

Enzyme activity and genetic polymorphisms in patients with type II diabetes mellitus

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Conflict of interest

None declared

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Abstract

Background. Diabetes mellitus (DM) has become more and more common and has a high morbidity and mortality rate worldwide. It is a multifactorial chronic disease affected by both genetic and environmental factors.

Objectives. To evaluate the association between antioxidant enzyme activities and their genetic variations and the level of malondialdehyde (MDA) in type II diabetes patients living in the Adiyaman province in the southeast part of Turkey.

Material and methods. One hundred patients diagnosed with type II DM (T2DM) and 100 healthy controls were included in the study. Malondialdehyde levels and antioxidant enzyme activities were measured spectrophotometrically. DNA isolation was performed and genotyping was carried out using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP).

Results. Our results revealed no significant differences in genotype distributions and allele frequencies of all polymorphisms between groups ($p > 0.05$). Significantly elevated MDA levels and a significant reduction in catalase (CAT) and paraoxonase (PON) enzyme activities were observed in patients compared to the control group in terms of study groups and genetic variations ($p < 0.05$). Moreover, CAT activity was reduced in TT genotype in terms of CAT –262 C/T polymorphism in patients ($p < 0.05$). Paraoxonase activity was observed to be lower in MM genotype in both groups ($p < 0.05$).

Conclusions. CAT –262 C/T polymorphism may be one of the factors that lead to severe clinical situation in DM. Our results suggest that TT genotype may be more prone to lipid peroxidation.

Key words: diabetes mellitus, oxidative stress, cat, malondialdehyde, paraoxonase

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Introduction

Diabetes mellitus (DM) has become more and more common and has a high morbidity and mortality rate worldwide. It is a multifactorial chronic disease affected by both genetic and environmental factors.^{1,2} Type II DM (T2DM) account for 90% of the DM cases. The reduction of insulin secretion or development of insulin resistance results in the impairment of macromolecule metabolisms, such as proteins, carbohydrates and lipids.^{3,4} The pharmaceutical development of insulin revolutionized the treatment of DM, but T2DM is still the most common disease globally with chronic complications.^{4,5}

The complications of DM can be expressed with 4 major mechanisms, including the production of reactive oxygen species (ROS), which is triggered by hyperglycemia, polyol pathway, the formation of advanced glycosylation end products (AGE), activation of protein kinase C, and hexamine pathway.⁶ Several factors, such as environmental factors, genetic risk factors, obesity, and oxidative stress, may all be associated with the development of T2DM. The oxidant/antioxidant balance in the organism shifts towards oxidation with a potential which results in the development of organopathies, such as cardiomyopathies, neuropathies and retinopathies.^{6,7} The excessive level of free radicals and ROS may result in several cellular injuries. In such cases, the antioxidant system may be insufficient to defend the cell against free-radical structures. The major components of the antioxidant system are enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), paraoxonase 1 (PON1), and glutathione peroxidase (GPx).^{7,8}

Our study aims to examine the association between CAT -262 cysteine (C)/threonine (T) and PON1 55 leucine (L)/methionine (M) genetic polymorphisms, CAT and PON1 antioxidant enzyme activities, and the level of oxidative damage marker malondialdehyde (MDA) in T2DM.

Material and methods

Sample collection

This study included a total of 200 individuals consisting of 100 healthy controls without any disorders (including DM) in their medical histories and 100 patients diagnosed with T2DM who were admitted to the Internal Clinic at Adiyaman University Training and Research Hospital, Turkey. The confirmation by Adiyaman University Ethic Committee (approval No. 2011/02-1) and a written consent from each subject have been provided after participants were properly informed. All experiments were performed according to the Declaration of Helsinki.

Biochemical analyzes

Fasting venous blood samples were collected into EDTA tubes and centrifuged at 3000 rpm for 10 min. After centrifugation, the plasma portion was separated and stored at -20°C until the analyses were performed. The enzymatic activities of CAT and PON1, and the level of MDA, the end product of lipid peroxidation, as an indicator of oxidative stress, were measured. Malondialdehyde forms a pink colored complex as a result of its incubation with thiobarbituric acid at a pH of 3.5 and 95°C in aerobic conditions. Based on this principle, the amount of MDA was determined with spectrophotometric measurement of this complex at 532 nm.⁹ The CAT enzyme activity was determined using spectrophotometric measurement of the substrate molecule hydrogen peroxide (H_2O_2) absorbance; it decreased over time as a result of its interaction with catalase at 240 nm.¹⁰

The PON1 activity was determined in alignment with the technique developed by Eckerson et al., which is based on monitoring the production of p-nitrophenol by the enzymatic hydrolysis of PON1 at 412 nm. A 100 mM Tris-HCl at pH 8, containing 2 mM CaCl_2 and 4 mM paraoxon was used as a buffer to measure its activity.¹¹

Molecular analyses

For the molecular analysis, leukocytes were separated from whole blood samples and DNA isolation was performed according to the method described by Poncz et al.¹² Genotype determination was carried out using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique.

The most commonly studied PON1 55 L/M polymorphism was determined using the PCR-RFLP method. The PCR amplification samples were digested with *Nla* III restriction endonuclease. Digestion products were separated on 2% agarose gel and visualized with ultraviolet light after ethidium bromide staining. Alleles of leucine and methionine for the PON1 55 position were assigned based on the presence of a 172 bp (undigested) fragment and 106 bp and 66 bp (digested) fragments, respectively.^{13,14} CAT -262 C/T polymorphism was similarly determined using PCR-RFLP method. The PCR amplification samples were digested with *Sma*I restriction endonuclease. Then, the digested products were separated on an agarose gel and visualized with ultraviolet light after ethidium bromide staining. The alleles of CAT -262 C/T polymorphism, threonine and cysteine were assigned based on the presence of a 340 bp (undigested) fragment and 185 bp and 155 bp (digested) fragments, respectively.¹⁵

The statistical analyses were performed using MedCalc v. 12.3 software (MedCalc, Ostend, Belgium). The Shapiro-Wilk test was used to determine whether all parameters were normally distributed. Abnormally distributed data are presented as median (min-max) and

mean ± standard deviation (SD), and group comparisons were conducted using the Mann–Whitney U test. The χ^2 and Fisher’s tests were used to evaluate the differences between the groups in terms of the genotype distribution and allele frequencies. Statistical significance for all analyses was set at a p-value <0.05.

Results

Biochemical analysis

In the current study, descriptive statistic data of biochemical analyses of study groups were given in Table 1. Malondialdehyde levels were found to be significantly higher in the diabetic group compared to the control (p < 0.0001). A significant reduction in plasma CAT and PON1 enzyme activities was observed in the diabetic group (p < 0.0001). Descriptive statistic data of biochemical analyses were displayed in Table 2.

Molecular analysis

There was no statistically significant difference between the diabetic and control groups with respect to genetic distribution and allele frequencies of CAT -262 C/T and PON1 55 L/M polymorphisms (p > 0.05). The genotype distributions and allele frequencies of the CAT -262 C/T and PON1 55 L/M polymorphisms between diabetic and control groups were displayed in Table 3.

In PON1 55 L/M polymorphism, the activity of the PON1 enzyme was lower for carriers of the MM allele, both in diabetic and control group compared to LL and LM genotypes (p = 0.0001) (Table 4A). In all genotypes, the MDA

Table 1. Descriptive statistic data of biochemical analyses

Parameter	T2DM group mean ±SD	Control group mean ±SD	p-value
HbA1c [%]	8.92 ±2.56	–	–
Insulin [mLU/L]	10.65 ±15.16	–	–
FBG [mmol/L]	10.95 ±5.62	5.15 ±0.40	<0.0001***
CHO [mg/dL]	208.6 ±45.95	180.2 ±41.79	0.0003***
TG [mg/dL]	185.1 ±108.8	115.9 ±63.25	<0.0001***
HDL-CHO [mg/dL]	40.36 ±9.7	42.59 ±10.46	0.236
LDL-CHO [ng/dL]	131.5 ±34.92	113.9 ±33.75	0.0038**
Age	56.75 ±10.86	36.65 ±14.67	<0.0001***
HOMA-IR	5.91 ±11.55	–	–
HOMA-β	36.14 ±33.82	–	–

Mann–Whitney U test; *p < 0.05; **p < 0.01; *** p < 0.001; T2DM – type 2 diabetes mellitus; HbA1c – glycosylated hemoglobin; FBG – fasting blood glucose; CHO – cholesterol; TG – triglycerides; HDL-CHO – high-density lipoprotein cholesterol; LDL-CHO – low-density lipoprotein cholesterol; HOMA-IR – homeostatic model assessment for insulin resistance; HOMA-β – homeostatic model assessment for β-cell function.

Table 2. Descriptive statistic data of biochemical analyses

Variable	T2DM group (n = 100)	Control group (n = 100)	p-value
	median (min–max)	median (min–max)	
MDA [nmol/L]	13.56 (4.25–43.31)	7.06 (1.36–10.76)	0.0001***
CAT [U/L]	19.50 (6.03–54.91)	47.16 (9.87–89.25)	0.0001***
PON [U/L]	21.42 (3.05–39.28)	43.35 (13.47–98.14)	0.0001***

Mann–Whitney U test; *p < 0.05; **p < 0.01; *** p < 0.001; T2DM – type 2 diabetes mellitus; MDA – malondialdehyde; CAT – catalase; PON – para-oxonase.

Table 3. The genotype distributions and allele frequencies of the CAT -262 C/T and PON55 L/M polymorphisms between diabetic patients and control group

Genotype	Control group (n = 100)		T2DM group (n = 100)		χ^2 test p-value	Interval OR (95% CI)	OR p-value
	n	%	n	%			
CAT							
TT	35	35	29	29	0.522	1	reference
CT	52	52	60	60		1.393 (0.75–2.58)	0.292
CC	13	13	11	11		1.021 (0.39–2.62)	0.965
Allele frequency					0.904		
T	122	61	118	59		–	–
C	78	39	82	41	1.087 (0.73–1.62)	0.683	
PON55							
LL	50	50	47	47	0.878	–	–
LM	38	38	39	39		1.092 (0.60–1.99)	0.774
MM	12	12	14	14		1.241 (0.52–2.96)	0.626
Allele frequency					0.669		
L	138	69	133	66.5		–	–
M	62	31	67	33.5	1.212 (0.74–1.71)	0.593	

χ^2 test; *p < 0.05; **p < 0.01; *** p < 0.001; T2DM – type 2 diabetes mellitus; CAT – catalase; OR – odds ratio; 95% CI – 95% confidence interval.

Table 4. Genotype comparison in terms of PON 55 L/M polymorphism (A) and CAT -262 C/T polymorphism (B)

Parameters	A. PON 55 L/M polymorphism			B. CAT -262 C/T polymorphism		
	genotype	T2DM group (n = 100)	control group (n = 100)	genotype	T2DM group (n = 100)	control group (n = 100)
		p-value	p-value		p-value	p-value
MDA [nmol/L]	LL-LM	0.122	0.736	CC-CT	0.962	0.780
	LL-MM	0.204	0.428	CC-TT	0.743	0.702
	LM-MM	0.944	0.407	CT-TT	0.602	0.931
CAT [U/L]	LL-LM	0.609	0.950	CC-CT	0.259	0.857
	LL-MM	0.237	1.000	CC-TT	0.492	0.618
	LM-MM	0.408	1.000	CT-TT	0.017*	0.348
PON [U/L]	LL-LM	0.184	0.646	CC-CT	0.195	0.522
	LL-MM	0.0001***	0.0001***	CC-TT	0.116	0.359
	LM-MM	0.0001***	0.0001***	CT-TT	0.546	0.634

Mann-Whitney U test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; T2DM – type 2 diabetes mellitus; MDA – malondialdehyde; CAT – catalase; PON – paraoxonase.

level was observed to be significantly higher ($p = 0.0001$ for LL and LM genotypes, $p = 0.002$ for MM genotype, respectively), while CAT ($p = 0.0001$ for LL and LM genotypes, $p = 0.001$ for MM genotype, respectively) and PON1 ($p = 0.0001$ for all genotypes) enzyme activities were observed to be significantly lower in patient group compared to control (Fig. 1).

No significant differences were observed regarding CAT -262 C/T polymorphism between genotypes in the control group ($p > 0.05$). However, CAT activity was seen to be significantly lower in TT genotype compared to CT genotype in the T2DM group ($p = 0.017$) (Table 4B). Moreover, in all genotypes, MDA level was observed to be significantly higher ($p = 0.001$ for CC genotype, $p = 0.0001$ for CT and TT genotypes, respectively), whereas CAT ($p = 0.004$ for CC genotype, $p = 0.0001$ for CT and TT genotypes, respectively) and PON1 ($p = 0.001$ for CC genotype, $p = 0.0001$ for CT and TT genotypes, respectively) enzyme activities were observed to be significantly lower in the T2DM compared to the control group (Fig. 2).

Discussion

Diabetes mellitus has a very high incidence of 10.9% in countries of the Middle East and North Africa, including Turkey as well. The incidence in Turkey is reported to be 14.85% and it constitutes a major health problem.¹⁶ According to the report by International Diabetes Federation (IDF) from 2015, there are 415 million diabetic patients in the world and it is estimated that this figure will reach 642 million in 2040.¹⁷ Diabetes is an oxidative stress state, in which free radicals are increased and/or antioxidant mechanisms are inhibited. The production of free radicals increases as a result of protein glycation and glucose autoxidation.⁶ Oxidative stress causes bodily complications such as nephropathy, cardiovascular diseases and neuropathy, all of which decrease the quality of life.⁹

Although the genetic predisposition is recognized in DM, the information on the specific genetic defects is limited.⁶ Therefore, we aimed to examine the oxidative stress state in T2DM by evaluating antioxidant enzyme activities and its association with their polymorphisms in our geographical region. There are 2 reasons why we carry out our study in the southeast region of our country. The population living in this region does not have the habit of eating a Mediterranean type diet and has of a different ethnic structure compared to some other regions of our country.

Malondialdehyde has been studied as a lipid oxidation indicator. We have found significantly high levels of MDA in diabetic patients compared to the control group, similarly to the previous studies, confirming the presence of severe oxidative stress in our patients.^{18,19} Likewise, the enzyme activities of CAT and PON1 were found to be significantly reduced in diabetic patients compared to the control group. Our enzymatic activity results are consistent with most recent studies.^{20,21} Unlike our study, Sozmen et al. and Memisogullari et al. reported increased CAT enzyme activity in diabetic patients.^{22,23} The impact of environmental factors (climate, air pollution, nutrition, lifestyle, etc.) and genetic background on DM development is indisputable. For example, Sozmen et al. carried out their study in a different region of Turkey in terms of eating habits (where the Mediterranean-type eating habit is preferred) and ethnicity, compared to our study (Adiyaman region). This may be the reason for the different results we have achieved regarding CAT enzyme activity. Therefore, the role of antioxidant defense mechanism in diabetic patients is controversial and requires further research, in which genetic background may be at least partly elucidated. Our study results may suggest that oxidative stress has developed in our patients and the antioxidant mechanism has become insufficient.

The issue of the effect of antioxidant enzyme polymorphisms on DM is unclear and controversial. Flekac et al. studied genetic polymorphisms of SOD and CAT

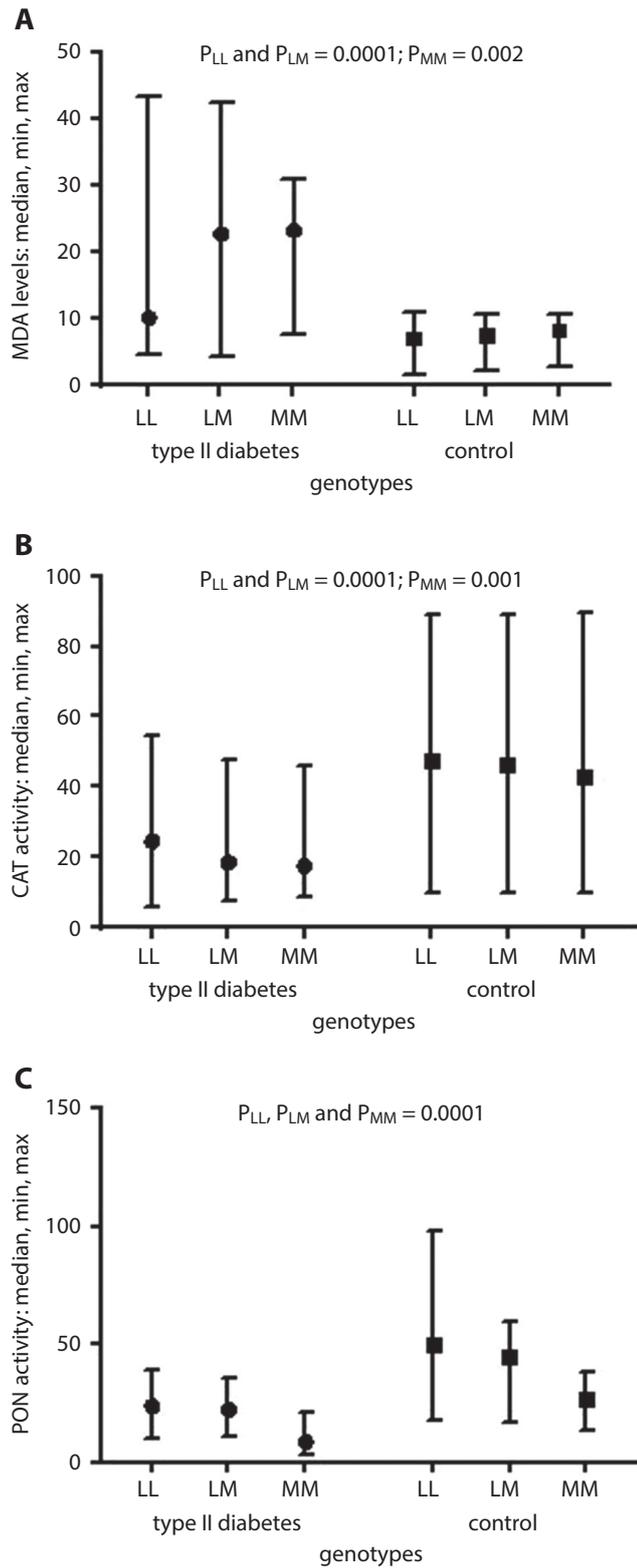


Fig. 1. PON 55 L/M polymorphism genotype comparison in terms of MDA level (A), and CAT (B), PON (C) enzyme activities between controls (circle) and patients (square) with T2DM

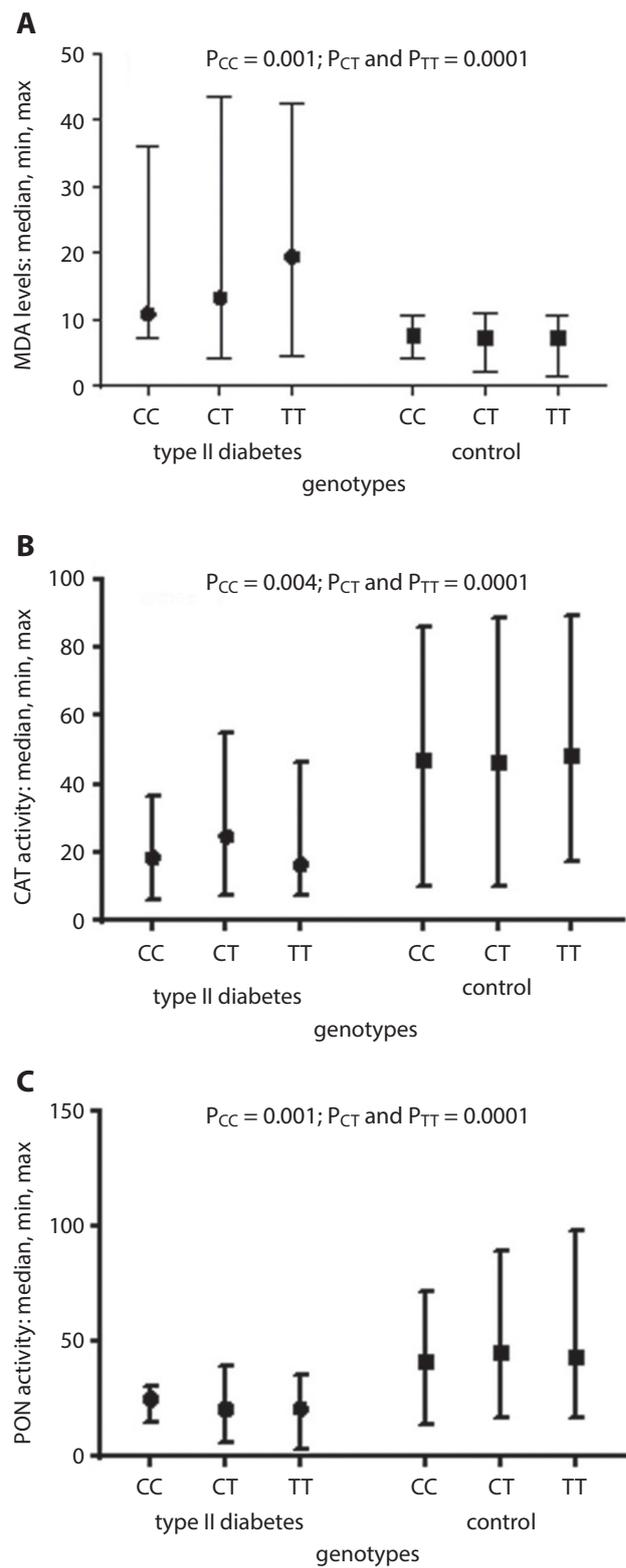


Fig. 2. CAT -262 C/T polymorphism genotype comparison in terms of MDA level (A) and CAT (B), PON (C) enzyme activities between controls (circle) and patients (square) with T2DM

in patients with DM, concluding that the superoxide dismutase (SOD) activity was higher in the CC and AA genotypes compared to the TT and CC genotypes of SOD Ala-9Val and SOD +35 A/C polymorphisms, respectively

($p < 0.05$). Moreover, they found significant differences regarding allele frequencies of SOD Ala-9Val polymorphism between control and diabetic groups. T allele expression level was observed to be significantly higher, whereas

C allele expression was significantly lower in the diabetic group compared to the control ($p < 0.05$). However, they did not observe any association with CAT polymorphism.²⁴ Kasznicki et al. did not observe any significant change in CAT and SOD enzyme activities between T2DM patients and controls as well as any association between their polymorphisms (CAT -262 C/T and SOD +35 A/C) and activity of these polymorphisms.²⁰ In our study, CAT activity was found to be significantly lower in TT genotype, which is in line with previous study performed by Flekac et al. compared to CT genotype in diabetic group, which suggests a significant association. CAT -262 C/T polymorphism may be at least partly responsible for the reduction of CAT enzyme activity which mediates oxidative stress state and leads to severe clinic situation in DM (secondary effect of DM developing over time such as frequent urination, fatigue, tiredness, blurred vision, frequent infections, itching and dryness of the skin, loss of weight, micro- and macrovascular damage which reduce quality of life over time). Moreover, in all genotypes of all polymorphisms examined in our study, CAT activity was observed to be significantly lower in the diabetic group when compared to the control. Any significant difference was not observed in terms of genetic distribution and allele frequencies of CAT -262 C/T between the diabetic and the control group. Tarnai et al. observed an elevation in the catalase enzyme activity in CT+TT genotype of C111T polymorphism in patients with T2DM.²⁵ Therefore, the relation between DM and polymorphisms of CAT and SOD is considered controversial because of previous studies with different results. There are studies in which the relationship between polymorphisms and enzyme activity has been shown,^{25,26} as well as studies in which such relationship was not found.^{27,28}

Gupta et al. studied serum PON1 activity and its different polymorphisms in T2DM patients in an ethnic population in northwestern India. They found significant reductions in the enzyme activity compared to the control group, similarly to our findings. Moreover, PON enzyme activity was reported to be significantly lower in all genotypes of diabetic patients compared to control group in terms of both PON1 192 Q/R and PON1 55 L/M polymorphisms. In addition, the enzyme activity was found to be highest in the RR genotype, whereas it was the lowest in the QQ genotype in both diabetic patients and controls.²⁹ Gupta et al. observed a higher enzyme activity in LL genotype compared to the LM genotype in the control group. However, they did not find a significant difference in the diabetic group.²⁹ Altuner et al. studied PON1 polymorphisms in the middle geographical region of Turkey (in Ankara). They reported reduced activities of PON1 in QQ and QR genotypes compared to RR genotype in terms of PON1 192 Q/R polymorphism both in control and diabetic groups. Similarly, they reported higher PON1 enzyme activity in LL genotype compared to MM in terms of PON1 55 L/M polymorphism in both diabetic and control groups, which

is in line with our results. They did not report any significant difference between the control and diabetic groups in terms of genotype distributions and allele frequencies.³⁰

In our study, PON1 enzyme activity was observed to be lower in MM genotype compared to LL genotype in terms of PON1 55 L/M polymorphism in both the T2DM and the control groups, which was in line with a previous study carried out in Turkey by Altuner et al.³⁰ In addition, we observed significantly lower PON1 enzyme activity in all genotypes in the T2DM group compared to the controls, which was in line a study performed by Gupta et al., whereas Altuner et al. did not report any significant difference between the groups in terms of genotype distributions and allele frequencies.^{29,30} As can be seen, there are similarities and differences between our study results and previous studies. This can be explained by the fact that different studies were performed in regions with different environmental conditions (climate, air pollution, nutrition, lifestyle, etc.) and in study populations with different genetic backgrounds and ethnicities. However, when the current studies are evaluated, PON1 55 L/M polymorphism does not seem to have a significant effect on the development of T2DM.

Conclusions

In this study, we concluded that there was a significant state of oxidative stress in diabetic patients. In addition, CAT enzyme activity was observed to be lower in TT genotype within the diabetic group, which suggests an association between CAT -262 C/T polymorphism and T2DM. Therefore, genetic testing in T2DM may be useful in the future to determine the relationship between genetic and environmental factors in development of the disease and its complications as well as the genetic predisposition, which requires further studies to confirm.

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