

Association between Serum Paraoxonase 1 Activities (PONase/AREase) and L55M Polymorphism in Risk of Female Infertility

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Abstract

Background: The risk of developing female infertility has been associated with gene polymorphisms that decrease the activity of enzymes involved in systemic Oxidative Stress (OS). In this study, *PON1* L55M polymorphism for association with susceptibility to infertility was investigated among Iranian female population.

Methods: Samples from 120 Iranian females [20 endometriosis; 30 Polycystic Ovary Syndrome (PCO); 70 controls] were analyzed and PCR-RFLP assay was used to determine the *PON1* rs854560 (L55M) frequencies. The paraoxonase (PONase) and arylesterase (AREase) activities of *PON1* enzyme were also assessed in order to investigate the association between serum *PON1* activities, female infertility, and *PON1* L55M polymorphism.

Results: The women with a MM genotype ($p=0.021$; OR=2.55) showed more possibilities of experiencing infertility than those with a LM genotype ($p=0.039$; OR=1.91). According to LSD test, endometriosis subjects had significantly lower paraoxonase enzyme activity compared to control group ($p=0.0024$; CI=95%). No significant difference was found in women with PCOS for both PONase and AREase activity in comparison with control group ($p=0.469$; CI=95%). Furthermore, *PON1* activities were the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls.

Conclusion: *PON1* L55M polymorphism may be associated with serum *PON1* activity and the risk of developing female infertility.

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Introduction

Female infertility is a complex disorder which may be caused by medical conditions including pelvic inflammatory disease, endometriosis, Polycystic Ovary Syndrome (PCOS), premature ovarian failure and uterine fibroids¹. PCOS is a heterogeneous female endocrine metabolic disorder affecting 5-10% of women characterized by its significantly complex clinical alignments². Endometriosis is a chronic, benign, estrogen-dependent condition characterized by the presence of endometrial tissue outside the uterus cavity associated with pelvic pain and infertility. The pathophysiologic mechanism of endometriosis is unknown and it affects 3-10% of women in reproductive years of their life and 20-50% of women with infertility³⁻⁵. In fact, PCOS and endometriosis appear to have a complex and multifactorial etiology in which a variety of genes interact with environmental factors to produce these conditions. Recent biochemical and genetic studies on the pathogenesis of PCOS and endometriosis were focused on the Single Nucleotide Polymorphisms (SNPs) affecting Oxidative Stress (OS)^{6,7}. The results of these

studies suggest that genetic polymorphisms in antioxidant genes including the SNPs affecting the activities of paraoxonase 1 (*PON1*) may contribute to female infertility⁸. Serum paraoxonase 1 is an antioxidant calcium-dependent esterase/lactonase, which circulates within High Density Lipoprotein (HDL) particles^{9,10}. *PON1* possesses antioxidant, anti-atherogenic, and anti-inflammatory properties and is involved in hydrolysis of several organophosphorus insecticides and nerve agents, inhibition of Low Density Lipoprotein (LDL) oxidation and increase of macrophage-associated cholesterol efflux¹¹. The activities of *PON1* are genetically regulated and the *PON1* gene polymorphisms have potent influences on its activities. *PON1* is a member of a multigene cluster including *PON1*, *PON2*, and *PON3*¹². Among all the SNPs of *PON1* gene, -909G/C [rs854572], -162A/G [rs705381], -108C/T [rs705379] in the promoter region and Q192R [rs662], L55M [rs-854560] in the coding region are the most studied polymorphisms^{13,14}. The L55M polymorphism influences the paraoxonase and arylesterase

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(ARE) activities and the stability of the protein¹⁵. In this case-control study, PONase and AREase activities were examined and the association of coding *L55M* polymorphism with female infertility in Iranian population was investigated. The *L55M* polymorphism could be a risk factor in determining susceptibility to female infertility.

Materials and Methods

Subjects

This case-control study analyzed a group of 50 infertile females including 20 with endometriosis who were diagnosed by laparoscopy and 30 with PCOS fulfilling the criteria for PCOS¹⁶, who attended the Isfahan Fertility-Infertility Center and Royan Institute. The control group consisted of 70 healthy age-matched volunteers who visited the clinic for a regular health check-up with proven fertility and no clinical or biochemical hyperandrogenism, no menstrual cycle irregularities, and no history of endometriosis and PCOS. Women from different regions of Iran were referred to this infertility center for check-up and they made the population of this study. There was no evidence of known paraoxonase-affecting diseases such as CHD, liver diseases, atherosclerosis, diabetes, hypertension, cancer and infections in all fertile and infertile females who were not genetically related. Blood sampling was done based on patient consent and an agreement was signed between the University of Isfahan and Isfahan Fertility-Infertility Center. Blood samples were obtained in the morning after overnight fasting and collected in EDTA and heparin coated tubes to analyze *PON1 L55M* polymorphism and PON1 activities. Blood and serum samples were stored at -20°C until analysis.

Genotyping analyses

For genotype analysis, genomic DNA was extracted from peripheral blood using a salting-out method described by Miller¹⁷ and was stored at -20°C till genotyping could be performed. Coding *L55M* (rs 854560) genotyping was carried out using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers for SNP were designed using the primer design software Oligo 7 and were listed as follows:

Forward primer: 5' TGAATTATTCTGAACCTATTA AAGAAGA 3'

Reverse primer: 5' AAGACTTAACTGCCAGTCCT AGA 3'

PCR was performed in 25 μ l reaction mixture containing 2 μ l of each forward and reverse primers (10 pM), 2.5 μ l of $\times 10$ solution buffer (20 mM Tris-HCl pH=8.6, 50 mM KCl, Cinnagen Inc, Iran), 0.5 μ l of four mixed dNTPs (10 mM, Cinnagen Inc, Iran), 0.75 μ l of MgCl₂ (50 mM, Cinnagen Inc, Iran), 0.3 μ l of 5u/ μ l Taq DNA polymerase (Cinnagene, Co., Iran), and 2 μ l (100 ng/ μ l) of genomic DNA. The PCR program was as follows: initial denaturation for 4 min at

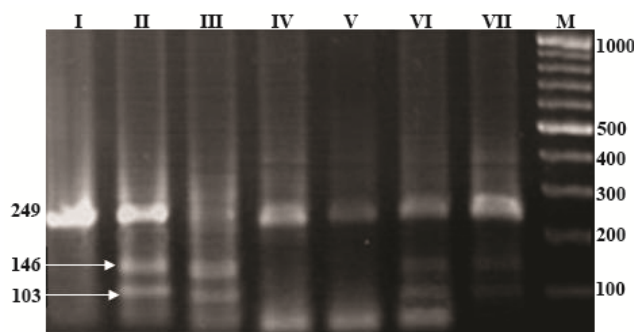


Figure 1. Electrophoresis pattern of *PON1 L55M* polymorphism using PCR-RFLP and *Hin1II* restriction enzyme. I) Undigested PCR products, II) LM (103, 146, and 249 bp fragments). III to VII) MM (103, and 146 bp fragments), LL (249 bp fragment), LL, and LM genotype, respectively. M) 100 bp ladder. Numbers are in base pair (pb).

94°C followed by 35 cycles of 40 s at 94°C, 35 s at 54-66°C, 72°C for 40 s, and a final extension step of 15 min at 72°C. The PCR products were run on 2% agarose gel and were visualized by ethidium bromide staining. Moreover, a restriction analysis was performed using 10 units of *Hin1II* enzyme (Fermentas, Vilnius, Lithuania) in buffer G (Fermentas) at 37°C for 16 hr. The restriction fragments were analyzed on 2% agarose gels and were visualized through ethidium bromide staining. Digested DNA fragments of 146 and 103 bp were detected in MM homozygotes (restriction site present), an undigested band of 249 bp was detected in LL homozygotes (restriction site absent) and heterozygous genotypes resulted in three different bands (146, 103 and 249 bp) through PCR-RFLP technique (Figure 1).

Analyses of PON1 activities

The serum was isolated by centrifugation at 800 g for 10 min at 4°C and was immediately frozen at -80°C in order to determine PON1 activities in blood samples. The PONase activity was measured using paraoxon (Sigma Chem, USA) as substrate and the increase in absorbance at 412 nm was determined, because of 4-nitrophenol formation. The activity was measured at 25°C during 3 min after 5 μ l of serum were added to each well containing 100 μ l of Tris/HCl (100 mMol, pH=8.0) buffer including 2 mMol CaCl₂ and 5.5 mMol of paraoxon. All results are expressed in U/ml which is defined as 1 nmol of 4-nitrophenol formed per minute. Arilesterase activity in serum was determined spectrophotometrically using the phenylacetate (Merck-Schardt) as a synthetic substrate. The reaction started by adding 100 μ l of phenylacetate (10 mM) as substrate solution to wells containing 5 μ l of serum (prediluted 1:10) and 1 mM CaCl₂ (Sigma, USA) in 50 mM Tris buffer pH=8. The phenol production was measured during 2 min at 270 nm and pH=8.0. The PON1's arilesterase activity is expressed as KU/ml which is defined as 1 μ mol arilesterase per minute. PON1's paraoxonase and arilesterase activities were determined in triplicate¹⁸.

Table 1. Association between *PON1 L55M* polymorphism and female infertility

1 st Polymorphism	2 nd Polymorphism	1 st and 2 nd mean difference	Standard deviation	p-value
MM	LM	0.23214 *	0.09928	0.021
	LL	-0.02381	0.12924	0.854
LM	MM	-0.23214 *	0.09928	0.021
	LL	-0.25595 *	0.12272	0.039
LL	MM	-0.02381	0.09928	0.854
	LM	0.25595 *	0.12924	0.039

* Significance difference with 95% confidence interval.

Table 2. Characteristics of cases and control samples (mean±SD)

	PCOS	Endometriosis	Fertile
Age	31.92±4.38	31.54±6.212	32.09±6.56
Body Mass Index (BMI)	28.03±5.41	24.89±4.02	24.66±3.86
Paraoxonase activity (U/l)	1682.88±2255.47	1259.90±1680.95	2529.02±3144.78
Arllesterase activity (KU/l)	227.67±131.11	212.88±119.79	255.58±145.53
Serum PON1 activity	7.39±6.89	5.91±6.78	9.89±5.71

Briefly, serum PON1 activity (PON1 phenotype) was determined by double substrate method. Phenotype is defined as the activity ratio of PONase activity divided by AREase activity.

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 (SPSS, Inc., Chicago, IL, USA) software. The Chi-square distribution (χ^2) was used to evaluate the PON1 genotype frequencies between the patient and the control groups. Multiple comparisons were done by employing one way analysis of variance (ANOVA) followed by a LSD (least significant difference) test. All data are presented as means ± standard deviation (SD). Statistical significance was assessed using the 5% significance level.

Results

Distribution of *PON1 L55M* genotype

The association between *PON1 L55M* polymorphism and female infertility in case and control subjects is shown in table 1. The distribution of genotypes were in Hardy-Weinberg equilibrium ($p=0.45$). The

results showed that there were significant differences regarding *PON1 L55M* polymorphism among fertile and infertile groups. The women with a MM genotype ($p=0.021$; OR=2.55) were statistically associated with infertility in comparison to those with a LM genotype ($p=0.039$; OR=1.91). There was no association between *L55M* polymorphism and each infertile group including patients with endometriosis and PCOS separately.

PONase, AREase activities and infertility

The group mean age, body mass index (BMI), and the serum PON1's paraoxonase and arilesterase activities are presented in table 2 for fertile, PCOs, and endometriosis groups. Paraoxonase enzyme activity was highly variable among different samples. According to LSD test, endometriosis subjects had significantly lower paraoxonase enzyme activity compared to control groups ($p=0.0024$; CI=95%) (Table 3). No significant difference was found in women with PCOS for both PONase and AREase activity in comparison with control group ($p=0.469$; CI=95%).

Table 3. Association between infertility and paraoxonase enzyme activity

Group 1	Group 2	Mean difference between two groups	Standard deviation	p-value
Endometriosis	Fertile	-3.86913 *	1.68803	0.024
	PCO	-1.14816	1.58038	0.469
Fertile	Endometriosis	4.86913 *	1.68803	0.024
	PCO	2.72097	2.01152	0.179
PCO	Endometriosis	1.14816	1.58038	0.469
	Fertile	-2.72097	2.01157	0.179

* Significance difference with 95% confidence interval.

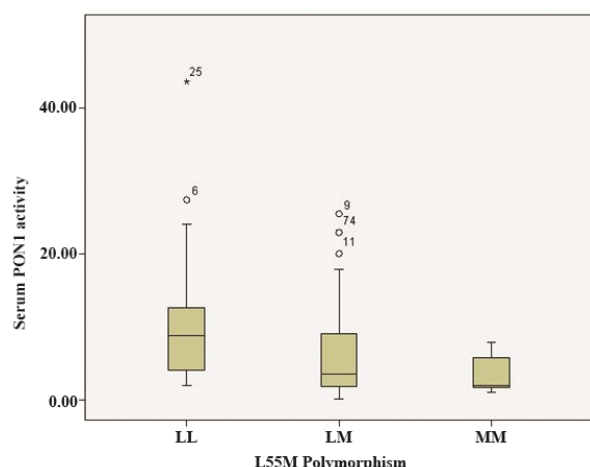


Figure 2. L55M genotypes (LL, LM and MM) and serum PONI activity in both controls and patients. Serum PONI activity was the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls.

Association between PONI L55M polymorphism and serum PONI activities

The association of PONI L55M polymorphism with PONI activities is shown in figure 2. Significant difference was observed among the genotypes of L55M polymorphism in patient and control groups. Serum PONI activity was the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls. It was found that in three genotypes, patients had significantly lower activity in comparison with controls.

Discussion

Serum PONI is a esterase/lactonase which has significant roles in protection of LDL and HDL against oxidation¹⁹⁻²³. Recently, PONI genotyping and analysis of serum PONI activity has been comprehensively studied to investigate associations with variety of diseases including cardiovascular disease, diabetes, infertility, Alzheimer, cancer, and Parkinson²⁰⁻²⁴. Genetic variants in PONI gene may modulate OS and thus affect susceptibility to female infertility. OS which was defined as imbalance between pro-oxidants and antioxidants has critical roles in normal functioning of the female reproductive system and development of female reproductive diseases such as endometriosis, and PCOS^{25,26}. The effects of OS on male infertility have been well described, but its impacts on female reproductive disorders are generally unknown. In this case-control study, PONI enzyme activities (PONase and AREase), and PONI L55M coding polymorphism were investigated in Iranian infertile women population for the first time. The global minor allele frequency of PONI L55M polymorphism (rs854560) is T=0.1827/915 according to the dSNP (www.ncbi.nlm.nih.gov/snp). This means that for rs854560, minor allele is 'T' and has a frequency of 18.27% in the 1000 genome

phase 1 population. It was found that serum PONI activity was significantly lower in endometriosis patients in comparison with controls. This result is similar to previous studies which have been performed in different populations^{27,28}. In another study, Verit *et al* reported a significant difference in two groups of endometriosis patients including women with moderate to severe endometriosis and women with minimal to mild endometriosis. PON-1 activity was significantly lower in women with moderate to severe endometriosis than in women with minimal to mild endometriosis and controls ($p<0.0001$)²⁹. The association between PONI activity and PCOS was suggested by Dursun *et al* for the first time. They reported a significant difference ($p=0.027$) in PONI activity between PCOS patients and control groups³⁰. In the present study, there was no significant difference in serum PONI activity of PCOS patients in comparison with the control group. The association of PONI L55M polymorphism and PONI activity was also evaluated in our study which indicated that PONI activity was affected by L55M polymorphism. PONase activity was the highest in LL genotype and lowest in MM genotype in both patients and control groups ($p=0.002$; CI=95%) and in all the three genotypes (LL, LM, MM); patients had lower PONase activity in comparison with controls. PONI's activity varies widely between individuals and is related to several other environmental factors. Our further analyses indicate that AREase activity was independent of L55M polymorphism which is similar to the observation by Chen *et al*³¹. Briefly, serum PONI activity or PONI phenotype was the highest in LL genotype followed by LM and then MM genotype (MM<LM<LL) in both patients and controls. In addition, it was found that MM genotype was more common in infertile females as compared to the controls (OR=2.55; $p=0.021$; χ^2 significance=0.029) and LM genotype was associated with low risk of infertility (OR=1.91; $p=0.039$) (Table 4). Furthermore, it was observed that the frequencies of PONI L55M polymorphism in this Iranian population were similar to those for European Caucasians but different from those seen in other Asian populations³². The findings of a case-control study which was performed by Sepehrvand *et al* confirmed this similarity of PONI L55M polymorphism frequency in other Iranian population^{33,34}.

Conclusion

In conclusion, results of this study support the hy-

Table 4. Distribution of PONI L55M genotypes in fertile and infertile groups

Genotype	Fertile, N ^a (f)	Infertile, N (f ^b)	OR ^c (CI ^d 95%)	p-value
LL	20 (28.6)	20 (40)		
LM	40 (57.2)	15 (30)	1.91	0.039
MM	10 (14.28)	15 (30)	2.55	0.021
Total	70	50		

a: Number; b: Frequency; c: Odd ratio.

pothesis that *PON1 L55M* polymorphism may have significant roles in serum PON1 activity and risk of developing female infertility. Our findings suggest further analyses are required to investigate other PON1 polymorphisms and their importance on the individual's susceptibility to infertility in larger groups.

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Conflict of Interest

All authors have seen and agreed with the contents of the manuscript and there is no conflict of interest to report.

References

- Gnoth C, Godehardt E, Frank-Herrmann P, Friol K, Tigges Jr, Freundl G. Definition and prevalence of subfertility and infertility. *Hum Reprod* 2005;20(5):1144-1147.
- Deligeorgoulou E, Kouskouti C, Christopoulos P. The role of genes in the polycystic ovary syndrome: predisposition and mechanisms. *Gynecol Endocrinol* 2009;25(9):603-609.
- Richter RJ, Jarvik GP, Furlong CE. Paraoxonase 1 status as a risk factor for disease or exposure. *Adv Med Biol* 2010;660:29-35.
- Bragatto FB, Barbosa CP, Christofolini DM, Peluso C, dos Santos AA, Mafra FA, et al. There is no relationship between Paraoxonase serum level activity in women with endometriosis and the stage of the disease: an observational study. *Reprod Health* 2013;10:32.
- Augoulea A, Alexandrou A, Creatsa M, Vrachnis N, Lambrinoudaki I. Pathogenesis of endometriosis: the role of genetics, inflammation and oxidative stress. *Arch Gynecol Obstet* 2012;286(1):99-103.
- Ji G, Gu A, Wang Y, Huang C, Hu F, Zhou Y, et al. Genetic variants in antioxidant genes are associated with sperm DNA damage and risk of male infertility in a Chinese population. *Free Rad Biol Med* 2012;52(4):775-780.
- Yeon Lee J, Baw C-K, Gupta S, Aziz N, Agarwal A. Role of oxidative stress in polycystic ovary syndrome. *Curr Womens Health Rev* 2010;6(2):96-107.
- H Sekhon L, Gupta S, Kim Y, Agarwal A. Female infertility and antioxidants. *Curr Womens Health Rev* 2010;6(2):84-95.
- Goswami B, Tayal D, Gupta N, Mallika V. Paraoxonase: a multifaceted biomolecule. *Clin Chim Acta* 2009;410(1):1-12.
- bin Ali A, Zhang Q, Lim YK, Fang D, Retnam L, Lim SK. Expression of major HDL-associated antioxidant PON-1 is gender dependent and regulated during inflammation. *Free Radic Biol Med* 2003;34(7):824-829.
- Camps J, Marsillach J, Joven J. The paraoxonases: role in human diseases and methodological difficulties in measurement. *Crit Rev Lab Sci* 2009;46(2):83-106.
- Précourt LP, Amre D, Denis MC, Lavoie JC, Delvin E, Seidman E, et al. The three-gene paraoxonase family: physiologic roles, actions and regulation. *Atherosclerosis* 2011;214(1):20-36.
- Kokouva M, Koureas M, Dardiotis E, Almpandou P, Kalogeraki A, Kyriakou D, et al. Relationship between the paraoxonase 1 (PON1) M55L and Q192R polymorphisms and lymphohaematopoietic cancers in a Greek agricultural population. *Toxicology* 2013;307:12-16.
- Ferk P, Gersak K. Association of -108 C>T PON1 polymorphism with polycystic ovary syndrome. *Biomed Rep* 2014;2(2):255-259.
- Abu Farha M, Fawzy M, Abdel Wahab A, Rashed L, Farouq A. Relation between paraoxonase 1 (PON1) gene polymorphism insulin resistance in polycystic ovary syndrome (PCOS). *Kasr Al-Aini J Obstet & Gynecol* 2011;1(2):75-82.
- Homburg R. The management of infertility associated with polycystic ovary syndrome. *Reprod Biol Endocrinol* 2003;1:109.
- Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988;16(3):1215.
- Sumegova K, Nagyova Z, Waczulikova I, Zitnanova I, Durackova Z. Activity of paraoxonase 1 and lipid profile in healthy children. *Physiol Res* 2007;56(3):351-357.
- Augoulea A, Mastorakos G, Lambrinoudaki I, Christodoulakos G, Creatsas G. The role of the oxidative-stress in the endometriosis-related infertility. *Gynecol Endocrinol* 2009;25(2):75-81.
- Gur M, Aslan M, Yildiz A, Demirbag R, Yilmaz R, Selek S, et al. Paraoxonase and arylesterase activities in coronary artery disease. *Eur J Clin Invest* 2006;36(11):779-787.
- Marsillach J, Camps J, Beltran-Debon R, Rull A, Aragones G, Maestre Martinez C, et al. Immunohistochemical analysis of paraoxonases-1 and 3 in human atherosclerotic plaques. *Eur J Clin Invest* 2011;41(3):308-314.
- Ozenoglu A, Balci H, Ugurlu S, Caglar E, Uzun H, Sarkis C, et al. The relationships of leptin, adiponectin levels and paraoxonase activity with metabolic and cardiovascular risk factors in females treated with psychiatric drugs. *Clinics (Sao Paulo)* 2008;63(5):651-660.
- Ikeda Y, Suehiro T, Inoue M, Nakauchi Y, Morita T, Arai K, et al. Serum paraoxonase activity and its relationship to diabetic complications in patients with non-insulin-dependent diabetes mellitus. *Metabolism* 1998;47(5):598-602.
- Elkiran ET, Mar N, Aygen B, Gursu F, Karaoglu A, Koca S. Serum paraoxonase and arylesterase activities in patients with lung cancer in a Turkish population. *BMC cancer* 2007;7(1):48.
- Ginsberg G, Neafsey P, Hattis D, Guyton KZ, Johns DO, Sonawane B. Genetic polymorphism in paraoxonase 1

- (PON1): Population distribution of PON1 activity. *J Toxicol Environ Health B Crit Rev* 2009;12(5-6):473-507.
26. Agarwal A, Gupta S, Sharma R. Oxidative stress and its implications in female infertility-a clinician's perspective. *Reprod Biomed Online* 2005;11(5):641-650.
 27. Jackson LW, Schisterman EF, Dey-Rao R, Browne R, Armstrong D. Oxidative stress and endometriosis. *Hum Reprod* 2005;20(7):2014-2020.
 28. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol* 2005;3:28.
 29. Verit FF, Erel O, Celik N. Serum paraoxonase-1 activity in women with endometriosis and its relationship with the stage of the disease. *Hum Reprod* 2008;23(1):100-104.
 30. Dursun P, Demirtaş E, Bayrak A, Yarali H. Decreased serum paraoxonase 1 (PON1) activity: an additional risk factor for atherosclerotic heart disease in patients with PCOS? *Hum Reprod* 2006;21(1):104-108.
 31. Chen Q, Reis SE, Kammerer CM, McNamara DM, Holubkov R, Sharaf BL, et al. Association between the severity of angiographic coronary artery disease and paraoxonase gene polymorphisms in the National Heart, Lung, and Blood Institute-sponsored Women's Ischemia Syndrome Evaluation (WISE) study. *Am J Hum Genet* 2003;72(1):13-22.
 32. Brophy VH, Jampsa RL, Clendenning JB, McKinstry LA, Jarvik GP, Furlong CE. Effects of 5' regulatory-region polymorphisms on paraoxonase-gene (PON1) expression. *Am J Hum Genet* 2001;68(6):1428-1436.
 33. Sepahvand F, Rahimi-Moghaddam P, Shafiei M, Ghaf-fari SM, Rostam-Shirazi M, Mahmoudian M. Frequency of paraoxonase 192/55 polymorphism in an Iranian population. *J Toxicol Environ Health A* 2007;70(13):1125-1129.
 34. Imai Y, Morita H, Kurihara H, Sugiyama T, Kato N, Ebihara A, et al. Evidence for association between paraoxonase gene polymorphisms and atherosclerotic diseases. *Atherosclerosis* 2000;149(2):435-442.