

Paraoxonase Gene Polymorphism and Activity in Type 2 Diabetes Mellitus with Microvascular Complications

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Abstract: Paraoxonase (PON1) is an antioxidant enzyme closely associated with HDL – cholesterol that protects LDL – cholesterol against oxidation. Less protection may therefore be supposed by decreased PON1 activity in diabetes mellitus (DM) patients. This study was undertaken to evaluate the association of PON1 gene polymorphism with diabetic nephropathy and the relationship of allelic polymorphism with PON1 activity in DM patients. The study was conducted on 36 patients with type 2 DM complicated with nephropathy, 24 patients without nephropathy, and 20 healthy subjects of matched age and sex to serve as control. Eight ml over night fasting venous blood were collected from every patient and control, distributed as such ; 5 ml in plain tube to separate serum for estimation of total cholesterol, triglyceride , LDL , HDL , glucose and PON1 activity, and 3 ml on EDTA vacutainer tube for estimation of glycated Hb (HbA1c) and PON1 genotyping. Obtained Results revealed that no gender or age influence was found on PON1 activity. Serum PON1 activity was significantly decreased in diabetic patients as compared with control. Also PON1 activity was significantly decreased in diabetic patients with nephropathy as compared with diabetic patients without nephropathy. The PON1 (55) LL genotype was the most frequent in healthy subjects, followed by the MM genotype, and then the LM genotype. In diabetic patients with nephropathy, the MM genotype was the most common, followed by the LL genotype, and then the LM genotype. In diabetic patients without nephropathy, the LL, MM, LM genotypes frequencies were 37.5%, 37.5%, and 25% respectively. The PON1 (192) QQ genotype was the most frequent in healthy subjects, followed by the RR genotype, and then the QR genotype. In diabetic patients with nephropathy, the RR genotype was the most common, followed by the QR genotype, and lastly the QQ genotype. In diabetic patients without nephropathy, the RR genotype was the most common, followed by the QQ genotype, and lastly the QR genotype. The PON1 activity in relation to genotyping showed insignificant difference in genotype LL, MM, LM, QQ, QR, and RR. Higher PON1 activity was found in diabetic patients with nephropathy, genotype LL than LM, and MM. In diabetic patients without nephropathy, the PON1(55)LL genotype showed significant increase in PON1 activity than MM and LM genotype. In diabetic patients without nephropathy, PON1 (192) higher activity was found in QQ, followed by RR, and lastly QR genotypes. In diabetic patients with nephropathy significant higher activity of PON1 was found in genotypes RR as compared with QQ, and QR genotypes.

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1. Introduction

Diabetes mellitus is a group of metabolic diseases (metabolic syndrome) caused by a complex interaction of genetic, immunological and environmental factors, and characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia is associated with long term damage, dysfunction and failure of various organs especially the eyes, kidneys, nerves, heart and blood vessels (ADA 2006). Diabetic nephropathy is one of the most serious complications in type 2 DM and the leading cause of end-stage renal disease (ESRD) in developed countries and associated with increased morbidity and mortality. The prevalence of diabetic nephropathy is projected to rise in the future as the

incidence of diabetes increases and the age of onset declines (ADA 2006).

Diabetic nephropathy has become the second most common leading cause of ESRD in Egypt after hypertensive nephropathy (Hassan et al., 2007).

Mechanisms underlying the development of diabetic nephropathy are complex. The main risk factors for the frequency, severity and progression of diabetic nephropathy include hyperglycaemia, hypertension, diabetes duration, age at onset, protein overload and smoking (Adler et al., 2003). There is also evidence to suggest that some individuals with diabetes have a genetic predisposition to diabetic nephropathy (Roman et al., 2004).

Human serum paraoxonase (PON1, EC3.1.8.1) a 43-kDa protein, catalyses the hydrolysis of

organophosphate esters, aromatic carboxylic acid esters and carbamates (**Li et al., 2005**).

Paraoxonase takes its name from the ability to hydrolyze paraoxon (PO), the highly toxic metabolite of the insecticide parathion (**Goldmacher et al., 2008**).

PON1 is a member of a family of proteins that also includes PON2 and PON3, the genes for which are clustered in a tandem on the long arm of human chromosome 7(q^{21,22}). PON2 is not present at all in human serum, while PON3 is present in very low levels (**Draganov and Ladu, 2004**).

PON1 is synthesized in the liver and is mainly associated with high-density lipoprotein (HDL). The enzyme decreases accumulation of the lipid peroxides in low density lipoprotein (LDL) due to its ability to reduce hydroperoxides and attenuates biological effects of mildly oxidized LDL (**Kinumi et al., 2005**).

In a given population, plasma PON1 activity can vary by more than 40-fold, and PON1 protein levels vary by more than 13-fold, raising the possibility of the PON1 polymorphism. This was elucidated once human PON1 DNA was isolated and sequenced (**Costa et al., 2003**).

A single nucleotide polymorphism in the coding region which was found to cause variability in the amino acid present at position 192, where either glutamine (Q) or arginine could be present. Another polymorphism was identified in the coding region that results in amino acid substitution at codon 55 where either leucine (L) or methionine (M) could be present (**Humbert et al., 1993**).

PON1 192 Q allele form is more efficient at metabolizing lipid peroxides (**Aviram et al., 2007**).

PON1 activity was found to be decreased in cardiovascular disease and in diabetes mellitus (**Karabina et al., 2005**). Several factors may take part in these changes. Firstly, oxidative stress is accelerated and thus lipid peroxidation may contribute to vascular wall impairment (**Gross et al., 2003**). Secondly, glycation of proteins including enzymes may decrease their activities in diabetes (**Kalousova et al., 2005**).

The present study was designed to evaluate serum paraoxonase activity and paraoxonase gene polymorphism in type 2 diabetic patients with and without microvascular complications (diabetic nephropathy).

2. Subjects and Methods:

The present study was conducted on 60 subjects with type 2 DM. 36 patients with microangiopathy (diabetic nephropathy), and 24 without diabetic nephropathy. Twenty apparently healthy subjects of matched age and sex were also included in the study.

Written consents were taken from all patients and control before enrollment in the study. The study was approved by The Ethical Committee of Research of Tanta University.

Diabetes and diabetic complications were defined according to the WHO criteria (**WHO, 1999**).

Patients were classified as having diabetic nephropathy if they had two out of three successive urine analysis of albumin/creatinine ratio >0.2 (normal <0.01, microalbuminuria 0.02-0.2, nephropathy >0.2).

Microangiopathy was confirmed by ophthalmoscopy or the present of peripheral neuropathy.

Exclusion criteria:

patients with macrovascular complications as IHD, or ischemic diseases of the lower limbs, or previous history of cerebral stroke

Sampling:

8 ml venous blood samples were collected after an overnight fast of 12-14 hours, distributed as such: 5 ml put in plain tube for serum separation and estimation of cholesterol, LDL, HDL, triglycerides and PON1 activity. 3ml put in EDTA tube for determination of glycosylated Hb and PON1 genotypes.

Serum cholesterol, HDL-C, triglycerides and fasting blood glucose were assayed on automated analyzer Hitachi 917 (Boehringer Mannheim Gm-bH, Mannheim) Germany; Commercial Kits were supplied by Roche diagnostics (Mannheim Germany). LDL cholesterol was calculated by the Friedewald formula (**Friedewald et al., 1972**).

Glycosylated Hb was measured by column chromatography. Albumin in urine was measured by an immunonephelometric method (**Rowe et al., 1990**). Reagents were supplied by Dade Behring Marburg. Paraoxonase (PON1) activity was measured spectrophotometrically (**Furlong et al., 1989**).

Serum was pre-incubated with 5×10^6 mol/L serine (Sigma-Aldrich) for 10 minutes at room temperature to inhibit serum butyrylcholinesterase activity, which is markedly elevated in diabetes and interferes with determination of paraoxonase activity. Paraoxonase activity was measured by adding 10 μ l of serum to 1ml tris/HCl buffer (100 mmol/L, pH 8.0, sigma-Aldrich) containing 2 mmol/L CaCl₂ and 5.5 mmol/L paraoxon (O, O-diethyl-O-P-nitrophenyl phosphate; sigma chemicals Co, USA). The rate of p-nitrophenol generation was determined on spectrophotometer RA50 (Bayer, USA) at 405 nm at temp 25°C and the paraoxonase activity was expressed in nmol/min/ml.

PON1 genotype determination:

Genomic DNA was extracted following the manufacture instructions of QIA amp DNA blood kit (Qiagen, Germany). Extracted DNA concentration was determined spectrophotometrically at 280nm. The genotypes of both point mutations (192 and 55) were determined by RFLP-PCR. The sequences of forward and reverse primers for both polymorphisms were:

restriction endonuclease

PON1-192F:5'-biotin-

CTATTTTCTTGACCCCTACTTA-3'

PON1-192R:5'-

CGCTAAACCCAAATACATCTCTCC-3' ^{384bp}
hsp92II (CATG)

PON1-55F:5'-biotin-

CTGACAGAAACTGGCTCTGAAG-3'

PON1-55R:5'-AAGCCAGTCCATTAGGTAGTAT-
3' ^{150bp A1wI(GGATC(N)4)}

As regard the genotypes of PON1 Q 192 R polymorphism, individuals homozygous for the Q allele had 150bp fragment, and those homozygous for the R allele had 89 and 61 bp fragments.

As regard the genotypes of PON1 L55 M polymorphism, individuals homozygous for the L allele had only 384bp fragment, and those homozygous for the M allele had 282 bp and 102 bp fragments.

Statistical analysis:

The SPSS 10.0 for windows was used for data management and analysis and the Microsoft power point for charts. Quantitative data were presented as mean±SD. For comparison of the two means, the student's t-test was used, while for the comparison of more than two means, one way analysis of variance (ANOVA) was used followed by Post Hoc test. Qualitative data were expressed as frequency and percentage P value was considered significant at 0.05 (Knapp, 1992).

3. Results**PON1 activity in controls, diabetics with nephropathy and diabetics without nephropathy**

- As shown in table (1), serum PON1 activity was significantly ($P < 0.001$) decreased in DM patients (mean 355 ± 79.63) as compared with control (mean 682.5 ± 91.60).
- As shown in table (2), also PON1 activity was significantly ($P < 0.001$) reduced in diabetics with

nephropathy (mean 177.8 ± 54.20) as compared with diabetics without nephropathy (mean 620.8 ± 85.64).

As shown in table (3), no gender or age influence on PON1 activity was found in healthy controls and in diabetics with and without nephropathy.

The effect of PON1 (55) gene polymorphism on PON1 activity in healthy controls and diabetics: As shown in table (4);

- The PON1 activity in control group showed insignificant difference in genotype LL (mean 706 ± 89.56), genotype MM (mean 661.7 ± 81.34) and genotype LM (mean 655 ± 78.56).
- Higher PON1 activity was found in diabetics with nephropathy genotype LL (mean 211.2 ± 63.3) than in LM genotype (mean 162.8 ± 54.35) and MM genotype (mean 156.4 ± 51.86) ($P < 0.05$).
- In diabetics without nephropathy the mean PON1 activity in different genotypes was (697.5 ± 84.62) for LL genotype and (578.3 ± 74.56) for LM genotype and (536.7 ± 68.23) for MM genotype ($P < 0.05$).

The effect of PON1 (192) gene polymorphism on PON1 activity in healthy controls and diabetics: As shown in table (4);

- The PON1 activity in control group showed insignificant difference in genotype RR, QQ and QR where the mean values were (682.5 ± 81.35), (693.8 ± 82.1), and ($660 + 80.63$) respectively.
- In diabetics without nephropathy, higher activity was found in QQ genotype (N:7) (mean 646.9 ± 79.8), and in RR genotype (N:10) (mean 644.4 ± 79.53), than in QR genotype (N:7) (mean 593.1 ± 76.54), but the difference is insignificant.
- In diabetics with nephropathy significant higher activity was found in RR genotype (mean 209 ± 56.42) as compared with QQ genotype (mean 153 ± 51.32) and QR genotype (mean 157.7 ± 52.4) with ($P < 0.05$).

As shown in table (5); there was a positive inverted correlation between PON1 activity and HAlc in healthy controls and diabetics with and without nephropathy

As shown in table (6); there was a positive correlation between PON1 activity and duration of diabetes in both studied groups (with and without nephropathy)

Table (1): PON1 activity in DM patients and controls

(nmol/mint/ml)	Control (20)	Diabetes (60)	P
PON1	682.5 ± 91.60	355 ± 79.63	< 0.001

Table (2): PON1 activity in diabetics with nephropathy as compared with diabetics without nephropathy (nmol/min/ml)

(nmol/min/ml)	DM without nephropathy	DM with nephropathy	P
PON1	620.8±85.64	177.8±54.20	<0.001

Table (3): The effect of gender on PON1 activity in controls, diabetics with nephropathy and diabetics without nephropathy

Gender	Control		DM with nephropathy		DM with out nephropathy	
	No	PON1	No	PON1	No	PON1
M	11	669.1±75.23	20	174.2±47.24	13	647.5±82.34
F	9	654.4±73.65	16	179.0±48.4	11	609.7±80.4

Table (4): PON1 activity in relation to genotypes in healthy controls and diabetics with and without nephropathy

genotype		Control (20)		DM with nephropathy (36)		DM with out nephropathy (24)	
		No	PON1	No	PON1	No	PON1
PON1 (192)	RR	8	682.5±81.35	15	209±56.42	10	644.4±79.53
	QQ	8	693.8±82.1	10	153±51.32	7	646.9±79.8
	QR	4	660±80.63	11	157.7±52.4	7	593.1±76.54
PON1 (55)	LL	10	706±89.56	13	211.2±63.3	11	697.5±84.62
	MM	6	661.7±81.34	14	156.4±51.86	7	536.7±68.23
	LM	4	655±78.56	9	162.8±54.35	6	578.3±74.56

Table 5: Correlation between PON1 activity and HA1c in healthy controls and diabetics with and without nephropathy

	Control		GI		GII	
	r.	p. value	r.	p. value	r.	p. value
HAIC & PON1 activity	0.142	0.632	0.425	0.011	0.403	0.035

Table 6: Correlation between PON1 activity and duration of diabetics in both studied groups (with and without nephropathy)

	GI		GII	
	r.	p. value	r.	p. value
PON1 activity & DM	0.369	0.016	0.425	0.020

4. Discussion

The molecular basis of the paraoxonase activity polymorphisms is a Messene mutation in the coding region of PON1 resulting in a glutamine (Q)/arginine (R) substitution at codon 192 (Humbert et al., 1993). Another coding region polymorphism, resulting in aminoacid substitution at position 55 Leu(L)/Met(M), has been associated with plasma

PON1 protein levels, with PON1M55 being associated with low plasma PON1 level. From the polymorphisms characterized in the promotor region, the C-108T substitution has the most significant effect on plasma PON1 levels, with the -107C allele providing levels of PON1 about twice as high as those seen with the -107T allele (Brophy et al., 2001).

Paraoxonase 1 (PON1) polymorphisms have been implicated as risk factors for

coronary artery disease, but the results of genetic association studies on the related phenotype of diabetic nephropathy are inconclusive (**ADA, 2006**).

The present study was designed to evaluate serum paraoxonase activity and the prevalence of paraoxonase gene polymorphism in type 2 diabetic patients with or without microvascular complications.

In the present work, diabetic patients with complications had higher age, longer duration of disease, increased HBA1C concentrations, increased LDL, triglycerides and albumin/creatinine ratio and decreased PON1 activity and HDL cholesterol. These results coincide with **Letellier et al., (2002)** who reported that, the type 2 diabetic patients had significantly elevated plasma triglycerides and low HDL cholesterol compared with the control subjects. In addition, Plasma PON1 activity was significantly decreased in the diabetic patients without vascular complications compared with the controls.

In present study we found significantly different proportion of allele distribution for coding region PON1-192 gene in type 2 diabetic patients with complications as compared with non-complicated diabetic patients or healthy subjects. Similar findings were reported by **Agachan et al., (2004)**.

In addition, the results of the present study proved that, there was statistically significant reduction in PON1 activity in diabetic patients in comparison to normal control subjects and in diabetic patients with complications as compared to those without complications. Similarly; **Flekac et al. (2008)** found statistically significant decrease in PON1 activity in patients with type 1 or type 2-diabetes as compared to normal control subjects. Similarly, **Karabina et al., (2005)** reported that, the PON1 activity significantly reduced in diabetic patients in comparison to healthy subjects.

Also; it was reported that paraoxonase activity decreased in patients with type-1 diabetes (insulin-dependent diabetes mellitus) or type 2 diabetes (**Mackness et al., 2004**).

Abbott et al., (1995) showed that paraoxonase activity, especially specific activity, in patients with diabetes was lower than that in controls independent of the A/B phenotype of the PON1 gene, indicating the 192Q/R polymorphism.

Lipoprotein abnormalities commonly present in type 2 diabetes, include hypertriglyceridemia and reduced plasma HDL cholesterol. In addition, low density lipoprotein (LDL) are converted to smaller, perhaps more atherogenic, lipoproteins termed small dense LDL (**Krauss, 1994**).

Low PON1 activity decreases the ability to

prevent lipid-peroxide formation with consequent acceleration of the oxidative stress. Overproduction of the reactive oxygen species in diabetic patients may be due to chronic hyperglycemia, hyperinsulinemia, elevated free fatty acids (FFA) and dyslipidemia (**Maritim et al., 2003**).

An early impairment of microvascular vasodilatory reserve is a prominent feature in the development of type 2 diabetes related vasculopathy. The defect appears to be endothelium dependent which may result from oxidative stress quenching the nitric oxide and neutralizing its many protective functions. Oxidative stress may also damage DNA, protein structure, and membrane properties (**Tooke, 2000**).

In diabetic patients, glycation of HDL or directly of PON1 in HDL may result in detachment of PON1 itself from the HDL and PON1 inactivation (**Karabina et al., 2005**).

PON1 is bound by HDL in lesser extent in diabetic patients as compared to healthy persons and its activity is then poorly stabilized (**Baum et al., 2006**).

Plasma lipids also modify composition, function and concentration of the HDL. Elevated plasma triglyceride-rich lipoproteins may substitute cholesterol esters in HDL by driving cholesterol ester transfer protein (CETP) with subsequent HDL depletion of cholesterol esters. As a result, both the conformation and function of HDL may be altered. PON1 on modified HDL may be disturbed to interact with substrates (**Bucala et al., 1994**).

In the present work, there was statistically significant proportional correlation between HDL and PON1 activity. This can be explained by the fact that PON1 is synthesized in the liver and is mainly associated with high-density lipoprotein (HDL) (**Tomas et al., 2004**). Thus reduced PNO1 activity in relation to decreased HDL-C can be explained.

Another explanation of this proportional correlation between HDL and PON1 activity could be the increased glycation of HDL, and its subsequent removal by the phagocytes in the subendothelium forming foamy cells, resulting in rapid progress to atherosclerotic vascular complications. The statistically significant inverse correlation between PON1 activity and HBA1C, and albumin/ creatinine ratio in our results is a good evidence of this explanation.

Similar results were reported by **Kordonouri et al., (2001)** who reported that, an inverse linear correlation was found between blood glucose levels and both serum concentrations of PON and its activity. Because oxidative stress may impair insulin action, reduced serum paraoxonase activity may contribute to insulin resistance, resulting in increased

HBA1C, cholesterol, LDL-C and triglycerides (**Abu Farha et al., 2010**).

In conclusion; data of the present study support the hypothesis that the Q/R192 gene polymorphism, rather than the L/M 55 is associated with diabetic status. The difference in allele frequency for this polymorphism between the diabetic patients and the controls may be the cause of the low paraoxonase activity found in type 2 diabetes mellitus.

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