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Association of -108 C>T *PON1* polymorphism with polycystic ovary syndrome

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Abstract. Polycystic ovary syndrome (PCOS) is possibly the most common endocrine disorder in premenopausal women. In this study, we aimed to investigate the association of the -108 C>T polymorphism in the *PON1* gene, which encodes the antioxidant enzyme paraoxonase-1, with PCOS. A total of 118 women with PCOS and 108 control subjects were included in this case-control study. The *PON1* polymorphism was genotyped, biochemical and clinical parameters were determined and the correlations between the parameters were statistically evaluated. The differences in the *PON1* allele and genotype distributions between PCOS patients and controls did not reach a statistical significance. The serum fasting glucose (GLU) levels did not differ significantly between the PCOS patients and the controls. However, the serum fasting insulin (INS) concentration, INS/GLU ratio and homeostasis model assessment (HOMA) index, although within the normal range, were significantly higher in the PCOS group. When considering PCOS patients and controls as separate groups or as a single group of patients, none of the analyzed biochemical or clinical parameters were found to be significantly correlated with the *PON1* polymorphism. Therefore, the -108 C>T *PON1* polymorphism was not found to be significantly associated with the presence of PCOS or with its particular clinical and biochemical characteristics in non-insulin resistant, non-obese patients.

Introduction

Polycystic ovary syndrome (PCOS) is possibly the most common endocrine disorder in premenopausal women. This syndrome is characterized by its significantly heterogeneous and complex clinical picture, including chronic anovulation, biochemical and/or clinical hyperandrogenism, insulin resistance, compensatory hyperinsulinism, abdominal obesity, ovarian dysfunction, polycystic ovary morphology, chronic inflammation and increased oxidative stress. Consequently, PCOS patients are at increased risk of developing diabetes mellitus type 2, infertility and cardiovascular diseases (1-3).

The pathogenesis of PCOS is considered to be multifactorial, including its molecular genetic basis. The familial segregation and clustering of PCOS cases has prompted the conduction of genetic studies. Accumulating evidence suggests an oligogenic model, with a complex mode of inheritance, in which predisposing and protecting polymorphic (genetic) variants interact with environmental factors, such as obesity and a sedentary lifestyle, during the postnatal or prenatal life, ultimately leading to the PCOS phenotype (4). Despite the progress in the elucidation of the genetic mechanisms underlying PCOS, universally accepted susceptibility genes for PCOS have not yet been established.

There are ongoing efforts to dissect the variants of genes from multiple pathways involved in the pathophysiology of PCOS, such as polymorphisms in genes involved in the adrenal and/or ovarian androgen biosynthesis, in the metabolism and action of steroid hormones, in the action, signalling and secretion of insulin, in energy homeostasis, as well as in gonadotropin action and regulation (5). Recent case-control genetic studies on the pathogenesis of PCOS were focused primarily on the single-nucleotide polymorphisms (SNPs) affecting inflammatory processes and oxidative stress, including the SNPs affecting the activity of paraoxonase-1 (PON1) (6-8).

Paraoxonase-1 is a serum high-density lipoprotein-associated antioxidant enzyme and, therefore, its activity may significantly affect the (anti)oxidative status. The PON1 activity was reported to be reduced in insulin-resistant disorders, including PCOS (9,10). In a recent study on 35 Saudi PCOS patients, PON1 activity and antioxidant status were significantly decreased compared to those in 30 healthy controls (10). Moreover, decreased PON1 activity was observed in two independent studies on 31 and 23 Turkish women with PCOS compared to 33 and 23 healthy controls, respectively (11,12). The decrease in serum PON1 activity may result in higher oxidative stress in PCOS patients, contributing to insulin resistance and atherosclerotic heart disease (9,12,13).

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Associations among the *PON1* gene (7q21.3), hyperandrogenism and insulin resistance in women with PCOS were previously reported (4). *In vitro*, the -108 C>T *PON1* polymorphism was shown to be responsible for ~23% of the *PON1* expression levels; the -108 T/T constructs exhibited reduced expression of the enzyme compared to the -108 C/C constructs (14). Furthermore, it was observed that -108 T *PON1* alleles predispose to lower transcriptional activity of the *PON1* gene *in vivo* and, consequently, to lower serum PON1 concentrations and activity in 139 Spanish premenopausal PCOS patients, compared to their 85 healthy control counterparts (9,13). Recently, the Leu55Met *PON1* polymorphism was investigated in an association study on 130 Polish PCOS patients and 70 healthy controls, but the results revealed similar genotype frequencies between the two groups and no significant association was identified between the *PON1* genotypes and the measured metabolic parameters, including insulin resistance (6).

The aim of the present study was to investigate the association of the -108 C>T polymorphism in the promoter of the *PON1* gene with the presence of PCOS, as well as the correlations between the selected clinical, biochemical and genetic parameters in our specific subphenotype group of Slovene PCOS patients.

Patients and methods

Patients. A total of 118 patients fulfilling the criteria for PCOS (2,15) were enrolled in the study group. The patients were included consecutively during a 4-year period, while undergoing treatment at the Department of Obstetrics and Gynaecology, Ljubljana University Medical Centre, Slovenia. All the patients exhibited menstrual cycle abnormalities (amenorrhoea or oligomenorrhoea) and polycystic ovaries (PCO), which was identified with an ultrasound scan. The morphological characterization of PCO was performed according to international consensus criteria [≥12 follicles measuring 2-9 mm in diameter and/or increased ovarian volume (>10 cm^3)] (16). Hyperandrogenism was assessed by the presence of hirsutism (Ferriman-Gallwey index score ≥8) and/or by increased serum free testosterone (FT) levels [calculated from serum total testosterone (TT) and serum sex hormone-binding globulin (SHBG) levels] (17), increased serum sulphated dehydroepiandrosterone (DHEA-S) and androstenedione (A) levels. Other possible causes of hyperandrogenism (late-onset congenital adrenal hyperplasia, Cushing's syndrome and androgen-secreting tumours) had been previously excluded by endocrinologists.

The control group consisted of 108 healthy age-matched volunteers who visited the clinic for a routine check-up, with proven fertility and no menstrual cycle irregularities, no clinical or biochemical hyperandrogenism, no PCO, no history of endocrinological or autoimmune disorders, who had not undergone surgery to the pelvic region. All the women were of European (Slovene) origin and were not genetically related.

A total of 226 patients were included in this study and they were the same patients that had already participated in our previous study on insulin gene polymorphism in PCOS (18).

The study protocol was approved by the National Medical Ethics Committee of the Republic of Slovenia (no. 97/05/01) and written informed consent was obtained from all the subjects who were enrolled in the study.

Biochemical and clinical analyses. Serum samples were obtained in the early follicular phase of the menstrual cycle, or randomly in amenorrhoeic patients. We measured the serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), SHBG, TT, DHEA-S, A, fasting insulin (INS) and fasting glucose (GLU) levels. The serum LH, FSH and SHBG concentrations were determined by chemiluminescent immunometric assay using LH-Immulite®, FSH-Immulite® and SHBG-Immulite®, respectively (Diagnostic Products Corporation, Los Angeles, CA, USA). The serum TT, DHEA-S and A levels were measured using commercial radioimmunoassay kits (TT: DiaSorin, Saluggia, Italy; DHEA-S: ICN Biomedicals, Costa Mesa, CA, USA; and A: Diagnostic Systems Laboratories, Webster, TX, USA). An immunoradiometric assay kit was used (DiaSorin) for determining serum INS levels, whereas the serum GLU levels were determined by the glucose oxidase method using a Beckman Glucose Analyser II (Beckman Coulter, Fullerton, CA, USA). The intra- and inter-assay coefficients of variation were 1.6-13.0%.

The body mass index (BMI) was calculated as mass (kg) divided by height squared (m^2) and insulin resistance was estimated by the INS/GLU ratio and by the homeostasis model assessment (HOMA) index, calculated as [INS (μU/ml) x GLU (mmol/l)] /22.5.

Genetic analysis. Genomic DNA was isolated from peripheral blood leukocytes using the commercial FlexiGene DNA kit (Qiagen, Hilden, Germany) for all the subjects, following the protocols recommended by the manufacturer. The -108 C>T *PON1* polymorphism was genotyped using polymerase chain reaction-restriction fragment length polymorphism. The 15-μl reaction mixture consisted of 90 ng genomic DNA, 0.2 μM of each of the two oligonucleotides (5'-GAC CGC AAG CCA CGC CTT CTG TGC ACC-3' and 5'-TAT ATT TAA TTG CAG CCG CAG CCC TGC TGG GGC AGC GCC GAT TGG CCC GCC GC-3') (14), 0.2 mM of each of the dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 0.3 μM formamide and 1.5 units of Taq DNA polymerase Gold® (Perkin Elmer, Foster City, CA, USA). The reaction conditions in a Primus PCR apparatus (MWG-Biotech AG, Ebersberg, Germany) were as follows: initial denaturation for 10 min at 95˚C, followed by 35 cycles of i) denaturation (45 sec, 94˚C), ii) annealing (45 sec, 63˚C) and iii) extension (1 min, 72˚C); and a final extension step for 7 min at 72˚C. The specific PCR products were 119-bp in length. Furthermore, a restriction analysis was performed using 5 units of Bsh1236I restrictase (Fermentas, Vilnius, Lithuania) in buffer R (Fermentas) at 37˚C for 16 h. The restriction fragments were analyzed on 4% agarose gels (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) using xylene cyanol dye. Digested DNA fragments of 52 and 67 bp were detected in C/C homozygotes (restriction site present), an undigested band of 119 bp was detected in T/T homozygotes (restriction site absent) and heterozygous genotypes resulted in three different bands (52, 67 and 119 bp).

Statistical analysis. The χ^2 test was used to compare the *PON1* allele and genotype frequencies between the patient

Table I. Biochemical and clinical parameters (means ± SD) in PCOS patients and control subjects.

Parameters	PCOS (n=118)	Controls (n=108)	P-value
Age (years)	24.4±4.4	25.3±3.8	NS
GLU (mmol/l)	4.2±0.7	4.2±0.6	NS
INS (mIU/l)	8.9±2.7	7.5±1.8	<0.001
INS/GLU	2.2±0.7	1.8±0.6	<0.001
HOMA	1.7±0.6	1.4±0.4	<0.001
SHBG (nmol/l)	44.4±19.1	61.0±14.7	<0.001
TT (nmol/l)	3.0±1.3	1.2±0.4	<0.001
FT (pmol/l)	39.8±18.3	12.8±5.3	<0.001
LH (IU/l)	10.1±6.5	2.4±0.7	<0.001
FSH (IU/l)	4.8±2.4	4.6±1.0	NS
BMI (kg/m^2)	22.3±3.1	21.2±1.1	0.001
DHEA-S (mg/dl)	355.6±255.4	333.1±209.7	NS
A (ng/dl)	250.1±110.3	165.2±65.5	<0.001

PCOS, polycystic ovary syndrome; NS, non-significant; GLU, serum fasting glucose concentration; INS, serum fasting insulin concentration; HOMA, homeostasis model assessment index; SHBG, serum sex hormone-binding globulin concentration; TT, serum total testosterone concentration; FT, serum free testosterone concentration; LH, serum luteinizing hormone concentration; FSH, serum follicle-stimulating hormone concentration; BMI, body mass index; DHEA-S, serum sulphated dehydroepiandrosterone concentration; A, serum androstenedione concentration.

and the control groups. The Student's t-test was used to compare the mean values of age, BMI, serum LH, FSH, TT, FT, DHEA-S, A, INS, GLU, SHBG levels, INS/GLU ratio and HOMA index between the PCOS and the control groups. The correlations between selected clinical, biochemical and genetic parameters were evaluated, considering all the patients as a whole or PCOS patients and controls separately. All the statistical analyses were performed using SPSS software for Windows, version 18.0 (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Biochemical and clinical profiles. Our PCOS patients had significantly higher BMI compared to that of the controls (P=0.001); 13% of the PCOS patients and none of the controls were considered obese (BMI>25 kg/m^2). All the women had normal serum GLU and INS concentrations. The serum GLU levels did not differ significantly between the PCOS patients and the controls, whereas the serum INS concentrations, INS/GLU ratio and HOMA index, although within the normal range, were significantly higher in the PCOS group (P<0.001 for all three parameters). The serum LH, TT, FT and A levels were significantly higher (P<0.001 for all four parameters), whereas the serum SHBG concentrations were significantly lower (P<0.001) in PCOS patients. Although higher mean serum FSH and DHEA-S levels were observed in PCOS patients compared to the control subjects, the difference did not reach a statistical significance.

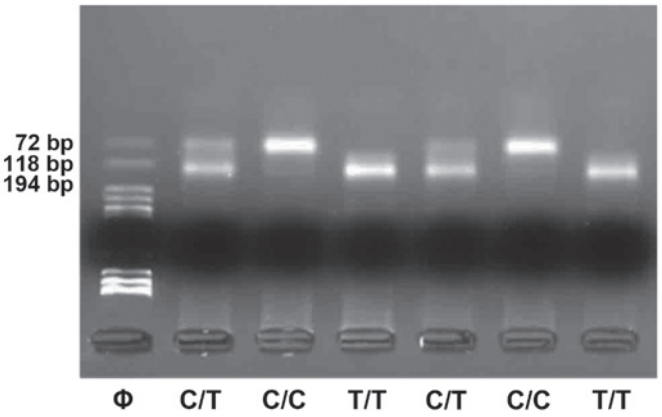

Figure 1. Genotyping of the -108 C>T *PON1* polymorphism using polymerase chain reaction-restriction fragment length polymorphism. The restriction fragments were detected on 4% agarose gels using xylene cyanol dye. The 52-bp fragment is not visible. Φ, marker ΦX174; bp, base pairs.

The measured biochemical and clinical parameters of the subjects are presented in Table I.

Genotyping of the -108 C>T PON1 polymorphism. The results of the molecular genetic analysis (Fig. 1) were as follows: The -108 C allele frequencies were 51.4 and 58.8%, whereas the -108 T allele frequencies were 48.6 and 41.2% in PCOS patients and controls, respectively. The distribution of the -108 C/C, C/T and T/T *PON1* genotypes in the patient and control groups was 27.3, 48.2 and 24.5% vs. 34.3, 49.1 and 16.6%, respectively. Despite an excess of the -108 T alleles and the -108 T/T genotypes in our PCOS patients, the differences in the allele and genotype distributions between the PCOS and control groups did not reach a statistical significance (P=0.119 and 0.285, respectively).

Correlations between patient biochemical, clinical and genetic characteristics. Women with the -108 *PON1* T/T homozygous genotype exhibited significantly higher serum INS levels compared to those with the -108 *PON1* C/T or C/C genotypes, when considering patients either as a whole or PCOS patients and controls separately. However, the correlations did not maintain a statistical significance following adjustment for age and BMI. None of the other analyzed biochemical and/or clinical parameters (serum TT, FT, SHBG, INS, GLU, DHEA-S and A levels, BMI, INS/GLU ratio and HOMA index) were found to be significantly correlated with the genetic polymorphism when considering patients as a whole or the PCOS patients and controls separately (data not shown).

Discussion

The results of the present study revealed no association of the -108 C>T *PON1* polymorphism with PCOS in the Slovene patients who were included in this study. In addition, we were unable to confirm any significant correlations of the evaluated biochemical and/or clinical parameters with the -108 C>T

PON1 polymorphism in our PCOS patients and/or control subjects.

Our results are contradictory to the initial results from Spanish women, in whom the homozygous -108 T/T genotype was significantly more prevalent among PCOS patients compared to healthy controls and resulted in lower PON1 expression (13). In addition, in a recent study on 30 Turkish PCOS patients and 30 age- and BMI-matched controls, the measured serum PON1 activity was found to be significantly lower in PCOS patients (19). However, one of the main characteristics of the PCOS patients in the Turkish study was their insulin resistance (19), whereas our PCOS patients were not insulin-resistant, although they exhibited significantly higher INS levels, INS/GLU ratio and HOMA index compared to their matched controls. Therefore, we were not able to directly compare the results between our study and the Turkish study. Furthermore, a study on Swiss non-diabetic patients demonstrated that the minor, -108 T *PON1* allele frequency was higher and the serum PON1 activity was lower in patients with abnormal serum GLU levels (suspected to have insulin resistance) compared to individuals with normal serum GLU levels. These observations indicated an active role of PON1 in predisposing to insulin resistance, as well as the probability that the -108 C>T *PON1* genetic polymorphism is linked with other gene products involved in glucose metabolism (20).

However, the results of our study are similar to those recently reported by a study on Chinese PCOS patients of the Chengdu area, where the -108 C>T *PON1* polymorphism was not found to be associated with PCOS in 346 patients (21). Our findings were also in accordance with the results of another study on Turkish PCOS patients without metabolic syndrome and insulin resistance, in whom PON1 activity appeared to be unaffected (22).

Accordingly, insulin resistance should be carefully considered when treating PCOS patients and optimizing their pharmacological treatment for ovulation induction and/or for improvement of the clinical presentation of PCOS in any individual patient.

The majority of the existing genetic studies on PCOS, including our own, are association studies, focused on candidate gene approach (23). A systematic review on different PCOS genetic association studies revealed an inconsistency in the results (24). These conflicting results may be partly attributed to the lack of universally accepted diagnostic criteria for PCOS and may also be due to the relatively small sample sizes with regard to the complex pathogenetic background of PCOS, which results, unlike monogenetic traits, from the interaction of several genetic variations with environmental factors (25). Appropriate male phenotype, affected reproduction, incomplete penetrance, genetic heterogeneity and variable expressivity of the syndrome, as well as gene-gene or gene-environment interactions should also be considered in PCOS patients (24).

Although not fully elucidated, the pathogenesis of PCOS remains an important issue and requires further investigations, which may overcome several obstacles and challenges regarding studying the genetic background of PCOS (4). Genome-wide association scans and functional genomics' approaches are becoming increasingly reasonable and realistic methodological tools to at least partly overcome the limitations of the existing genetic studies in PCOS (24,26,27). Strict and uniform diagnostic criteria, improved application of the candidate gene approach using haplotype-based analyses, intermediate phenotypes, replication of positive results in large cohorts, more family-based studies, gene selection from expression studies and whole-genome (DNA microarrays), proteomic and metabolomic approaches, may enhance the possibilities of identifying PCOS genetic risk factors (23,28,29).

In conclusion, we observed no significant association of the -108 C>T *PON1* polymorphism with the presence of PCOS or with the investigated clinical and biochemical characteristics of the syndrome in Slovene non-insulin resistant and non-obese PCOS patients, suggesting that this polymorphism may not affect oxidative stress (if any) in this specific PCOS subgroup. In order to obtain highly clinically useful results from further PCOS genetic association studies, PCOS patients should be carefully classified into subphenotypes and appropriate therapeutic approaches should be selected accordingly.

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