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The analysis of *PON1* gene expression and rs662 polymorphism in Iranian patients suffering from cardiovascular diseases

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ABSTRACT

In addition to lifestyle and diet, cardiovascular disease can be caused by genetic factors related to heart function issues and the blood biochemical content. The *PON1* gene encodes paraoxonase1 as an HDL-depended enzyme inhibiting LDL oxidation and relative side effects. The polymorphism of *PON1* can significantly influence the activity and the levels of serum PON1, as well as the occurrence of cardiovascular disease. Therefore, this study analyzed *PON1* gene expression and Q192R polymorphism among Iranian cardiovascular patients. This case-control research included 40 patients and 40 controls. The Q192R polymorphism of the *PON1* gene was analyzed by real-time PCR. In addition, *PON1* gene polymorphism was analyzed by the developed methodology for PCR and RFLP. The data was statistically analyzed by t-test ($P < 0.05$) via SPSS version 23.0. The expression of *PON1* was considerably reduced in the patients ($P < 0.05$), compared to the control population. Additionally, there was a significant correlation between the expression of the gene, LDL level, and the age of the patients ($P < 0.05$). The low *PON1* gene expression can indicate that the enzyme is a key factor positively affecting cardiovascular diseases among the Iranian population. Additionally, cardiovascular diseases in the Iranian population are significantly associated with the *PON1* gene polymorphism.

Keywords: Paraoxonase1, *PON1* gene, Cardiovascular Disease, rs662, Real-time PCR, RFLP.

1. INTRODUCTION

Cardiovascular diseases are one of the main causes of death throughout the world (Packer et al., 2020). Various environmental and genetic factors can influence cardiovascular disease (Glovaci et al., 2019). It is estimated that 12.2 million people in the United States suffer from cardiovascular diseases, the leading cause of death in the United States (Cross et al., 2020). Also, modern

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lifestyles gradually increased cardiovascular diseases among the populations of developing countries (Saina et al., 2023). Iran is also one of the developing countries facing a sharp increase in this disease (Sarrafzadegan and Mohammmadifard, 2019).

Oxidation of low-density lipoproteins (LDL) in the artery wall is responsible for the initiation and progression of atherosclerosis. On the other hand, HDL can prevent the development of atherosclerosis by reducing the oxidation of LDL. Evidence shows an inverse relationship between plasma HDL concentration and coronary heart disease (CHD), so HDL is an inhibitor for the progression of atherosclerosis (Boren et al., 2020). Studies on animal models show that increasing the HDL concentration through various pathways, including increasing the expression of apolipoprotein A1, reduces the progression of atherosclerosis (Kunachowicz et al., 2023).

Human paraoxonase (aryl dialkyl phosphatase, EC.3.1.8.1) is a glycosylated enzyme with 354 amino acids and 45 kDa molecular mass (Kotur-Stevuljević et al., 2020). Serum paraoxonase activity in humans is very diverse, including about 40 different types of structures. Genetic factors, environmental factors, chemical substances, smoking, drinking alcohol, or even the consumption of certain medicinal compounds and specific pathological and physiological factors can influence paraoxonase activity. Paraoxonase 1 (PON1) is an HDL-dependent enzyme that participates in protecting cells and biomolecules against the effect of LDL by reducing peroxidation products during the LDL oxidation process (Taler-Verčič et al., 2020). PON1 is synthesized in the liver and then released into the blood plasma. Human serum PON1 is complexed with blood phosphate-binding protein (HPBP) before being released into the blood circulation. This enzyme is mainly bound to HDL and rarely found VLDL- and chylomicron-bound (Kotur-Stevuljević et al., 2020; Akhavan et al., 2015).

In several models of atherosclerosis studies, *PON1* gene expression in mice reduced oxidized LDL. Other changes, such as a decrease in the level of peroxides, LDL oxidase, and the oxidative stress of the macrophages, as well as an increase in glutathione, have also been observed. In addition, it has been reported that some polymorphisms in the *PON1* gene are associated with a higher risk of heart disease. *PON1* gene polymorphism can independently increase the risk of heart disease. (Godbole et al., 2020; Khalil et al., 2021; Ochoa-Martínez et al., 2021; Kumar et al., 2021; Reza et al., 2022). There are also many studies to demonstrate low *PON1* expression can cooperate in the progress of neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (Khalaf et al., 2023; Marawne et al., 2022; Abdel-Salam et al., 2020). PON1 can play a crucial

role to detoxify organophosphates and other nuerotoxins, causing neurodegeneration. Also, peroxidase and terriesterase functions of PON1 can inhibit acetyl coline over-production, caused by free radicals and oxons, during neurodegeration. Also, PON1 has antioxidant and anti-inflammation properties. So, PON1 can prevent neuroinflammation leading to neurodegerative disorders (Tutunchi and Akhavan, 2016; Reichert et al., 2020; Khalaf et al., 2023).

In the present study, the level of *PON1* gene expression in cardiovascular Iranian patients is compared with normal Iranian people, and analyzed the association between some clinical factors and the *PON1* gene expression among the Iranian population. In addition, the frequency of Q192R polymorphism in the *PON1* gene and the correlation between *PON1* gene expression levels have been studied.

2. METHODOLOGY

2.1. Sampling

After overnight fasting, blood samples from 80 individuals with cardiovascular disease, with an average age of 59.04 ± 15.68, were collected for this case-control study at the ICU of Shariati Hospital, Tehran, Iran. 40 cardiovascular patients and 40 age- and gender-matched controls attended the study. Additionally, blood biochemical factors such as triglyceride, cholesterol, HDL, LDL, and FBS were analyzed. The study was approved by the Research Ethics Committee of the Islamic Azad University, East Tehran Branch (Approval Code: 22330553941001; Approval Date: 2021.08.29). All studies abided by and conformed to the requirements from the Guideline for the Clinical Research Development Units (CRDU) and Hospital Research Development Committee (HRDC) in Iran. Although the study has been approved by the Research Ethics Committee, meeting the professional and legal requirements is the sole responsibility of the PI and other project collaborators.

2.2. PON1 Gene Expression

The RNX-Plus kit (SinaClon Co., Tehran, Iran) was used to extract the total RNA (SinaClon Co., Tehran, Iran). RNA quality was measured by using A260/280 and A230/280 absorbance ratios. RT-PCR and real-time PCR were employed to analyze *PON1* gene expression. Specific primers for these PCR tests were designed by Primer Express software v3 (Table 1). *PON1* gene expression quantitative data was standardized with the GAPDH gene. The samples were amplified in a 20 µL PCR reaction mixture containing 10 µl ROX/SYBR Master 2X (Thermo Fischer, Massachusetts,

USA), 0.5 µl cDNA, and 1.2 µl of each primer. The PCR conditions were as follows: initial denaturation at 95 °C for 10 s, followed by 45 cycles including denaturation at 95 °C for 5 S; annealing at 55°C for 1 min, then extension at 72 °C for 30 S.

Table 1. The primers used in the study.

Name	***Sequence (5`-3`)***	***Tm (˚C)***	***Amplicon size (bp)***
PON-1 F	GACAGGAGACCTTTGGGTTG	59.55	95
PON-1 R	GGATTCGAAGCACCTCTGAT	59.24	
GAPDH F	ATGGAGAAGGCTGGGGCT	62.05	124
GAPDH R	ATCTTGAGGCTGTTGTCATCATTCTC	61.62	
Q192R-F	ATGTGTTGCTGTGGGACCTGAG	66	238
Q192R-R	CCTGAGAATCTGAGTAAATCCACT	68	

2.3. Q192R Polymorphism Analysis

The DNA samples were extracted by using the DNP kit (SinaClon Co., Tehran, Iran). The quality and quantity of extracted DNA were analyzed by agarose gel electrophoresis and spectrophotometry, respectively. The RFLP method was used for finding SNPs in the *PON1* gene. First, PCR was utilized to amplify the *PON1* gene by Q192R-F and Q192R-R (Table 1). The PCR reaction was 25 µL containing 5 µl genomic DNA, 1 µl each primer and 12.5 µl PCR master mix (including 2.5 mM dNTP, 1.0 mM $MgCl_2$, 1X Taq buffer, and 0.33 U/µL Taq DNA polymerase; SinaClon Co., Tehran, Iran). Also, the PCR program was 40 cycles, including a pre-denaturation step for 3 min at 95°C followed by a denaturation step at 95°C for 30 S, annealing at 62°C for 30 S, and polymerization at 72°C for 3 min with final extension at 72°C for 5min.

The 238 bp PCR product was digested using *Bsp*PI (*Alw*I; Thermofischer, Massachusetts, USA) restriction enzyme at 55 °C for 16 hours. Agarose Gel electrophoresis was used for further analysis of the digestion product. A single 238 bp band was considered as QQ (AA genotype), three bands with 238, 175 and 63 bp lengths as QR (AG genotype), and two bands with 175 and 63 bp lengths as RR (GG genotype).

2.4. Statistical Analysis

The data were statistically analyzed by SPSS v23. Where applicable, the continuous variables were presented as means standard deviations. An odd ratio (OR) and 95% confidence coefficient were used to determine the association between various groups, alleles, and genotypes. PON1 gene expression quantitative data was analyzed by computing 2-ΔCT and performing a t-test ($P < 0.05$).

3. RESULTS

We studied the relationship between blood biochemical factors such as triglyceride, cholesterol, HDL, LDL, and FBS and the *PON1* gene expression, also Q192R gene polymorphism, among 80 Iranian people, including 40 normal individuals as the control and 40 cardiovascular patients, including 57.5% male and 42.5% female. The average ages of these studied individuals were 60.55±15.98 and 58.12±14.27 years.

The assessment of blood biochemical factors showed no significant differences in serum FBS and LDL levels across the groups (P = 0.16 and 0.16 > 0.05, respectively; however, the factors were higher in patients (129.95±55.92 and 92.17±31.07, respectively) than normal people (88.1±12.17 and 85.4±25.11, respectively). On the other hand, the cholesterol and triglycerides in patients were significantly higher (204.45±47.99 and 233.07±69.22 respectively; P < 0.05) than in normal individuals (139.62±46.72 and 97.22±22.24 respectively), while HDL in cases was much lower than controls (61.55±33.43 and 108.05±39.38 respectively; Table 2).

Table 2. The clinical and biochemical characteristics of the studied population.

Variables	***Cases***	***Control***	***P Value***
Age (years) (mean±SD)	60.55±15.98	58.12±14.27	0.13
Male	57.5%	52.5%	0.1
Female	42.5%	47.5%	0.16
FBS	129.95±55.92	88.1±12.17	0.24
LDL	92.17±31.07	85.4±25.11	0.19
HDL	61.55±33.43	108.05±39.38	0.005*
Cholesterol	204.45±47.99	139.62±46.72	0.0001*
Triglyceride	233.07±69.22	97.22±22.24	0.038*

Note: FBS=Fast Blood Sugar; LDL= Low-density Lipoprotein ;HDL= High-density lipoprotein.

3.1. *PON1* Gene Expression

Real-time PCR and ΔΔCT techniques were respectively utilized to analyze *PON1* gene expression and RQ. First, the Chi-square test checked the data normality, and then the Mann-Whitney test was used for significance analysis. *PON1* gene expression in patients significantly decreased, lower than control group (P<0.05; Fig 1).

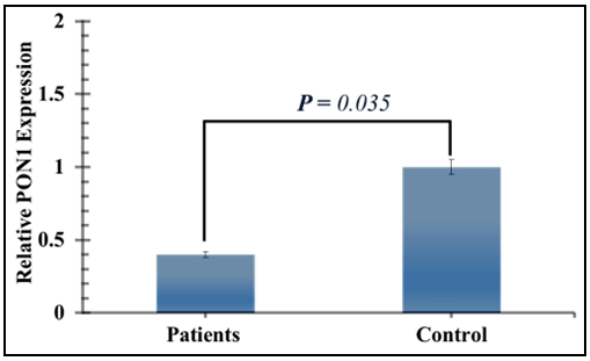

Figure 1. The comparison of the PON1 gene expression between control and cardiovascular patients.

Descriptive statistics analysis was employed to calculate the ASYMP sig number for the association between patient biochemical factors, gender, and age with *PON1* gene expression level. According to the statistical analysis results, there is no statistically significant correlation between gender, blood cholesterol levels, HDL, and FBS with the *PON1* gene expression (P>0.05). However, *PON1* gene expression was decreased in older studied groups, so there is a significant correlation between *PON1* expression and age (P<0.05); as the more senior individuals were, the less *PON1* was expressed (Fig 2a).

Moreover, serum LDL was the other factor showing a significant correlation with *PON1* gene expression (P<0.05); serum LDL levels in patients significantly increased when the RQ raised (Fig 2b).

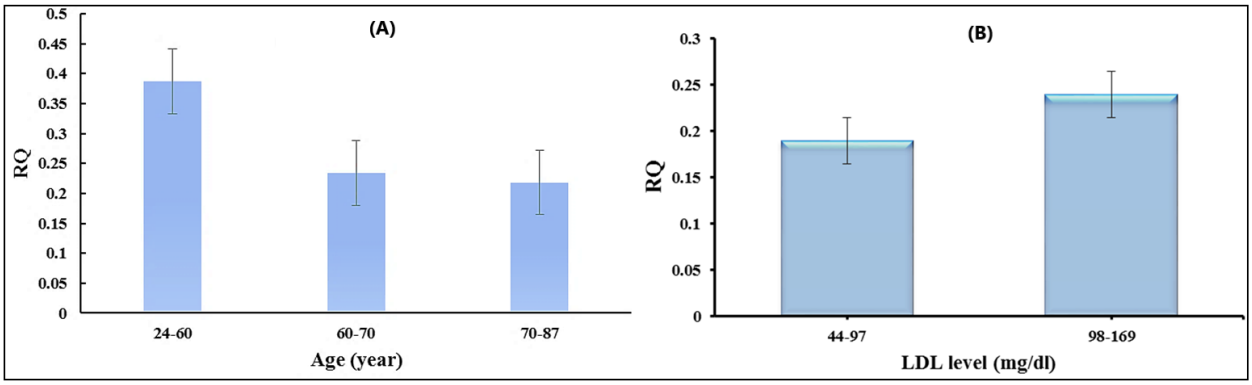

Figure 2. The PON1 gene expression correlation with age (A) and serum LDL level (B).

3.2. Q192R Polymorphism Analysis

The *PON1* gene PCR product was digested using the *Bsp*PI enzyme, and the samples were subsequently electrophoresed on a 3% agarose gel. Homozygous patient samples GG/RR created two bands of 63 and 175 base pairs with polymorphism and mutation, causing glutamine to change to arginine. Normal homozygous (AA/QQ) samples had a 238 bp band in normal samples,

meaning no enzymatic digestion happened because there was no mutation. If any samples represented three bands on the agarose gel, the patients would be heterozygous (AG/RQ); of course, the sample was not observed (Fig 3).

Moreover, the results indicated that the average RQ of the polymorphic and non-polymorphic samples were 0.05 (18%) and 0.37 (82%), respectively (Table 3). So, the *PON1* gene expression decreased more significantly in mutants than in the normal genotype (Fig 4).

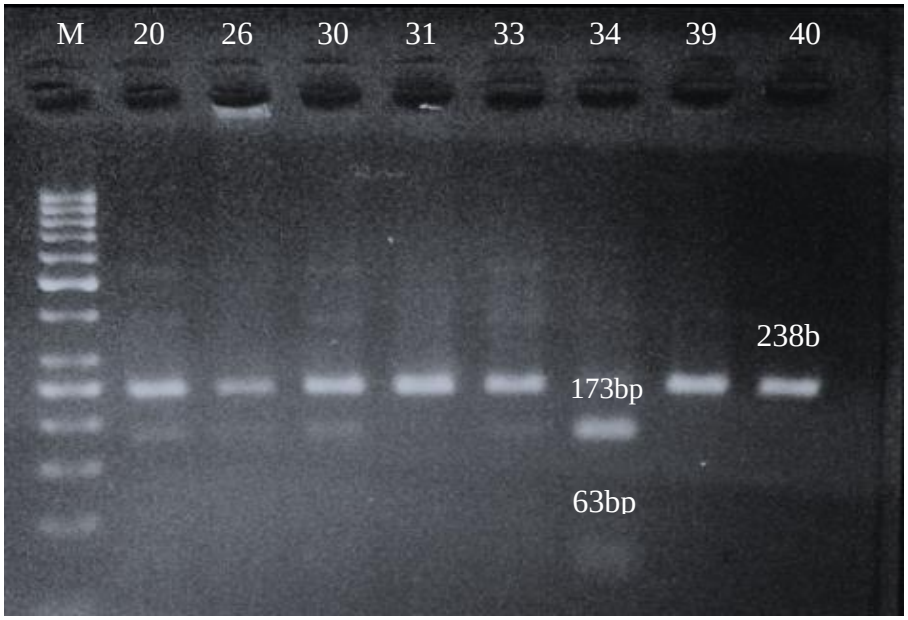

Figure 3. The enzymatically-digested PCR product of *PON1* on agarose gel (3%). M) Molecular Ladder.

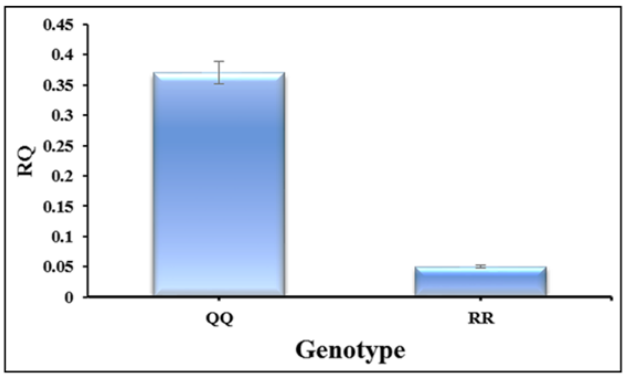

Figure 2. The PON1 gene expression among patients with or without the Q192R polymorphism (QQ: non-polymorphic, RR: polymorphic).

Table 3. The frequency and band size of genotypes in the studied samples.

Genotype	***Frequency (%)***	***Size (bp)***
Homozygous AA/QQ	82%	238
Heterozygous AG/RQ	0	175, 63, 238
Homozygous GG/RR	18%	175, 63

4. DISCUSSION

The *PON* multigene family are all adjacent and located on chromosome 7q21.22. PONs play a role in reducing oxidative stress and preventing atherosclerosis. The association between lipoprotein oxidation and cardiovascular diseases has been studied for over 30 years. Specifically, the vital role of PON1 has been repeatedly studied in antioxidant activities by protecting LDL against lipid peroxides and thus attenuating the development of atherosclerosis (Chistiakov et al., 2017; Shunmoogam et al., 2018; Su et al., 2019). Also, HDL plasma level is directly related to PON1 protein level. HDL creates a hydrophobic environment to protect the N-terminal part of PON1, which is also required for PON1 interaction with the substrate. Besides, lipoproteins and their metabolism, biological macromolecules, drug treatments and lifestyle are effective on PON1 activity. It has also been reported that any disruptions in PON1 functions and *PON1* expression can remarkably cause several cardiovascular diseases, including arteriosclerosis, diabetes, high cholesterol and metabolic syndrome, particularly in elders (Lopez et al., 2017; Adhe-Rojekar et al., 2018; Chen et al., 2019).

The present study demonstrated that the *PON1* gene expression in cardiovascular patients significantly decreased compared to normal people. This means the PON1 enzyme can play a crucial role in cardiovascular diseases. Also, a significant correlation was observed between the age and LDL level with the *PON1* gene expression. So, the PON1 enzyme plays a protective role in preventing atherosclerosis, as demonstrated in previous studies (Ashiq and Ashiq, 2021; Wysocka and Zwolak, 2021). It has also been reported that PON1 activity can be reduced by lead pollution, thus, people can be predisposed to atherosclerosis (Abdulwaliyu et al., 2021). Besides, serum PON1 levels decreased by lower *PON1* gene expression. Hence, low serum PON1 level can cause lipoprotein oxidation; consequently, the lipid sections of the oxidized lipoproteins are deposited as atheroma in the arteries, leading to coronary artery diseases and atherosclerosis (Durrington et al., 2023). The results represented that lower *PON1* gene expression in most samples from cardiovascular patients can be one of the effective factors in the occurrence of cardiovascular diseases. Low *PON1* expression could be caused by a mutation in the gene regulatory sequences (Zeng and Zeng, 2019), so a polymorphism study can be advantageous to find the mutation (Ramedani et al., 2015).

Polymorphism techniques are widely used to analyze mutations and differences in alleles and loci of a specific gene and diversity among populations or individuals of species. Besides, the

methods can be employed to find single nucleotide polymorphism in a gene involved in diseases and disorders (Abdilla et al., 2023; Barzin et al., 2016). Restriction fragment length polymorphism-PCR (RFLP-PCR) is a molecular method analyzing changes in homologous DNA sequences, known as polymorphisms (Hashim and Al-Shuhaib, 2019). According to RFLP-PCR results, heterogeneity in the *PON1* gene was mainly observed among cardiovascular patients, while normal people were homologous for the gene. Also, polymorphic samples showed lower *PON1* expression.

In a study on cardiovascular patients in north India, low *PON1* gene expression is related to cardiovascular diseases. Q192R polymorphism in the *PON1* gene is significantly associated with cardiovascular diseases among Indian patients. Also, the homozygous QQ genotype of Q192R SNP causes a nine-fold higher risk of cardiovascular diseases within the population (Kumar et al., 2021); As well as this, our study indicated that the polymorphism is correlated with cardiovascular patients in Iran and homozygous QQ genotype can increase the risk of cardiovascular diseases, about seven-fold, among Iranian patients.

In another study among north Indian cardiovascular patients, Raza et al. (2022) showed that Q192R polymorphism in the *PON1* gene can significantly increase cardiovascular diseases among the north Indian population, as same as Iranian people. They reported that QQ genoype of the SNP can significantly increase the risk of cardiovascular diseases about 11 fold among north Indian patients.

Also, in a study on Mexican cardiovascular patients, Ochoa-Martínez et al. (2021) demonstrated a significant correlation between heterozygous phenotype (QR) of Q192R polymorphism and the occurrence of cardiovascular diseases (Kumar et al., 2021). Their results are completely different with our findnings and also what resulted in Indian studies (Kumar et al., 2021; Raza et al., 2022).

On the other hand, Godbole et al. (2020) indicated that hemozygous RR phenotype of Q192R polymorphism in the *PON1* gene is more related to cardiovascular diseases in Western Indian patients, and can develop the diseases more than other phenotypes. However, low PON1 gene expression plays a crucial role in medical conditions. These results are different from our findings about Iranian cardiovascular patients. Because, QQ phenotype can lead to higher risk of the cardiovascular diseases among Iranian population.

Among Chinese population, PON1 expression can decrease the risk of the cardiovascular diseases, and also QQ genotype of Q192R is significantly associated to develop the diseases, more than other genotypes (Huang et al., 2022). This results can confirm our findings about Iranian population.

Another study on Turkish population repoted that There is a correlation between the PON1 QQ genotype and a higher rate of cardiovascular diseases (Solmaz Avcikurt et al., 2021). This results are similar our findings on Iranian cardiovascular patients.

5. CONCLUSION

Finally, it can be concluded that low *PON1* expression can be a crucial factor causing cardiovascular diseases among Iranian patients. *PON1* gene expression can be reduced by numerous factors, particularly mutation in gene regulatory regions, causing polymorphism; however, the results indicated that the Q192R polymorphism cannot be the only factor involved in *PON1* gene expression, so other SNPs also need to be analyzed.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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