

Association of peroxonase-1 (PON-1) polymorphisms with glucose metabolism, insulin resistance, and reproductive hormones in Greek women with polycystic ovary syndrome.

Abstract: Association of paraoxonase-1 (PON-1) polymorphisms with glucose metabolism, insulin resistance and reproductive hormones, in Greek women with PCOS.

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Objective: To find the association of PON-1 polymorphisms with glucose metabolism, insulin resistance and reproductive hormones, in Greek women with PCOS.

Setting: Outpatient gynaecological clinic of Aretaieion University Hospital.

Patients: 87 women with PCOS were recruited for this study, the mean age of which was 23.91 \pm 4.78 years and the mean value of the BMI was 27.95 \pm 6.84.

Methods: Venous blood samples were obtained for genetic study (PCR-RFLP technique to examine the Leu55Met, Gln192Arg and -108C/T polymorphisms of PON-1 gene), hormonal profile, glucose and insulin assays, on days 3 to 7 from cycling patients.

We evaluated insulin resistance with homeostasis model assessment (HOMA) index.

The quantitative index of insulin sensitivity QUICKY was also calculated.

Finally, we calculated the free androgen index (FAI).

Data were analyzed with the Graph Pad in Stat and frequency and susceptibilities of mutations were compared by employing the chi-square test (χ^2 -test).

Results: The **Leu55Met polymorphism** in PON-1 gene was not found in our study.

Concerning the **Gln192Arg polymorphism**, we found that the fasting insulin levels and HOMA index, were significantly higher in women with the QR/RR genotype than those with the QQ genotype.

As regards the **-108C/T polymorphism in the promoter of PON-1 gene**, we found that the presence of the -108T allele results in higher levels of Testosterone and higher insulin resistance as found by the higher HOMA index.

Conclusions: The Gln192Arg polymorphism of PON-1 gene and even more the -108C/T polymorphism in the promoter of the gene, were found implicated in insulin resistance in PCOS women. However, both genotypes had only minor effect on reproductive hormones and BMI.

Introduction:

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, affecting approximately 4-7% of women of fertile age (1).

It is characterised by menstrual irregularity, ovarian and adrenal androgen overproduction (2), hirsutism and acne (3).

PCOS is also associated with an increased risk of impaired glucose tolerance and type 2 diabetes mellitus (4, 5).

The aetiology of PCOS is complex and is not yet resolved, but it is known that insulin resistance plays a basic role in the pathogenesis of the syndrome (6) and obesity is frequently found in these patients (7).

The increase in serum insulin levels resulting from insulin resistance facilitates androgen secretion from the ovaries and the adrenals in PCOS patients (6) and obesity worsens the insulin resistance of these women. Amelioration of insulin resistance by weight loss (7) or by insulin-lowering drugs (8) improves hyperandrogenism in PCOS women.

Familial aggregation provides evidence supporting a genetic basis for PCOS (9), but the precise genetic mechanisms are still unknown. It is also important, that hyperandrogenism and insulin resistance co segregate in families of PCOS patients (10, 11) suggesting a common genetic origin of these disorders.

Currently, PCOS is considered a polygenic trait that might result from the interaction of susceptible and protective genomic variants under the influence of environmental factors.

The genetic basis of the PCOS implicates several molecular defects and candidate genes like, genes involved, in biosynthesis and metabolism of androgens, in the secretion and action of insulin, in gonadotrophin action and regulation, in obesity and insulin resistance and finally genes involved in chronic inflammation.

Recent studies correlate the predisposition for PCOS with gene polymorphisms that affect insulin action (12, 13) and **peroxonase -1(PON-1) gene** was added in this steadily increasing list of candidate genes for PCOS pathogenesis (14, 15).

The PON gene has at least 3 members, PON1, PON2 and PON3, located in chromosome 7q21.3-22.1 (16).

Three polymorphisms in PON1 genes have been studied and have been correlated with PCOS or higher BMI and increased indexes of insulin resistance, namely, the -108C/T, Leu55Met and Gln192Arg (17, 18, 19, 20).

The PON1 gene is expressed mainly in the liver and encodes for serum paraoxonase, which is an antioxidant HDL-associated enzyme. Liver PON1 mRNA expression is influenced by genetic and environmental factors and both androgens

and proinflammatory mediators decrease liver PON1 expression (21), which also contributes to the pathogenesis of PCOS (22, 23, 24). Therefore, we thought that the above mentioned PON1 polymorphisms, hyperandrogenism and proinflammatory genotypes might contribute to reduced PON1 expression, resulting in a higher oxidative stress in these women.

Because oxidative stress may impair insulin action (25), reduced serum paraoxonase activity may contribute to insulin resistance and this is supported by the finding of reduced serum paraoxonase activity in insulin-resistant disorders such as type-2 diabetes mellitus (26, 27) and cardiovascular atherosclerotic disease (28, 29).

Since the diversity observed, in clinical and hormonal characteristics among women with PCOS is well-documented, the present study was undertaken to examine the relationship between the above mentioned polymorphisms in PON1 gene and the clinical and hormonal characteristics in Greek women with PCOS.

Subjects and methods

Subjects

Eighty-seven women with PCOS were consecutively recruited for this study in three outpatient teaching endocrinology and gynaecology clinics. Mean age was 23.91 ± 4.78 years. The biochemical and the hormonal characteristics of the patients are shown in Table 1. The diagnosis of PCOS in these patients was based on the criteria established at the 1990 PCOS conference (National Institutes of Health National Institute of Child Health and Development, Bethesda, Maryland) (Dunaif A, 1992). For at least 2 months before the study, women had not taken steroid preparations (including oral contraceptives) or medications known to alter insulin secretion and/or action. Exclusion criteria for the participation in the study included pregnancy, hypothyroidism, hyperprolactinemia, Cushing syndrome, and non-classical congenital adrenal hyperplasia. No patient had diabetes, or renal, neoplastic, metabolic, hepatic, cardiovascular, or malabsorptive disorders. Body mass index (BMI) was calculated for each patient (Table 1).

Study protocol

The procedures used in this study were in accordance with the guidelines of the Helsinki Declaration on human experimentation. The patients and parents of adolescent patients were informed about the study and gave their consent. The study was approved by the ethics committee of all three institutions and institutional review board approval was obtained.

At admission, venous blood samples were obtained for genetic study and for hormonal profile, glucose, and insulin assays, on days 3 to 7 from cycling patients. In case of amenorrhic patients blood samples were obtained after a progesterone-induced menstruation. All patients underwent an oral glucose tolerance test (OGTT). After an overnight 12-h fast, blood samples were obtained at 0 min. Glucose (75gr) was then administered orally, and blood samples were obtained at 30, 60, 90, and 120 min for measurement of glucose concentrations. On the same day, an abdominal ultrasound with a 5-MHz transducer was performed to assess the morphology and volume of the uterus and ovaries.

Hormone and Biochemical assays

LH was measured by Micro particle Enzyme Immunoassay (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 0.5 mIU/mL, and total coefficient variability (CV) of 5.2-10.0 %.

FSH was measured by Micro particle Enzyme Immunoassay (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 0.4 mIU/mL, and total coefficient variability (CV) of 5.1-10.1 %.

Prolactin was measured by Micro particle Enzyme Immunoassay (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 0.6 mIU/mL, and total coefficient variability (CV) of 3.4-6.3 %.

TSH was measured by Micro particle Enzyme Immunoassay (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 0.03 mIU/mL, and total coefficient variability (CV) of 5.1-10.3 %.

Testosterone was measured by Micro particle Enzyme Immunoassay (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 0.1 ng/mL, and total coefficient variability (CV) of 6.0-13.7 %.

DHEA-S was measured by solid phase, competitive chemiluminescent immunoassay (Diagnostic Products Corporation (DPC), Los Angeles, USA), with a sensitivity of 30 ng/mL, and coefficient of 6,8-9,5% for intra-assay, coefficient of 8,1-15,0% for interassay and 8,0-15,0% of total variability.

Δ_4 Androstenedione was measured by ELISA (IBL GmbH, Hamburg, Germany) with sensitivity of 0.02 ng/mL, and coefficient of 4,7-9,1% for intra-assay and of 9,6-12,0% for interassay variability.

17-0H Progesterone was measured by ELISA (Bio Source Europe SA, Nivelles, Belgium), with a sensitivity of 0,05 ng/mL, and coefficient of 4,3-8,1% for intra-assay, coefficient of 7,5-9,5% for interassay variability.

SHBG was measured by solid phase, competitive chemiluminescent immunoassay (Diagnostic Products Corporation (DPC), Los Angeles, USA), with a sensitivity of 0,2 nmol/L, and coefficient of 4,1-7,7% for intra-assay, coefficient of 5,8-13,0% for interassay and 6,0-13,0% of total variability.

Insulin was measured by an Micro particle Enzyme Immunoassay (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 0.1 μ IU/mL, and coefficient of 2.6-4.1% for intra-assay and of 2.0-2.9% for interassay variability.

Glucose was measured by an enzymatic method (ABBOTT LABORATORIES, diagnostics division, IL, USA), with sensitivity of 1.5 ng/dL, and total coefficient variability (CV) of 2.74-1.5 %.

Biochemical indexes

Evaluation of insulin resistance with homeostasis model assessment (**HOMA**) index (Fasting serum insulin ($\mu\text{U/ml}$) \times fasting plasma glucose (mmol/l) divided by 22.5) was performed (Bonora et al, 2000).

The quantitative index of insulin sensitivity **QUICKI** was calculated from the next formula: $\text{QUICKI} = 1/[\log(\text{insulin } 0) + \log(\text{glucose } 0)]$.

The **glucose response to the OGTT** was also analyzed by calculating the area under curve (AUC), according to the Tai procedure for the metabolic curves (Tai 1994, Diabetes care 17, 152-154).

The ratio of $T \times 100/\text{SHBG}$ was used to calculate the free androgen index (**FAI**) (Morley et al 2002).

Molecular genetic studies

DNA was extracted by whole blood samples using the Qiamp Blood Kit (Qiagen, Germany). We used the PCR-RFLP technique to examine, the Leu to Met substitution in the amino acid 55 of the PON1 gene, the Gln to Arg substitution in the amino acid 192 and the -108 C to T substitution in the promoter of the PON1 gene as previously described (Rea et al 2004).

Statistical analysis

Data were analyzed with the Graph Pad In Stat (version 3.00, Graph Pad Software, Inc., San Diego, CA, USA).

Continuous data were expressed as mean \pm SD.

Statistical significance was set at $p < 0.05$.

Frequency and susceptibilities of mutations among the examined groups were compared by employing the chi-square test (χ^2) test.

Results:

1. Frequency of PON1 polymorphisms in Greek women with PCOS

Eighty-seven Greek women with PCOS were examined, the mean age of which was 23.91 \pm 4.78 years and the mean value of the BMI was 27.95 \pm 6.84.

- a) The **Leu55Met substitution** in PON1 gene was not found in our study. All specimens that were examined had the LL genotype, meaning that all specimens were homozygous to the wild type allele.
- b) The **Gln192Arg substitution** in PON1 gene in our study was found in homozygous state (RR genotype) in 8 patients (9.2%), whereas in heterozygous state (QR genotype) in 27 patients (31%) and 52 women (59.8%) had the wild type (QQ genotype).
- c) The **-108C/T substitution** in the promoter of PON1 gene in our study the polymorphism was found in homozygous state (TT genotype) in 18 patients (20.7%), whereas in heterozygous state (CT genotype) in 30 patients (34.5%) and 39 women (44.8%) had the wild type (CC genotype).

2. Association of PON1 polymorphisms with clinical, hormonal and biochemical characteristics of PCOS patients

i) As regards the **Gln192Arg polymorphism** of PON1 gene, no difference was found in BMI between patients with QQ genotype and those with QR/RR genotype (table 2).

No statistically important differences were also found in the levels of FSH, LH, PRL, TSH, 17 OH-P, T, D4A, DHEA-S and SHBG between the 2 groups.

The fasting insulin levels were significantly higher in the QR/RR genotype vs. the QQ genotype ($p= 0.0026$).

The fasting glucose levels were higher in the QR/RR genotype vs. the QQ genotype but the difference was not statistically important.

Therefore, PCOS women with QR/RR genotype of PON1 gene, were more insulin sensitive, as evidence by a significant higher HOMA index (70.40 \pm 44.90 vs.52.89 \pm 34.87, $p= 0.046$).

ii) Concerning the **-108 C/T polymorphism** in the promoter of PON1 gene, no difference was found in the BMI and also in the levels of FSH, LH, TSH, PRL, 17OH-P, D4A, DHEA-S, SHBG between patients with CC genotype and those with CT/TT genotype (table 2).

Testosterone levels were significantly higher in patients with CT/TT genotype vs. those with CC genotype ($p= 0.049$).

Also, those with CT/TT genotype were presented with higher insulin resistance as found by the higher HOMA index (69.31+/-46.63 vs. 51.16+/-30.24, p=0.036) and the higher fasting insulin levels, they had (11.61+/-5.95 vs. 19.59+/-15.24, p=0.0016). No statistically important differences were found in fasting glucose levels and QUIKI index between the 2 groups.

Discussion

PCOS is a highly complex and heterogeneous syndrome. Many PCOS women show increased insulin resistance and hyperinsulinemia, which in turn aggravates hyperandrogenism (30, 31, 32).

However, the pathogenesis of insulin resistance in PCOS patients is believed to be multifactorial.

Taken into account the existing diversity, the present study was designed to answer questions whether PCOS women, carriers of the PON1 polymorphisms, have differences in glucose metabolism and insulin resistance indices and reproductive hormone profile, compared to PCOS women with the wild-type genes.

None of the subjects tested in our population was found to carry the **Leu55Met** polymorphism, as all samples had the LL genotype. The absence (or the extremely rare presence) of M allele in the Greek population of women with PCOS could be attributed to the ethnic differences often encountered in the frequency of several polymorphisms (17).

Concerning the **Gln192Arg** substitution of PON1 gene, we found that the fasting insulin levels were significantly higher in women with the QR/RR genotype than those with the QQ genotype.

The fasting glucose levels were higher in women with the QR/RR genotype vs. those with the QQ genotype, but not in statistically significant level and the HOMA index was slightly higher in women with the QR/RR genotype.

It has been reported that insulin resistance is related to decreased activity of PON1 in women with PCOS. Our results can not confirm this observation because the HOMA index was slightly higher in patients with the QR/RR genotype and these women have increased activity of PON1 compared to those with the QQ genotype (Agacham et al 2004).

As regards the **-108C/T polymorphism** in the promoter of PON1 gene, our results are in agreement with the study of San Millan et al (2004:J.Clin.End.Met.89:2640-2646) confirming that the presence of the -108T allele results in higher levels of free testosterone.

The -108T allele is found more often in non diabetic patients with disturbances in fasting glucose concentration and has been related to insulin resistance (34). As oxidative stress is able to influence insulin activity (35), the decreased activity of PON1 enzyme may contribute to insulin resistance. This hypothesis is supported from the finding that in disturbances related to insulin resistance like diabetes mellitus type-2, it is observed a decreased activity of PON1 (36).

In recent studies, the decreased activity of PON1 has been related to insulin resistance (34). In our study, in accordance to previous ones, we found that PCOS women with the -108T allele showed higher levels of insulin resistance as proved from the increased HOMA index. This group of PCOS patients should also have decreased PON1 activity according to previous studies which relate the genotype with the activity of peroxonase (17).

In conclusion, the Gln192Arg polymorphism of PON1 and even more the -108T/C polymorphism, were found implicated in insulin resistance in PCOS patients. However, both genotypes had only minor effect on reproductive hormones.

Therefore, further studies are needed to clarify better the role of PON1 polymorphisms, in determination of the phenotype, as well as the clinical and hormonal characteristics in women with PCOS.

Table 1. Clinical and hormonal characteristics of PCOS patients

Characteristics	Mean values \pm S.D.
Age (yr)	23.91 \pm 4.78
BMI (kg/m ²)	27.71 \pm 6.82
FSH (IU/lt)	5.59 \pm 1.27
LH (ng/ml)	8.92 \pm 6.2
PRL (ng/ml)	14.97 \pm 8.22
TSH (ng/ml)	1.98 \pm 1.1
17 OH-P (nmol/lt)	1.21 \pm 0.56
T (nmol/lt)	0.82 \pm 0.25
D4A (nmol/lt)	2.84 \pm 0.96
DHEA-S (nmol/lt)	2583.95 \pm 1344.6
SHBG (nmol/lt)	35.25 \pm 15.56
FAI	2.59 \pm 1.60
Fasting glucose (nmol/lt)	95.17 \pm 11.97
Fasting insulin (μ U/ml)	14.95 \pm 12.05
HOMA	57.87 \pm -36.54

Table 2: Clinical and hormonal characteristics according to different genotype groups (mean +/- SD).

Characteristics	Q192R			-108C/T		
	QQ	QR/RR	p	CC	CT/TT	p
Age (years)	23.44± 4.9	24.64±4.6	0.308	23.7±5.37	24.1±4.32	0.364
BMI (kg/m ²)	28.31±6.96	27.23±6.93	0.496	28.04±6.58	27.87±7.22	0.912
FSH (IU/lt)	5.38±1.39	5.54±1.58	0.660	5.52±1.39	5.36±1.54	0.640
LH (ng/ml)	8.79±6.43	8.92±6.15	0.933	9.15±6.03	8.48±6.63	0.655
PRL (ng/ml)	14.36±8.59	15.85±7.17	0.452	15.98±9.09	13.25±7.03	0.162
TSH	2.10±1.21	1.89±0.86	0.437	2.11±1.14	1.93±1.04	0.479
17OH-P (nmol/lt)	1.30±0.59	1.17±0.66	0.373	1.32±0.57	1.17±0.67	0.248
T (nmol/lt)	0.88±0.32	0.76±0.21	0.059	0.83±0.32	0.96±0.38	0.049
D4A (nmol/lt)	2.86±0.96	3.14±1.98	0.423	3.02±1.05	2.89±1.76	0.708
DHEA-S (nmol/lt)	2643.6±1359.5	2348.8±1007.2	0.308	2668.7±1370.9	2362.3±1058.5	0.259
SHBG	34.76±14.58	37.83±16.78	0.376	34.19±15.41	37.84±15.45	0.280
Fasting glucose (nmol/lt)	94.52±12.55	98.63±12.54	0.144	95.11±11.71	97.39±11.69	0.413
Fasting insulin (µU/ml)	12.44±7.51	21.23±17.91	0.0026	11.61±5.95	19.59±15.24	0.0016
QUICKI	0.32±0.027	0.30±0.036	0.525	0.33±0.029	0.32±0.034	0.419
HOMA	52.89±34.87	70.40±44.90	0.046	51.16±30.24	69.31±46.63	0.036
FAI	2.75±1.81	2.62±1.78	0.757	2.38±1.23	2.69±1.41	0.272

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