Original Article

The Effect of Paraoxonase-1 (PON1) Concentration and Gene Polymorphism (L55M, Q192R Common Codons Gene Variant) with T2D Development Case–Control Study in Iraqi Populations

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Abstract

Background: Type 2 diabetes mellitus (T2D), also referred to as non-insulin-dependent diabetes mellitus (NIDDM), is a prevalent and chronic metabolic condition marked by high levels of sugar in the bloodstream, a condition known as hyperglycemia. Paraoxonase-1 (PON1) plays a role in inhibiting lipid peroxidation and has been linked to conditions marked by elevated oxidative stress, such as cardiovascular ailments and diabetes. **Objective:** We conducted a study to explore the connection between PON1 levels and genetic variations (Q192R, L55M) in relation to the occurrence of T2D within Iraqi populations. **Materials and Methods:** In this case–control study, included 200 subjects diagnosed as (T2D) and 200 subjects without any health problems as our study participants. The technique employed specific allele discrimination real-time PCR, which utilizes probe-based RT-PCR, to investigate the genotypes of PON1 rs662 (Q192R T>C) and rs854560 (L55M A>T). Additionally, we conducted a standard biochemical analysis according to established protocols for other biochemical measurements. **Results:** The R and M alleles exhibited a significant association with susceptibility to T2D, demonstrating odds ratios (OR) of 1.40 (95% confidence interval [CI]: 1.04–1.82) and 1.56 (95% CI: 1.16–2.12), *P* value associated with these associations were 0.03 and 0.003, respectively. When compared with controls, T2D patients had significantly higher frequencies of both R and M alleles. Furthermore, the concentration of PON1 was notably lower in the patient group when compared to the healthy. Also, T2D subjects displayed increased LDL and reduced HDL levels. **Conclusion:** Our findings suggest an association between the development of T2D and the minor allele frequency (MAF) of both Q192R and L55M polymorphisms in the PON1 gene in Iraqi populations.

Keywords: Allele discrimination/SNPs real-time PCR technique, common codons variation, PON1 gene, T2D

Introduction

Type 2 diabetes mellitus (T2D), also referred to as non-insulin-dependent diabetes mellitus (NIDDM), is a prevalent and chronic metabolic condition marked by high levels of sugar in the bloodstream, a condition known as hyperglycemia. It exerts a wide impact on people worldwide, affecting a significant majority of individuals. Type 2 diabetes (T2D) accounts for over 90% of all cases of diabetes mellitus. In this condition, the pancreatic islet β-cells either do not produce enough insulin or face difficulties in responding to insulin effectively, particularly in peripheral tissues like muscle, liver, and adipose tissues; this phenomenon is referred to as insulin resistance.[1] Diabetes is linked to increased oxidative stress within the human body, which exhibits the susceptibility to vascular diseases. This arises from a multifactorial, genetically influenced disorder.[2] Previously, genetic research suggested that more than 25 mutations are associated with T2D. These mutations predominantly arise from various sites of the entire human genome, including both coding and non-coding regions, which are involved in the control

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of insulin secretion.[3] Investigating the genetic differences (known as SNPs) in antioxidant enzymes responsible for detoxifying harmful compounds can provide an idea of the genetic etiology of diabetes. Recent studies into biochemistry and genetics demonstrate that PON1 plays crucial roles in antioxidants.[4] The paraoxonase (PON) gene family includes three members (PON1, PON2, and PON3) encoded by three adjacent but distinct genes that are located on the long arm of human chromosome 7q21.3–22.1.[5] Each member of the PON family plays a crucial role in reducing oxidative alteration of low-density lipoprotein (LDL). and cell membranes, consequently aiding in the prevention of atherosclerosis.[6] PON1 (EC 3.1.8.1). PON1 is a protein molecular weight of roughly 43 kDa and is made up of 354 amino acids. Its configuration is that of a six-bladed β-propeller, where each blade comprises four β-sheets.[7] Human PON1 enzymes possess hydrophobic N-terminal segments. Yet, these segments exclusively play a role in associating with blood plasma HDLs.[8] PON1 is primarily synthesized in the liver and subsequently released into the bloodstream. PON1 can break down about 19% of the lipid peroxides and approximately 90% of the cholesteryl hydroperoxides present in oxidized-LDL particles. This implies a significant role against atherosclerosis (atheroprotective) by effectively removing peroxidized LDL.[9] Prior genetic research has indicated a connection between PON1 and diabetes mellitus.[10,11] Numerous research studies have demonstrated the identification of over 200 single nucleotide polymorphisms (SNPs) located on the PON1 gene. These genetic variations Contribute to the diversity seen in both the concentration and activity of PON1 among individuals.[6] The variations in SNPs might impact processes such as splicing and polyadenylation effectiveness, message stability, or the binding of transcription factors.[12] Two frequent and effective single nucleotide variations exist within the PON1 gene: a change from changing the amino acid (glutamine Q to arginine R at position 192, denoted as Q192R (rs662), and a shift from changing the amino acid (leucine L to methionine M) at position 55, which is represented as L55M (rs854560).[13] accordingly, the protein known as PON1 and the levels of paraoxonase in the body That attach with benefit lipoprotein (HDL) from individuals with DM show significantly decreased levels in blood (2.8-fold and 1.7-fold, respectively), in comparison to the control subjects.[14] In the current investigation, we examined the levels of enzymes and the prevalence of specific genetic variations at codon positions (Q192R and L55M) in individuals affected by type 2 diabetes, with individuals who do not have any disease.

Materials and Methods

Study population and design

The current study design as a case–control study included 200 individuals who were recently diagnosed with T2D (ranging from 35 to 69 years consisting of 103 men and 97 women). ADditionally, there were 200 control participants (ranging from 35 to 69 years including 100 men and 100 women).

The specimens were collected from the cases that were reviewer by Al-Sadr Medical City (diabetes and endocrinology center) in Iraq at over the course of time in the Najaf province from April 2021 to December 2022. The control group consisted of individuals who volunteered for the study and met the specified criteria for inclusion in that group as follows: No previous record of diabetes mellitus, fasting plasma glucose levels less than 110 mg/dL, in addition, glycemic control level (HbA1c equal to or lower than 5.8%). All individuals in the cases groups did not have type 1 diabetes, liver diseases, renal failure, or chronic illnesses, and none of them were pregnant. The evaluations comprised a questionnaire designed to evaluate details about demographics, medical background, and individual habits. Anthropometric assessments, like the calculation of body mass index (BMI), involve dividing a person's weight in kilograms by the square of their height in meters. The diagnosis of T2D cases was made by a specialized physician following the guidelines of the American Diabetes Association (ADA), which specify T2D diagnosis criteria as fasting blood glucose (FBG) levels equal to or exceeding (126 mg/dL), and glycemic control level (HbA1C) ≥6.2%.[15] The research was designed according to the ethical principles outlined in the declaration and received approval from the Biochemistry Department's scientific committee at the College of Medicine, University of Kufa, Iraq, as well as from the Ministry of Health's Al-Najaf Health Directorate and the Information Center for Research & Development in Najaf Province. Participants provided informed consent after receiving a clear explanation of the study's objectives.

Ethical Approval

The research was designed according to the ethical principles outlined in the declaration and received approval from the Biochemistry Department's scientific committee at the College of Medicine, University of Kufa, Iraq, as well as from the Al-Najaf Health Directorate. Participants provided informed consent after receiving a clear explanation of the study's objectives.

Biochemical analyses

At the clinic's laboratory obtained blood samples, while the analysis of biomarkers was conducted within the laboratories of Biochemistry Department's in the Faculty of Medicine at the University of Kufa.

Approximately 10 mL of complete venous blood were collected from all subjects following an overnight fasting period. Five milliliters of blood samples were obtained

in vacutainer tubes containing EDTA for the purpose of conducting HbA1C and genotyping analyses. The remaining 5 mL of blood, which was collected in a vacutainer gel tube, was used for various standard laboratory tests to assess the levels of the following parameters.

1. Determination of quantity fasting blood glucose (FBG) by enzymatic method.
2. Estimation PON1 concentration, by ELISA technique.
3. Determining insulin levels using the ELISA method.
4. Estimation of the lipids levels of total cholesterol (TC), triglycerides (TGs), and HDL-C measurement by enzymatic method.
5. Calculated LDL cholesterol was calculated according to "Friedwald's equation": LDL-C = Total cholesterol – (HDL-C + TG/2.2)
6. Detection of HbA1c levels was performed using a commercially available kit that utilized an affinity technique (on the turbidimetric inhibition immunoassay (TINIA) automatically on the COBAS INTEGRA 400 plus, with an accuracy rate and failure rate both under 5%.

Genotyping analysis

PON1 genotype investigation: rs662 SNP and rs854560 are located on chromosome 7, at PON1 gene. All subjects were genotyped for Q192R and L55M polymorphism of PON1 gene by utilizing real-time PCR for allele discrimination and SNP Analysis, employing the subsequent procedures.

DNA extraction

DNA extraction from frozen whole blood using genomic DNA (gDNA) purification kits (Product Code: 10023, Add Prep Genomic, Korea) was conducted. The concentration of DNA was assessed using UV spectrophotometry with a Nanodrop. The ultraviolet light absorbed by DNA fluctuation is based on the wavelength employed. In the current study, an application of a Nanodrop instrument from Bio drops, UK, was utilized to perform estimation, which was conducted at wavelengths of 260 and 280 nm. These findings are illustrated in Table 1 and Figure 1. The DNA's integrity was assessed by undergoing 1% (w/v) agarose gel electrophoresis.

Table 1: The purity of DNA

DNA	Mean ± SD
Purity of extracted DNA (A260/A280)	1.86 ± 0.04
Concentration of extracted DNA	72.88 ± 6.16

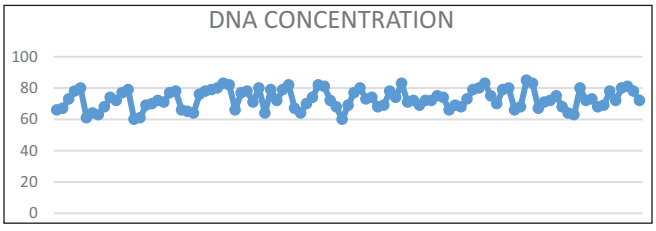

Figure 1: Concentration of extracted DNA samples

Primer and probs design

Real-time PCR amplification: The genomic DNA extracted was subjected to amplification through specific primer and TaqMan probe sets, which were designed to amplify and detect the target genetic variation. Primer and probe sequences were designed by using applied biosystems (AB) and Primer3Plus online design tools, and their accuracy was validated through the National Centre for Biotechnology Information (NCBI). These amplification components were supplied by Alpha Company based in Canada.

For the rs662 T>C and rs85450 A>T the primers and probs that are designed for target regions in PON1 gene are illustrated in Table 2

Procedure of amplification allele discrimination real-time PCR

The details of real-time PCR for allele discrimination and single nucleotide polymorphism detection protocol for the analysis and amplification of SNP rs662 and SNP rs854560. This information outlines the volumes of reagents, temperature conditions, and cycling parameters used in the real-time PCR assay for these two SNPs.

SNP rs662 analysis

- Initial denaturation: 95°C for 5 min (1 cycle)
- Denaturation: 95°C for 30 s (40 cycles)
- Annealing: 59°C for 30 s (40 cycles)
- Extension: 72°C for 30 s (40 cycles)

SNP rs854560 analysis

- Initial denaturation: 95°C for 10 min (1 cycle)
- Denaturation: 95°C for 30 s (40 cycles)
- Annealing: 59.3°C for 20 s (40 cycles)
- Extension: 72°C for 30 s (40 cycles)

Table 2: The primers and probs sequence used in this study

Gene/ SNP	Primer and probs	Primers and probs sequences
rs854560 A>T	Forward	F-ACAACCTGTACTTTCTGTTCTCTTTTCTG
	Reverse	R-CAGAGCTAATGAAAGCCAGTCCAT
	Wild	HEX-AGTATCTCCAAGTCTTC-BHQ
	Mutant	FAM-CAGTATCTCCATGTCTTC-BHQ
rs662 T>C	Forward	F-CGACCACGCTAAACCCAAATAC
	Reverse	R-TGTGGGACCTGAGCACTT
	Wild	HEX-TCTCCCAGGATTGTAAGTAG-BHQ
	Mutant	FAM-CTCCCAGGATCGTAAGTA-BHQ

Principle

This experimental approach involved the use of fluorescent probes to assess the allelic diversity in DNA samples being analyzed using two separate probes, with each probe tagged with a unique fluorescent marker, namely Hex, and FAM, which were utilized to distinguish among genetic variances that could vary by as little as a solitary change single DNA base pair. Expressing whether something is present or not of a specific allelic variant according to the Ct value acquired through the different allele-specific probe. The amplification plots display the rise in fluorescence throughout the PCR cycles. These graphs highlight instances where data was collected. The selection of a precise data collection location for analysis was established using the TaqMan real-time PCR Method for allele discrimination and SNP Analysis, conducted on the Mx3005P QPCR System. This setup was configured via the Analysis Selection/Setup screen.[16] The probe targeting the commonly gene allele (referred to as the wild-type allele) had a 6-HEX (VIC) reporter dye attached to the 5 ends, whereas the probe designed for the less common gene variant (mutated allele) was marked with FAM (6-FAM) at its 3 ends.[17]

Allelic discrimination (AD) analysis

To investigate the genotype of an unidentified DNA sample, its fluorescence is measured and then compared to both dyes according to the baseline fluorescence levels. If the fluorescence exhibits a significantly higher intensity when using the dye that marks the presence (wild allele), but not with the dye that marks the mutated allele, normally each sample is having two normal alleles. Conversely, If the fluorescence exceeds the baseline for the dye representing the mutated allele while staying constant for the dye indicating the normal allele, the sample is categorized as exhibiting homozygosity for the mutated allele. In situations where the sample displays a moderate fluorescence intensity for both dye varieties, it is labeled as having one normal and one mutated allele heterozygous. automatic detection of allele discrimination using the

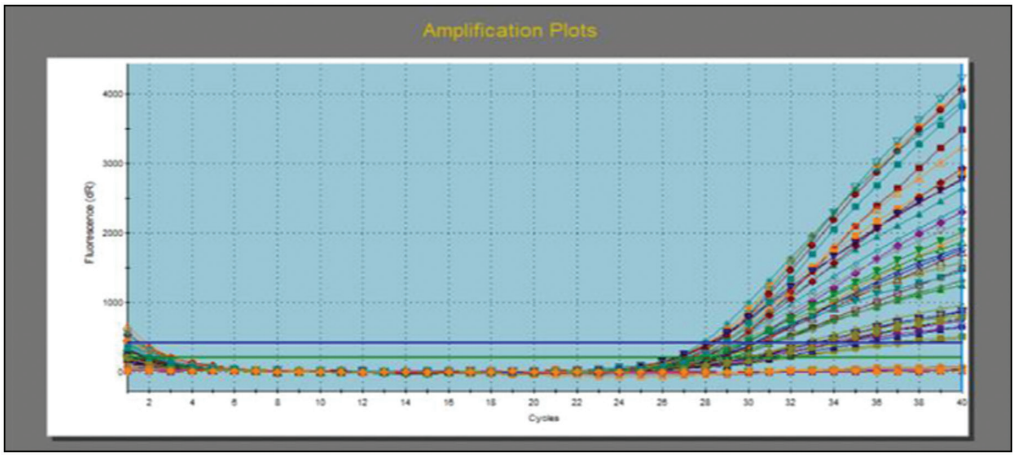

Figure 2: Depicted the amplification plot of the PON1 gene variant rs662 T>C

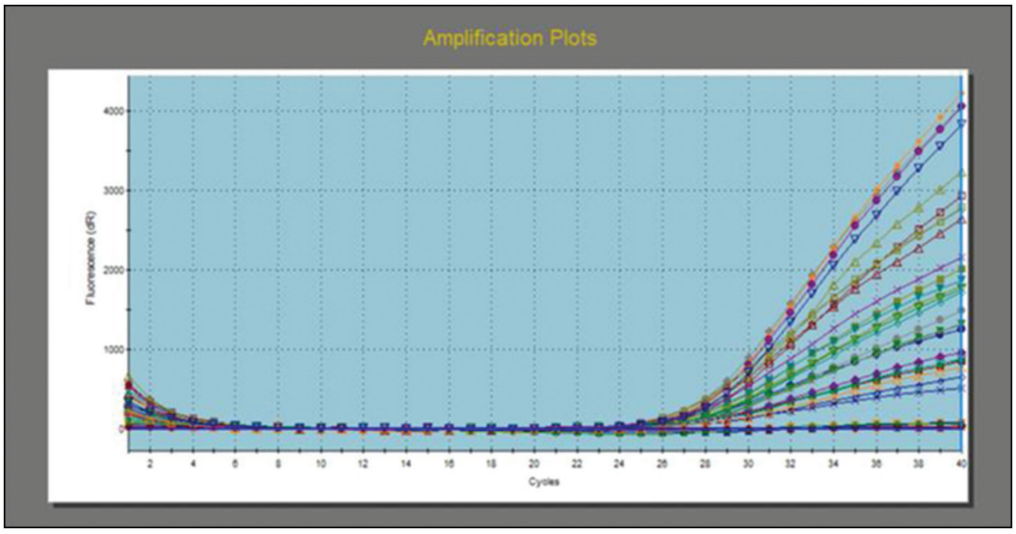

Figure 3: Depicted the amplification plot of the PON1 gene variant rs854560 A>T

M×3005P QPCR systems depicted in Figures 2 and 3: There was not any extra treatment to construct the plot.[18]

Statistical analysis

Multinomial logistic regression analysis was conducted using IBM SPSS version 25 software to assess the impact of genotypes and allele frequencies on T2D Expressed in different inheritance patterns, which encompass recessive, codominant, dominant, additive, and allelic models. Quantitative data were summarized using median and range, while qualitative data were presented as frequency and percentage. To compare two means, an independent samples *t* test was employed for significance testing, and for comparisons involving more than two variables, ANOVA tests were applied. Categorical data were described using counts and percentages. Odds ratios (ORs), *P* value, and (CI 95%) were computed. Furthermore, the data underwent adjustments for variables like gender, age, and BMI, resulting in the recalculation of corresponding 95% confidence intervals (CIs) and ORs, followed by a reevaluation of *P* value. The Hardy–Weinberg equilibrium (HWE) was assessed to ensure that the genotype frequencies met the expected values and calculated https://scienceprimer.com/hardy-weinberg-equilibrium-calculator. It is used to determine if a population is experiencing evolutionary changes or experiencing variations from the expected genetic equilibrium. In this study, the established level for significance was set at 0.05, where results with (*P* value of less than 0.05) were reflected as significant, and those with a *P* value less than 0.001 were considered highly significant.

Results

Clinical and biochemical parameters as shown in Table 3 are compared to the healthy control groups. The findings indicated significant variances across all factors except for age and BMI (*P* = 0.4, 0.2, respectively). Individuals diagnosed with T2D demonstrated significant elevations in their TC, triglycerides (TG), LDL, and very LDL (VLDL) concentrations, along with increased fasting blood sugar (FBS), insulin, HOMA IR, and HbA1c levels. Furthermore, T2D patients displayed significantly reduced levels of high-density lipoprotein (HDL) and PON1 compared to the control group, with a *P* value less than 0.05.

Genotype and allele frequency of PON1 gene

The allele frequencies of PON1 gene variation, as well as the distribution of PON1 Q192R and PON1 L55M

Table 3: Clinical and biochemical parameters of controls and T2D

Group			IQR	Median	*P* value
Age	Control	48.00	56.00	53.00	
	Patients	47.00	57.00	52.00	0.4
BMI	Control	30.00	35.00	33.00	
	Patients	30.10	35.19	33.00	0.2
PON1	Control	1.76	2.03	1.88	
	Patients	1.11	1.41	1.27	**0.0001**
FBS	Control	83.25	93.00	88.00	
	Patients	162.25	200.00	181.00	**0.0001**
HbA1c	Control	4.90	5.40	5.20	
	Patients	7.80	9.20	8.30	**0.0001**
Insulin	Control	7.30	8.60	8.10	
	Patients	6.55	12.70	9.35	**0.0001**
HOMAIR	Control	1.60	1.80	1.70	
	Patients	2.93	5.28	4.20	**0.0001**
Chol	Control	154.00	172.00	164.00	
	Patients	167.00	219.00	185.50	**0.0001**
TG	Control	114.25	132.00	124.00	
	Patients	182.25	276.00	232.00	**0.0001**
HDL	Control	38.00	44.75	41.00	
	Patients	32.00	38.00	35.00	**0.0001**
VLDL	Control	23.00	26.00	25.00	
	Patients	36.00	55.00	47.00	**0.0001**
LDL	Control	88.00	106.75	97.00	
	Patients	93.00	131.75	104.50	**0.0001**

Based on our findings, we noted significant increase (FBS, HbA1c, HOMA IR, insulin, cholesterol. TG, LDL and VLDL) in patients when compared with controls; also we noted a notable decrease in HDL and PON1 protein levels in type 2 diabetes individuals when compared with controls groups (*P* value = 0.0001) and are represented in bold

Table 4: Assessing genotype and allele frequency differences of the Q192R PON1 gene in control and type 2 diabetes mellitus participants

Rs662 Q192R T>C	Control, *N* = 200	Patients, *N* = 200	Non-adjusted OR (CI 95%)	*P* value	Adjusted	*P* value
Genotype						
QQ (TT)	91 (45.5%)	75 (37.5%)	–			
QR (TC)	61 (30.5%)	62 (31%)	0.811 (0.51–1.3)	0.34	0.8 (0.499–1.28)	0.36
RR (CC)	48 (24%)	63 (31.5%)	0.63 (0.38–1.02)	0.06	0.61 (0.34–1.00)	0.05
Allele						
Q	243 (60.75%)	212 (55.25%)	–			
R	157 (39.25%)	188 (47%)	1.4 (1.04–1.82)	0.03		

Table 5: Assessment of genetic variations and allele distributions of the L55M PON1 gene in both healthy individuals and those with type 2 diabetes mellitus

L55M	Healthy 200 Sub.	Cases 200 Sub.	Non-adjusted OR (CI 95%)	*P* value	Adjusted	*P* value
Genotype						
LL	100 (50%)	69 (34.5%)	–			
LM	91 (45.5%)	114 (57%)	0.55 (0.36–0.83)	0.001	0.28(0.14–1.02)	0.001
MM	9 (4.5%)	17 (8.5)	0.36 (0.15–0.86)	0.02	0.2 (0.9–1.1)	0.002
Allele						
L	291 (72.75%)	252 (63%)	–			
M	109 (27.25%)	148 (37%)	1.56 (1.16–2.12)	0.003		

genotype frequencies, are presented in Tables 4 and 5. The genotyping distribution of these studied polymorphisms conformed to Hardy–Weinberg equilibrium in all participants. Regarding the PON1 polymorphism, we noted a significant association between the R allele frequency and T2D when compared to the control group, with an odds ratio (OR) of 1.40, 95% CI = (1.04–1.82), and *P* value of 0.03. The frequency of the R allele was significantly higher in individuals with T2D compared to the control group (47.0 vs. 39.25%, respectively), and it exhibited an association with the presence of T2D. The QQ genotype (Gln/Gln) was the most prevalent in both diabetic patients (37.5%) and controls (45.5%), whereas the QR genotype (Gln/Arg) was less common in diabetic patients (31%), and the RR genotype (Arg/Arg) was less prevalent in controls (24%). However, no significant divergence was observed in the Q192R polymorphism genotypes between diabetic individuals and the control group ($P > 0.05$).

Regarding the PON1 55 polymorphism, the LL genotype (Leu/Leu) was the most prevalent variant among individuals in the control group, making up 50% of the control group. Conversely, in diabetic patients, the LM genotype (57%) was more common than the LL genotype. Nevertheless, when comparing the genotype distribution of PON1 55 polymorphisms between diabetic patients and the control group, no statistically significant differences were observed. A significant finding emerged when examining the frequency of the M allele, which demonstrated an association with T2D when contrasted with the control group. The odds ratio (OR) stood at 1.56, with a 95% CI ranging from 1.16 to 2.12, accompanied by a corresponding *P* value of 0.003. Notably, the M allele frequency was significantly higher among individuals with T2D in comparison to the control group (37.0 vs. 27.25%, correspondingly), and it is enhancing the T2D development as explained in Table 5.

Discussion

Diabetes is associated with elevated levels of endogenous inflammation and oxidative stress, leading to an enhanced susceptibility to vascular diseases.[2] It is recognized as a complex condition influenced by multiple factors and a combination of genetic factors.[2] The genetic variation of SNPs in antioxidant enzymes responsible for detoxifying harmful compounds can provide an idea into the genetic etiology of diabetes. Recent researchers in the fields of biochemistry and genetics have highlighted the crucial role of PON1, a protein associated with HDL, in enhancing powerful overall antioxidant mechanisms in the human body.[4] Based on our findings, we noted a notable decrease in HDL and PON1 protein levels in type 2 diabetes individuals when compared to control groups (*P* value = 0.0001) [Table 3]. Moreover, a study conducted by Mackness *et al.*[19] discovered that individuals diagnosed with T2D displayed significantly lower serum PON1 levels in comparison to those who were in good health. The reduced PON1 levels observed in individuals with T2D could potentially impact the effectiveness of HDL in preventing LDL oxidation. This

suggests that PON1 might hinder the functional capacity of HDL in these individuals, potentially inducing their susceptibility to T2D.[20] The connection between the enzyme PON1 and diabetes mellitus seems to reciprocal connection. Diabetes mellitus noticeably reduces PON1 levels, and conversely, an individual's PON1 genotype can influence their susceptibility to developing diabetes mellitus.[11] While multiple research studies have indicated a correlation between PON1 genetic variations and the susceptibility to various illnesses across diverse population groups.[21-23]

To detect the relation between Q192R polymorphism of PON1 gene and T2D development in Iraqi populations. The current research reveals a link between the R allele and the occurrence of T2D. the minor allele frequency (MAF) (R) carriers exhibit a 1.4-fold higher risk for developing T2D among Iraqi individuals *P* value of 0.03 [Table 4], regardless of adjustments made for BMI, age, and gender don't change the results. Several studies have explored the relationship between specific PON1 gene variants and the risk of type 2 diabetes.[24,25] Some contradictory findings have no association between PON1 polymorphism and T2D.[19,20] Multiple ethnic studies have reported a significant correlation between T2D and PON1 gene variant in individuals from Saudi Arabia and Egypt.[26,27] However, there was no significant connection detected within the southern Iranian population.[28] These different results could stem from various factors such as racial disparities, geographical variations, lifestyle choices, and economic conditions. We also attribute the disparity between our research and previous studies to differences in the sample size used. Our examination of the L55M polymorphism reveals that the L allele exerts a suppressive influence, whereas the M allele has a promoting effect on the development of T2D. Specifically, individuals carrying the M allele have a significantly higher prevalence of T2D compared to those with the L allele, with an odds ratio (OR) = 1.56 and a 95% CI =1.16–2.12 (*P* value = 0.003), as shown in Table 5. Those possessing the (T) allele face approximately a 1.56-fold increased risk of developing T2D in comparison to those without this allele. Importantly, these findings remained consistent despite after adjusting for variables such as gender, age, and BMI. These results agree with prior research, much of which employed a case–control methodology. Notably, there exists a significant correlation between this specific SNP and T2D. for example, a study conducted on populations in southern Iran investigating the PON1 55 polymorphism revealed that the L allele (A) was more prevalent in the control group, while the M (T) allele was more common than the (A) allele in individuals with T2D.[29] Our findings also agree with the results presented in the study conducted by Flekač *et al.*[30] in the Czech Republic, which involved individuals with diabetes and a control group. This research observed a higher prevalence of the (T) allele, specifically from the L55M polymorphism, among the diabetes group in comparison with healthy subjects. Additionally, the study indicated that the (T) alleles had a more pronounced influence on the advancement of diabetes. Furthermore, it is essential to note that our study's findings opposed to the findings of the study by Gupta *et al*.[31] was found no linking between the rs854560 polymorphism alleles and the onset of the type 2 DM in populations originating from North-West India. Also in the study by Mahrooz,[32] no significant correlation was found in the distributions of both genotype and allele frequencies for rs854560 polymorphism between individuals with diabetes and the control group in Iranian populations.

Conclusions

The MAF for the SNPs rs662 T>C and rs854560 A>T (corresponding to alleles C and T respectively) within the PON1 gene variation could potentially be associated with an increased likelihood of developing T2D in this current study of individuals from the Iraqi population. PON1 concentration in the current study significantly decreased in patients' groups when compared with control groups.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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