimi şeklinde iki polimorfizm tanımlanmıştır. Çalışmamızda, koroner arter hastalığı olan Türk hastalarda PON55 and PON192 polimorfizmlerinin frekansları ile bu polimorfizmlerin plazma lipit düzeylerine etkilerinin incelenmesi amaçlanmıştır. **Gereç ve Yöntemler:** KAH tanısı anjiyografik olarak konulmuş 135 hastada ve 110 sağlıklı kişide PON55 and PON192 genotipleri ve plazma lipitleri incelendi. **Bulgular:** Hastalarda PON55 MM genotipinin frekansının kontrollere göre anlamlı olarak daha fazla olduğu saptandı. Hasta ve kontrol grubu arasında PON192 genotiplerinin dağılımı açısından anlamlı bir fark bulunmadı. Hem PON55 hem de PON192 genotiplerinde hastaların plazma HDL-K düzeyleri, bu genotiplere karşılık gelen kontrol olgularındakine göre anlamlı olarak daha düşük bulundu. Her iki polimorfizm için de genotiplerle hastalığın ciddiyeti arasında bir ilişki olmadığı görüldü. **Sonuç:** PON55 ve PON192 genotiplerinin Türk toplumunda koroner arter hastalığı riskinin ve ciddiyetinin belirlenmesinde uygun bir gösterge olmadığı sonucuna varılmıştır.

Anahtar Kelimeler: Koroner hastalık; kolesterol, HDL; pon1 proteini, insan; polimorfizm, genetik; arildialkilfosfataz

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The paraoxonases (PON 1, 2, 3) are a group of enzymes that display arylesterase activity and cluster on the long arm of chromosome 7.[1,2] Among paraoxonases, PON1 has been studied more extensively and is understood by far better than PON2 and PON3.[3] In addition to its role in hydrolyzing organophosphorus compounds, it has been shown to play an important role in lipid metabolism and thus in cardiovascular disease and atherosclerosis.[4-9] PON1 is widely distributed among tissues and also among serum, where it is associated with high-density lipoprotein-cholesterol (HDL-C). Variations in the size and shape of HDL-C could strongly affect the binding affinity and stability of PON1 and may cause reduced antioxidative capacity in HDL.[10]

PON1 was suggested to lower the risk of coronary heart disease (CHD) by preventing low-density lipoprotein-cholesterol (LDL-C) and HDL-C oxidation during atherosclerotic process. The relation between PON activity and coronary artery disease (CAD) has been attributed to polymorphisms in the gene coding for this enzyme.[1,2]

Serum levels of PON activity vary widely among individuals, which may partly account for differences in susceptibility to organophosphate poisoning. The molecular basis for this difference appears to be related to the polymorphisms in the PON gene.[11] The PON1 gene has two common polymorphisms in the coding region, which lead to a leucine→methionine substitution at position 55 (PON55) and a glutamine→arginine substitution at position 192 (PON192). They independently influence PON1 activity and have been defined as the molecular basis of interindividual variability in PON activity.[2,12]

The frequencies of the PON1 alleles vary greatly across human populations. The distributions of two polymorphisms were significantly different between whites and blacks. The lowest frequency of the PON1 Met55 allele was reported in Chinese populations and the relatively high frequency of the PON1 Arg192 allele in blacks.[13,14] Numerous recent studies have investigated the association between PON1 R/Q polymorphism and coronary artery disease (CAD). The results of these studies are conflicting.[15-17] Approximately half of them have demonstrated a higher risk of CAD associated with the R192 allele, and other studies have failed to establish a relationship between PON1 R192 and CAD.[4-9,18-22]

The aim of this study was to investigate the frequency of PON55 and PON192 polymorphisms in Turkish patients with angiographically-defined coronary artery disease, and to determine whether this polymorphism was effective on serum lipid levels, especially on HDL-C.

MATERIAL AND METHODS

SUBJECTS

In the present study, 135 subjects with angiographically-defined CAD (99 male, 36 female, aged 40-86 years) and 110 healthy controls (72 male, 38 female, aged 39-88 years) were screened for the PON1 L/M55 and Q/R192 polymorphisms.

Subjects who were referred to the Department of Cardiology, Istanbul Faculty of Medicine for coronary angiographic examination and defined as having CAD (more than 50% stenosis in at least one major coronary artery) were enrolled in the study. The severity of CAD was expressed by the number of affected vessels (one, two or three vessel disease) and by the Duke scoring system; a prognostic index that includes the number of diseased major vessels, the presence of left main coronary artery disease, the percentage narrowing of the major vessels, and involvement of the left anterior descending coronary artery, particularly when the proximal segment shows severe stenosis (≥95%). The Duke score ranges from 0-100 (0=no disease, 100=the most severe disease).[23] Details of patients recorded at the time of admission included age, gender, height and weight, habits, history of ischemic heart disease, hypertension and medication. Exclusion criteria for all subjects were diabetes mellitus, renal or liver disease, malignancy, thyroid disease, connective tissue disease or any acute medical condition within the 3 months prior to the study. Clinical and demographic characteristics of patients and controls were reported in

TABLE 1: The demographical and biochemical parameters of the controls and the coronary artery disease patients (median±standard error of mean).

	Controls (n=110)	CAD patients (n=135)	p
Age	55.0±1,12	59.0±0.88	0.057
Sex (F/M)	38/72	36/99	0.182
Smokers, n (%)	16 (14.5%)	79 (58.5%)	<0.001
Alcohol consumption, n (%)	14 (12%)	22 (16.3%)	0.546
BMI (kgm-2)	26.0±0,41	29,7±0.74	<0.001
TG (mg/dL)	120,0±8,52	160.50±7.70	<0.001
TC (mg/dL)	212,0±4,49	202.5±3.79	0.056
HDL-C (mg/dL)	48,0±1,41	38.0±0.78	<0.001
LDL-C (mg/dL)	127.0±3,91	30.0±3.19	0.812

CAD patients vs controls.

BMI: Body mass index; CAD: Coronary artery disease; F: Female; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein cholesterol; M: Male; TC: Total cholesterol; TG: Triglyceride.

Table 1. Those receiving lipid-lowering drugs or any treatment known to influence lipid metabolism or glucose tolerance were excluded. Age-matched healthy persons lacking any symptoms of CAD and any family history for cardiovascular disease, not receiving lipid-lowering drugs or any treatment known to influence lipid metabolism or glucose tolerance were selected as the control group, following a physical examination by a cardiologist. Body mass index (BMI) was calculated as the ratio between weight and height squared (kg/m^2). Local ethics committee approved the study protocol, and informed written consent prior to participation was obtained from all subjects.

DNA ANALYSIS

Genomic DNA was isolated from peripheral blood leukocytes by phenol extraction method. For the determination of PON1 L/M55 polymorphism a fragment of 170 bp in PON1 gene was amplified by polymerase chain reaction (PCR). The oligonucleotide primers used for amplification was, forward 5′-GAAGAGTGATGTATAGCCCCAG-3′ and reverse 5′-TTTAATCCAGAGCTAATGAAAGCC-3′. For amplification, 200 ng of genomic DNA was used in a final volume of 50 µl containing 1 µl of each 40 pmol primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.0, 50 mM KCl and 0.25 units of *Taq* DNA polymerase. The PCR conditions were denaturation at 95°C for 5 min one cycle, followed by 40 cycles (94°C for 45 sec, 51°C for 72 sec and 72°C for 45 sec) and finally by an extension at 72°C for 10 min. The PCR product was digested with NlaIII (New England BioLabs, UK); separated by electrophoresis on a 12% polyacrylamide gel, and visualized by silver staining. The resulting fragments were 126 and 44 bp for the M allele (presence of the restriction site) and 170 bp for the L allele (absence of the restriction site).

For the determination of Q/R192 polymorphism a fragment of 99 bp in PON1 gene was amplified by PCR. The oligonucleotide primers used for amplification was, forward 5′-TATAT-GTTGCTGTGGGACCTGAG-3′ and reverse 5′-CACGCTAAACCCAAATACA TCTC-3′. The amplification conditions were similar to those for PON1 L/M55 polymorphism. The PCR conditions were denaturation at 94°C for 5 min one cycle, followed by 36 cycles (94°C for 1min, 61°C for 1min and 72°C for 1min) and finally by an extension at 72°C for 10 min. The PCR product was digested with AlwI (New England BioLabs, UK), was separated by electrophoresis on a 12% polyacrylamide gel, and was visualized by silver staining. The resulting fragments were 69 and 30 bp for the R allele (presence of the restriction site) and 99 bp for the Q allele (absence of the restriction site).

LIPID ANALYSIS

Serum total cholesterol (TC), triglyceride (TG), HDL-C and LDL-C concentrations were measured by the DPP Modular System autoanalyzer (Roche, Switzerland) in fasting venous blood samples.

STATISTICAL ANALYSIS

The NCSS 2000 statistical package was used for the power analysis. The size of the study population achieved 80.71% power to detect an effect size (W) of 0.20 using two degrees of freedom of the Chi-square distribution (α=0.05, β=0.20).

The differences between actual and expected frequencies of the polymorhisms employed the Hardy-Weinberg equilibrium (HWE) equation, as implemented in the DeFinetti Program version

2.50. The statistical significance for deviations from HWE was determined using the Pearson χ^2-test. Since the odds ratio (OR) enables the comparison of two variables symmetrically and a is a powerful way to show statistical associations between two data values, the ORs were calculated for genotypes and were given with 95% confidence intervals (CIs). The wild-type genotype/allele served as a reference category. Comparison of individual clinical variables between genotypes were assessed with the χ^2-test. The differences were considered to be significant if the value of probability (p) did not exceed 0.05.

After testing the normal distribution of data with the Kolmogorov-Smirnov test, the pair wise comparisons were performed by Mann-Whitney U test by SPSS 12.0 version for Windows. Results of the measured parameters were expressed as median±standard error of mean (SEM) for CAD patients and controls, as well as for different genotypes in each group. Spearman's correlation coefficient was computed to determine the relations between genotypes and the Duke score. PON55 and -192 genotypes, age, sex, BMI, smoking and alcohol consumption were used as covariates and the effects of these covariates on CAD risk were calculated by multivariate logistic regression analysis in patients and controls.

RESULTS

The BMI and serum triglycerides levels of the patients with CAD were significantly higher and HDL-C levels were significantly lower in comparison to the controls (Table1).

The genotype and allelic distribution of the subjects with respect to the PON1 coding region polymorphisms showed that 31.85% of CAD patients were genotyped as PON55 LL, 42.96% as PON55 LM, and 25.18% as PON55 MM (Table 2). There was a significant difference in the distribution of PON55 MM genotypes between CAD patients and controls (p=0.042, OR=2.06; 95% CI=1.02-4.18). M allele frequency was significantly higher than L allele frequency in CAD patients (p=0.028, OR=1.52; 95% CI= 1.04-2.16).

TABLE 2: The genotype and allele distribution of the controls and coronary artery disease patients with regard to PON1 L/M55 and PON1 Q/R192 polymorphisms.

	Controls (n=110) n (%)	CAD patients (n=135) n (%)	p
PON1 L/M55			
LL	47 (%42.72)	43 (%31.85)	Reference
LM	45 (%40.90)	58 (%42.96)	0.236
MM	18 (%16.36)	34 (%25.18)	0.042
LM+MM	63 (%57.26)	62 (%68.14)	0.079
M allele frequency	81 (%36.6)	128 (%46.6)	0.030
PON1 Q/R192			
QQ	43 (%39.09)	57 (%42.22)	Reference
QR	51 (%46.36)	59 (% 43.70)	0.624
RR	16 (%14.54)	19 (%14.07)	0.780
QR+RR	67 (%.60.90)	78 (%57.77)	0.619
R allele frequency	83 (%37.7)	97 (%35.9)	0.616

CAD, coronary artery disease.

Among CAD patients, 42.22% were genotyped as PON192 QQ, 43.70% as PON192 QR, and 14.07% as PON192 RR. A similar genotype frequency was observed among the healthy controls. There was no statistical significance in the allelic distribution between CAD patients and controls (p=0.68).

The effects of genotype distribution on serum lipids of controls and CAD patients were examined individually for each polymorphism (Table 3 and 4). The comparison of serum lipid levels between controls and patients with regard to corresponding genotype carriers for PON55 polymorphism showed that TC levels were lower in LM carrier CAD patients, TG levels were higher in LM and MM carrier CAD patients, HDL-C levels of CAD patients were lower in all genotypes and LDL-C levels were higher only in MM carrier CAD patients (Table 3).

Except for serum HDL-C and TG levels, the comparison of the serum lipids according to corresponding genotypes for PON192 polymorphism did not reveal a statistical significance between CAD patients and controls. While the serum HDL-C levels of QQ, QR and RR carrier CAD patients were significantly lower than the corresponding genotype carrier controls, TG levels were higher in QQ carrier CAD patients (Table 4).

TABLE 3: Serum lipid levels of controls and coronary artery disease patients with respect to PON1 L/M55 genotypes (median ± standard error of mean).

	Controls (n=110)			CAD patients (n=135)				
	(A) LL (n=47)	(B) LM (n=45)	(C) MM (n=18)	(D) LL (n=43)	(E) LM (n=58)	(F) MM (n=34)	p	
TC (mg/dL)	205.0±5.99	223.5±7.19	167.0±1.21	199.0±7.06	202.5±6.62	207.0±5.07	A-D	0.172
							B-E	0.023
							C-F	0.193
TG (mg/dL)	125.0±1.03	123.0±1.73	87.0±1.37	150.0±1.26	157.0±8.16	170.0±2.29	A-D	0.148
							B-E	0.025
							C-F	0.006
HDL-C (mg/dL)	50.0±2.12	50.5±2.27	43.0±3.87	37.5±1.73	38.0±1.17	38.0±1.11	A-D	< 0.001
							B-E	< 0.001
							C-F	0.027
LDL-C (mg/dL)	127.0±5.11	138.0±6.93	112.0±7.95	122.5±5.39	132.5±5.82	132.0±4.56	A-D	0.806
							B-E	0.268
							C-F	0.019

Controls vs CAD patients with regard to corresponding genotype

(A): LL carrier controls; (B): LM carrier controls; (C): MM carrier controls; (D): LL carrier patients; (E): LM carrier patients and (F): MM carrier patients

CAD: Coronary artery disease; F: Female; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein cholesterol; M: Male; TC: Total cholesterole; TG: Triglyceride.

TABLE 4: Serum lipid levels of controls and coronary artery disease patients with respect to PON1 Q/R192 genotypes (median ± standard error of mean).

	Controls (n=110)			CAD patients (n=135)				
	A) QQ (n=43)	(B) QR (n=51)	(C) RR (n=16)	(D) QQ (n=57)	(E) QR (n=59)	(F) RR (n=19)	p	
TC (mg/dL)	220.0±7.82	210.0±7.01	210.5±11.3	201.5±6.51	196.0±5.4	204.5±12.4	A-D	0.143
							B-E	0.085
							C-F	0.822
TG mg/dL	123.0±12.9	129.5±15.8	102.0±16.9	159.5±8.6	161.0±8.49	177.0±23.9	A-D	0.039
							B-E	0.101
							C-F	0.770
HDL-C mg/dL	54.5±2.29	45.0±2.33	46.5±4.01	37.5±1.17	37.0±1.32	38.0±1.91	A-D	<0.001
							B-E	< 0.001
							C-F	0.017
LDL-C mg/dL	132.0±7.03	125.5±6.10	121.5±8.89	131.5±5.41	130.0±4.2	130.5±10.5	A-D	0.965
							B-E	0.948
							C-F	0.473

Controls vs CAD patients with regard to corresponding genotype

(A): QQ carrier controls; (B): QR carrier controls; (C): (RR) carrier controls; (D): QQ carrier patients; (E): QR carrier patients and (F): RR carrier patients

CAD: Coronary artery disease; F: Female; HDL-C: High-density lipoprotein-cholesterol; LDL-C: Low-density lipoprotein cholesterol; M: Male; TC: Total cholesterole; TG: Triglyceride.

The distribution of Duke score according to genotypes in PON55 and PON192 polymorphisms of CAD patients was also evaluated (Figure 1a and 1b). Duke score of the patients showed no difference between genotypes in both polymorphisms (Table 5). In order to examine the interaction of PON55 and PON192 polymorphisms on severity of CAD the correlation analysis was also done but no relation was found between genotypes and Duke scores (p=0.397 and p=0.695, respectively).

The logistic regression model predicting CAD status by using age, sex, BMI, smoking, alcohol

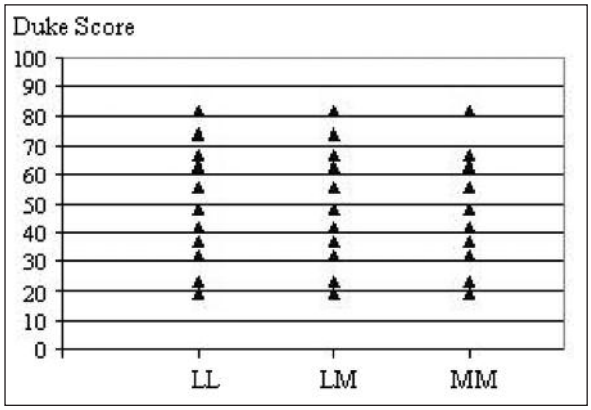

FIGURE 1a: The distribution of Duke scores according to PON1 L/M55 genotypes in coronary artery disease patients.

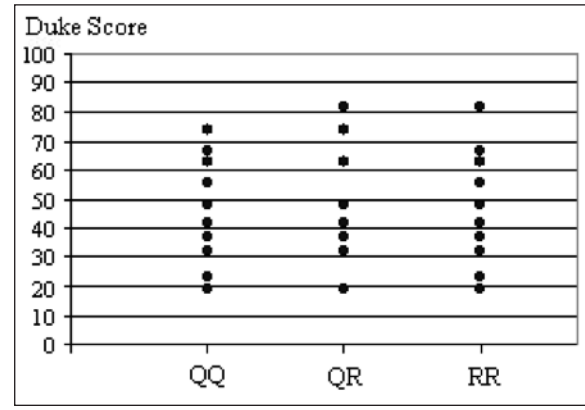

FIGURE 1b: The distribution of Duke scores according to PON1 Q/R192 genotypes in coronary artery disease patients.

consumption and PON genotypes as covariates was evaluated ($p<0.001$). The model showed that the predictors for the CAD status were age, sex, BMI and smoking but neither of the studied PON genotypes ($p=0.823$ for PON55, $p=0.878$ for PON192) nor alcohol consumption ($p=0.207$).

TABLE 5: The of Duke scores of coronary artery disease patients with respect to PON1 L/M55 and PON1 Q/R192 genotypes (median ± standard error of mean).

Duke Score for PON1 L/M55		p
LL n=43	39.5±2.56	Reference
LM n=58	37.0±2.21	0.505
MM n=34	48.0±3.10	0.245
Duke Score for PON1 Q/R192		
QQ n=57	37.0±2.39	Reference
QR n=59	42.0±2.38	0.704
RR n=19	37.0±4.47	0.700

LM. MM genotypes and QR. RR genotypes vs corresponding reference genotypes.

DISCUSSION

The relation between PON activity and CAD has been attributed to polymorphisms in the gene coding for this enzyme.[2] PON gene polymorphisms have been studied widely but the results of those studies are conflicting. PON55 polymorphism has been associated with CAD risk in some but not all reports. In a study of 408 Caucasian non-insulin-dependent diabetics, homozygosity for the L allele has been reported as an independent risk factor for CAD.[13] In another study, no association was found between PON 55 polymorphism and CAD in Asian Indians and Chinese.[24] The results of this case -control study with age- and sex-matched controls showed that the frequency of polymorphic M allele was significantly higher in CAD patients than in the healthy controls but there was no association between the M allele and CAD severity. Kaman et al. also studied the PON55 polymorphism in an angiographically-defined Turkish population and did not find an association between the severity of CAD and PON55 polymorphism.[25] In contrast to the results of the present study, they reported that the frequency of M allele was significantly higher among control subjects than among CAD patients. The difference in the frequency of polymorphic M allele between these two studies including Turkish populations may arise from the different sample sizes. Although the study population of the report by Kaman et al. was larger than the population in the present study, the CAD patients with diabetes were excluded in the present study but not in the study by Kaman et al.[25]

The studies investigating the relation between the PON192 polymorphism and CAD have also shown conflicting results even among the studied populations within the same country.[6,17,18,22,26-28] In the present study, the evaluation of PON192 genotype distribution between healthy controls and CAD patients did not show any significant difference. The results of two different studies on

PON192 polymorphism in Turkish populations also showed no significant difference in the distribution of PON192 genotypes or alleles between CAD patients and controls.[29,30]

PON is located on the surface of apoprotein-AI and J containing HDL-C and has a pivotal role in preventing LDL-C oxidation.[31,32] PON polymorphisms were suggested to be associated with plasma lipid levels and therefore with CAD.[8,9,16] The results of studies on the association between PON polymorphisms and plasma lipids are conflicting. While some studies report that the M allele carriers of PON55 have higher plasma concentrations of total cholesterol, LDL-C and apoprotein-B levels than the non-carriers, others report LL carriers have an increased concentration of HDL-C and higher apoprotein-AI levels.[29,33,34] Although the results are not consistent among populations in the same country, an association for PON192 has been shown in CAD patients of French, American, Japanese and Indian populations.[4,7,16,17] QQ carriers for PON192 were reported to have lower apo-B /apo-AI ratios in addition to lower plasma total cholesterol, LDL-C and apolipoprotein-B levels.[35,36] However, other studies showed no association between these PON polymorphisms and plasma lipoproteins.[37,38] A study investigating the association between PON192 polymorphism and serum lipid levels in Turkish CAD patients also failed to show any significant results.[29] In the present study, regarding the comparison of serum lipid levels between CAD patients and the controls with regard to polymorphic allele, the most significant results were in HDL levels. In both polymorphisms, we found that the HDL-C levels of patients were significantly lower than the levels in controls in all genotypes. Since bearing either polymorphic M or R allele has a significant effect on HDL-C levels and all CAD patients bearing either of the genotypes have lower HDL-C levels than the controls, we thought that serum HDL-C level was independent from PON55 and PON192 polymorphisms.

Considering that the frequency of polymorphic M allele was significantly higher in CAD patients than in healthy controls and evaluating the results of serum lipids other than HDL-C, it seems that the M allele carriers of PON55 have higher serum concentrations of TG and LDL-C and lower TC levels compared to corresponding controls. Since these results are obtained either in homozygote or heterozygote CAD patients, suggesting a relation between the M allele of PON55 and serum lipids would be inaccurate.

We also investigated the interaction between PON polymorphisms and severity of CAD in the present study. Our results showed that the severity of CAD was not associated with PON55 or with PON192 genotypes. Our results are in agreement with the results of Kaman et al. who investigated both PON55 and 192 polymorphisms in a Turkish population with CAD.[25]

When the results of the present study is assessed together with similar studies including Turkish populations we may conclude that PON55 and PON192 genotypes are not useful markers for the estimation of either cardiovascular risk or the severity of coronary artery disease in the Turkish population.

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