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Effect of Paraoxonase Gene Polymorphisms on Paraoxonase Levels and Insulin Resistance Index in Women with Polycystic Ovary Syndrome

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Abstract: Paraoxonase1 (PON1) is an antioxidant high-density lipoprotein (HDL)-associated enzyme. Because oxidative stress may impair insulin action, decreased serum paraoxonase activity may contribute to explain the insulin resistance associated with polycystic ovary syndrome (PCOS). We evaluated the possible association of PON1 -108 C/T and L55M polymorphisms with PCOS and their influence on serum paraoxonase and insulin resistance index in randomly selected Egyptian women with PCOS. Ninety-four PCOS patients and 60 healthy age- and body mass index (BMI)- matched controls were genotyped for PON1 -108 C/T and L55M variants using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and were evaluated for markers of hyperandrogenism, Paraoxonase activity, glucose and insulin levels and calculation of insulin resistance by homeostasis model assessment. Serum paraoxonase activity was significantly reduced in the PCOS compared to control group (P= 0.003). Homozygosity for -108 T (P= <0.001) and for -55 M (*P* =0.035) alleles was more prevalent in PCOS patients than in controls. Both polymorphisms were associated with increased risk for PCOS; the OR for -108 TT and TC genotype was found to be 3.06, 95% CI = 1.44- 6.53 (P=0.002) and the OR for -55 MM and ML genotype was 2.23, 95% CI = 1.06- 4.72 (P=0.034). In PCOS patients and controls as a whole, women with -108 TT genotype (compared to carriers of –108C alleles) and women homozygous for 55M alleles (compared to carriers of the 55L allele) presented with reduced paraoxonase activity (P= 0.001 and P=0.054), higher BMI (P= 0.014 and P=0.006), inrceased fasting insulin (P= 0.018 and P=0.004) and increased fasting insulin resistance index (P= 0.037 and P=0.003) respectively. PON1 -108 C/T and L55M polymorphisms were found to be associated with PCOS. These polymorphisms are important determinant of serum paraoxonase activity and contribute to insulin resistance in women with PCOS.

Key words: Paraoxonase, Polycystic ovary syndrome, Polymorphism, Insulin resistance

INTRODUCTION

The polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women of fertile age (Carmina and Lobo, 1999). Insulin resistance with compensatory hyperinsulinemia and a metabolic profile similar to insulin resistance/metabolic syndrome are frequent metabolic abnormalities in PCOS that may further worsen the endocrine manifestations of hyperandrogenism and ovulatory dysfunction (Sam and Dunaif, 2003). Amelioration of insulin resistance by weight loss or by insulin-lowering drugs improves hyperandrogenism in PCOS women (San Millan *et al.*, 2004).

Serum paraoxonase-1 (PON1) is an antioxidant high-density lipoprotein (HDL)-associated enzyme encoded by the PON1 gene, which is mainly expressed in the liver (Bin Ali *et al.*, 2003). Because oxidative stress may impair insulin action (Rudich *et al.*, 1997), reduced serum PON1activity may contribute to explain the insulin resistance associated with PCOS (Dursun *et al.*, 2006). Reduced serum PON1activity has been found in other insulin-resistant disorders such as type 2 diabetes mellitus and cardiovascular atherosclerotic disease (Mackness *et al.*, 2004). The –108C/T polymorphism in PON1 is responsible for ~23% of PON1 expression levels in some cell systems, in which –108TT variant showed reduced PON1 expression compared with –108CC variant (Brophy *et al.*, 2001). San Millan *et al.* (2004) found that homozygosity for –108T alleles is more prevalent in PCOS patients compared with healthy controls and hypothesized that the resulting decrease in serum paraoxonase activity might result in a higher oxidative stress in women suffering from PCOS.

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A common polymorphism in the PON1-coding region is a leucine (L) to methionine (M) substitution at codon 55 (Humbert *et al.*, 1993). The PON1 -55 polymorphism does not affect the catalytic efficiency of substrate hydrolysis by the enzyme, but the PON1 -55M allele is correlated with decreased mRNA and protein levels (Brophy *et al.*, 2000). Previous studies suggested that the L55M polymorphism in PON1 was not associated with PCOS (San Millan *et al.*, 2004), but subjects homozygous for M55 alleles presented with a higher body mass index (BMI) and increased index of insulin resistance (Brophy *et al.*, 2001, Barbieri *et al.*, 2002 and San Millan *et al.*, 2004).

The aim of the present study was to investigate the possible association of -108C/T and L55M polymorphisms in the PON1 gene with the risk of PCOS and to evaluate the contribution of these two polymorphisms to serum paraoxonase activity and insulin resistance in Egyptian women with PCOS.

MATERIAL AND METHODS

Subjects:

This study was conducted on 94 PCOS patients (mean age 27.6 ± 4.8 years), with body mass index (BMI) = 31.4 ± 7.3 kg/m^2, they were recruited from those attending the endocrinology out patients clinic in Kasr El-Aini Hospital, Cairo University. PCOS was diagnosed by the presence of chronic anovulation, hyperandrogenemia, and exclusion of other common causes of hyperandrogenism (congenital adrenal hyperplasia and androgen-secreting tumors) in accordance with the criteria proposed in 1990 by the National Institutes of Health (NIH) and revised in 2003 (The Rotterdam ESHRE/ASRM Sponsored PCOS Consensus Workshop Group, 2004). Sixty healthy nonhyperandrogenic women with matched age and BMI were enrolled as controls. All the controls had normal fasting glucose concentrations and normal blood pressure. The patients and controls had not taken hormonal medications, including contraceptive pills for the last 6 months. Oral consent was obtained from each patient and control.

Sampling:

Blood samples were collected after a 12-14 h fast. Blood samples were immediately centrifuged, and serum was separated and frozen at -70°C until assayed for total testosterone, androstenedione, dehydroepiandrosterone-sulphate (DHEAS), a complete lipid profile, insulin and glucose levels and PON1 activity. EDTA samples were collected for the determination of PON1 Genotypes. DNA was stored at –20°C until analyzed.

Biochemical and Hormonal Analysis:

Glucose, triglycerides, total cholesterol and HDL were assayed on automated analyzer Hitachi 917; commercial kits were supplied by Roche diagnostics (Boehringer Mannheim, GmbH D-68298, Mannheim, Germany). The LDL level was calculated using the Friedewald equation.

Serum concentrations of testosterone, androstenedione, prolactin and DHEAS were measured by chemiluminescent enzyme immunoassay (Immulite 2000; Diagnostic Products Corporation, Los Angeles, CA, USA). The free testosterone concentration was calculated from total testosterone and sex hormone-binding globulin concentrations (Vermeulen *et al.*, 1999).

Insulin concentration was measured on Elecsys analyzer (reagents supplied by Roche Diagnostics, Mannheim, Germany) (Clark, 1999).

Calculation of insulin resistance by homeostasis model assessment (HOMA-IR), was estimated as fasting insulin concentration (µU/mL) x fasting glucose concentration (mg/dl) /405 (Yokoyama *et al.*, 2003).

PON1 activity was assessed by the rate of enzymatic hydrolysis of paraoxon (Sigma Chemical Co.) to *p*-nitrophenol. The amount of *p*-nitrophenol generated was monitored with a continuously recording spectrophotometer by the increase in absorbance at 405 nm and 25°C (Gan *et al.*, 1991).

Determination Of PON1 Genotypes:

Genomic DNA extraction was performed using the standard Salting out technique (Trowsdale, 1993). PON1 -108 C/T and L55M genotyping was conducted by polymerase chain reaction -restriction fragment length polymorphism (PCR-RFLP) method.

- The –108C/T polymorphisms were determined according to Brophy *et al.* (2000) using the following primers: forward, 5'GACCGCAAGCCACGCCTTCTGTGCACC-3'; reverse, 5'TATATTTAATTGCAGCCGCAGCCCTGCTGGGGCAGCGCCGATTGGCCCGCCGC-3'. The product was digested with BstUI (New England BioLabs Inc.), and the digestion products were resolved by electrophoresis in a 3% agarose gel.

- The L55M polymorphisms were determined following a protocol developed by Humbert *et al.*, (1993) using the following primers: forward,

5'-GAAGAGTGATGTTATAGCCCCAG-3'; reverse,5'ACTCACAGAGCTAATGAAAGCCA -3'. The product was digested with NlaIII (New England BioLabs), and the digestion products were resolved by electrophoresis in a 5% agarose gel.

Statistical Analysis:

Results were analyzed using SPSS Statistical Package (version 10). Numeric data were summarized as means and standard deviations, categorical measurements were summarized as percentages. Odds ratio and their 95% confidence intervals were calculated for all potential risk factors. Comparison between categorical measurements was done using the chi--square test .P-values <0.05 were considered significant.

***Results*:**

Clinical and biochemical variables in PCOS patients and controls are shown in Table (1). As compared with controls, PCOS patients had significantly increased total and free testosterone, androstenedione and DHEAS concentrations, fasting insulin level and HOMA value. The mean serum PON1 activity was significantly lower in the PCOS group compared to the controls (P=0.003). The lipid profile and fasting glucose level were comparable between the two groups.

Distribution of the -108 C/T and L55M Polymorphisms of the PON1 Gene:

PCOS patients were more frequently homozygous for the –108 T variant (PCOS=39.4% *vs*. controls=10%) (P= <0.001) and homozygous for -55 M variant (PCOS=39.5% *vs*. controls=21.6%) (*P* =0.035) compared with healthy controls (Table 2).

Both polymorphisms were associated with increased risk for PCOS; the OR for -108 TT and TC genotype was found to be 3.06, 95% CI = 1.44- 6.53 (P=0.002) and the OR for -55 MM and ML genotype was 2.23, 95% CI = 1.06- 4.72, (P=<0.001). In PCOS patients and controls as a whole, women with -108 TT genotype (compared to carriers of –108C allele) and women homozygous for 55M allele (compared to carriers of the 55L allele) presented with reduced paraoxonase activity, increased markers of hyperandrogenism (hirsutism scores and total testosterone, free testosterone and androstenedione concentrations), higher BMI and fasting insulin and increased insulin resistance index (Table 3).

Table 1: Clinical and biochemical variables in PCOS patients and controls.

Variable	PCOS (n = 94)	Controls (n = 60)	*P*
Age (years)	27.6 ± 4.8	29.4 ± 4.9	NS
BMI (kg/m^2)	31.4±7.3	29.8±6.5	NS
Hirsutism score	14.1 ± 6.9	2.4 ± 1.8	<0.001
Total testosterone (ng/dl)	68 ± 29	40 ± 23	<0.001
Free testosterone (ng/dl)	1.5 ± 0.4	0.7 ± 0.2	<0.001
DHEAS (µg/dl)	232.3 ± 13.9	162.0 ± 76.9	<0.001
Basal androstenedione (ng/dl)	430 ± 180	210 ± 113	<0.001
Fasting glucose (mg/dl)	89 ± 14	87 ± 16	NS
Fasting insulin (µU/ml)	18 ± 6	13 ± 5	<0.001
HOMA	3.7 ± 2.4	2.4 ± 1.8	<0.001
Total cholesterol (mg/dL)	179 ± 45	171 ± 50	NS
HDL cholesterol (mg/dL)	43 ± 18	45± 13	NS
LDL cholesterol (mg/dL)	157 ± 42	149 ± 29	NS
Triglycerides (mg/dL)	96 ± 41	89± 36	NS
PON1(U/L)	182 ± 107	239 ± 126	0.003

BMI: body mass index, DHEAS: Dehydroepiandrosterone sulfate, HOMA: homeostasis model assessment, PON1: paraoxonase, NS: non significant Values are means ± SD

Table 2: PON1 -108 C/T and L55M genotype frequencies in PCOS and controls

Genotype		PCOS (n=94) Frequency (%)	Controls(n=60) Frequency (%)	P
-108 C/T	TT	37 (39.4%)	6 (10 %)	<0.001
	CT	35 (37.2%)	25 (41.7%)	0.703
	CC	22 (23.4%)	29 (48.3%)	0.002
	TT+CT	72(76.6%)	31(51.7%)	0.002
L55M	MM	37(39.5%)	13(21.6 %)	0.035
	LM	33 (35.0%)	21 (35.0 %)	0.873
	LL	24(25.5%)	26 (43.4 %)	0.034
	MM+LM	70(74.5%)	34(56.6%)	0.034

Table 3: Clinical and biochemical characteristics in all participants according to PON1 -108 C/T and L55M genotypes.

Parameters	-108 C/T polymorphism			-55L/M polymorphism		
	CC+CT genotype	TT genotype	*P*	LL + LM genotypes	MM genotype	*P*
Number of women	111	43		117	37	
BMI (kg/m^2)	28.2 ±7.7	31.7±8.1	0.014	28.7±6.8	32.6±9.1	0.01
Total testosterone(ng/dl)	49 ± 27	70±41	<0.001.	47.7 ±22	64.3±28	0.01
Free testosterone (ng/dl)	0.8 ± 0.7	1.6±0.9	<0.001	0.9 ± 0.5	1.5 ± 0.9	0.04
Androstenedione (ng/dl)	343±129	456±180	<0.001	321±120	437±154	0.011
Fasting glucose (mg/dl)	89 ± 17	93±12	0.16	87±10	91±14	0.161
Fasting insulin (µU/ml)	13±6	16±9	0.018	14±7	18±8	<0.004
HOMA	2.7±1.9	3.5±2.6	0.037	2.8±1.8	3.9 ±2.2	0.003
PON1 (U/L)	244±116	178±83	0.001	225±118	183±103	0.045

Values are means ± SD

Discussion:

The number of genes associated with PCOS is increasing rapidly, suggesting that PCOS may result from the interaction of multiple genomic variants with environmental factors such as obesity and a sedentary lifestyle (San Millan *et al.*, 2004). PCOS is currently considered to be a part of the metabolic syndrome (Dursun *et al.*, 2006), so genomic variants associated with the metabolic syndrome should be considered candidate genes to explain PCOS inheritance (Legro *et al.*, 2002 and Yildiz *et al.*, 2003). The PON1 gene is expressed mainly in the liver and encodes for serum paraoxonase, which is an antioxidant high-density lipoprotein-associated enzyme. Liver PON1 mRNA expression is affected by genetic and environmental factors, and both androgens and proinflammatory mediators decrease liver PON1 expression (Bin Ali *et al.*, 2003). Of interest, both androgen excess and proinflammatory genotypes contribute to the pathogenesis of PCOS (Peral *et al.*, 2002 and Escobar-Morreale *et al.*, 2003).

In the present study we evaluated whether -108 C/T and L55M polymorphisms at PON1 gene are associated with the risk for PCOS and whether the two polymorphisms are associated with variations in serum paraoxonase activity and insulin resistance indexes. Our results showed that -108 TT and -55 M M variants were found to be more prevalent in PCOS in comparison with controls and were associated with increased risk for PCOS development. In agreement with our results, association of homozygosity for –108T alleles with PCOS was found in previous Spanish (San Millan *et al.*, 2004 and San Millán *et al.*, 2006) and German studies (Knebel *et al.*, 2009). On the other hand, San Millan *et al.* (2004) found no differences in the distributions of 55 L > M polymorphism between Spanish women with PCOS and controls.

As expected from their association with PCOS, -108 TT and -55MM genotypes presented with increased markers of hyperandrogenism (hirsutism scores and total testosterone, free testosterone, and androstenedione concentrations). In our work the PCOS group presented with reduced paraoxonase activity as compared to the controls. In accordance with our findings, reduced serum paraoxonase in PCOS patients was reported by Dursun *et al.* (2006) and Fencki *et al.* (2007). On the contrary, the serum paraoxonase activities of the PCOS and control groups were not actually different in the study of San Millan *et al.* (2006). Our results might be explained by the hypothesis that the association of homozygosity for –108T alleles with PCOS together with hyperandrogenism and proinflammatory genotypes, might contribute to reduced PON1 expression in PCOS patients, possibly resulting in a higher oxidative stress in these women (San Millan *et al.*, 2004). In the present work we found that -108 TT (as compared to carriers of –108C allele) and - 55MM (as compared to carriers of the 55L allele) genotypes were associated with reduced paraoxonase activity in PCOS and controls. Similarly, San Millan *et al.* (2006) found that women homozygous for –108T alleles of PON1 present with reduced serum paraoxonase activities irrespective of the presence or absence of PCOS. However, the PON1–L55M polymorphism was not investigated. According to Brophy *et al.* (2000) individuals with PON1-55MM show, on average, a lower level of enzyme activity than do individuals with PON1-55 LL. The difference in expression level has been suggested to be due to linkage to other elements, such as regulatory-region polymorphisms (i.e., –108T) associated with lower PON1 expression (Leviev and James, 2000 and Victoria *et al.*, 2001).

Our results showed that -108 TT and -55MM genotypes were associated with higher BMI and increased index of insulin resistance in PCOS patients and controls as a whole. Increased BMI and index of insulin resistance in subjects homozygous for M55 alleles were suggested by Barbieri *et al.* (2002), Deakin *et al.*, (2002) and San Millan *et al.* (2004). Considering that oxidative stress may impair insulin action (Rudich *et al.*, 1997) and induces chronic inflammation (Ceriello and Motz, 2004), which further contributes to insulin resistance, type 2 diabetes, atherosclerosis and cardiovascular disease (Fernandez-Real and Ricart, 2003), reduced serum PON1 activity may contribute to insulin resistance. This hypothesis is supported by the finding

of reduced serum paraoxonase activity in insulin-resistant disorders such as type 2 diabetes mellitus (Sakai *et al.*, 1998) the association of homozygosity for –108T alleles of PON1 with PCOS might contribute to explain the insulin resistance and the increased risk for atherosclerosis associated with this syndrome (Solomon, 1999). In conclusion, the PON1–108C/T and L55M polymorphisms play a causative role in the development of PCOS. These polymorphisms are important determinant of serum paraoxonase activity and contribute to insulin resistance in women with PCOS.

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